Plasma micro-RNA-22 is associated with disease activity in well-established rheumatoid arthritis

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Abstract

Objective

Micro-RNAs (miRNAs) are an endogenous small, single-stranded, non-coding RNAs with a 18-25 nucleotide long and have been reported as potential extracellular biomarkers of various diseases. They mainly decrease the gene expression by inhibiting the translation or cause mRNA destabilisation. The aim of our study was to identify miRNAs whose concentration may be associated with severity of rheumatoid arthritis (RA).

Methods

A total of 74 unrelated individuals, 50 with RA and 24 in a control group were enrolled to the study. Real-time PCR was used to evaluate the plasma concentration levels of 8 miRNAs: miR-26a, miR-125b, miR-20b, miR-22, miR-221, miR-17, miR-93, miR-106b.

Results

The logistic regression results showed that miR-22 (p=0.0003) and miR-26a (p=0.049) may be the most important molecules distinguishing RA patients and healthy controls. Moreover, the quantity of miR-22 was different between rheumatoid factor (RF)-positive and RF-negative patients (p=0.04).

Conclusion

In this study we demonstrated for the first time that plasma concentration of miR-22 may be considered as a potential molecular marker associated with disease activity.

Key words

epigenetic, micro-RNA-22, molecular marker, rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a systemic, chronic autoimmune disease and mainly leads to joins destruction due to the outcome of ongoing inflammation. The aetiopathology of RA still remains unclear, however compilation of environmental, genetic, proteomic and epigenetic factors has a significant role in RA development. It is important to diagnose RA in an early stage because consequences of disease are irretrievable. Undiagnosed patients have a progressive manifestation often resulting in a disability and premature mortality (1, 2). When anti-citrullinated protein antibody (ACPA) and rheumatoid factor (RF) are negative, it may be problematic to meet the classification criteria developed by The American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) in 2010 (3). Therefore, finding the new biomarkers of disease morbidity and severity may be useful for more effective diagnosis, especially in seronegative patients with RA.

Epigenetic modifications such as DNA methylation, histone proteins alterations or modifications in genes expression caused by noncoding RNAs (ncRNAs) have been reported as important factors associated with pathogenesis of RA (4). Micro-RNAs (MiRNAs) are an endogenous small, single-stranded, ncRNAs with a 18-25 nucleotide long. They may reduce the gene expression by inhibiting the translation or cause mRNA destabilisation by binding the seed sequence of the 3' untranslated region (UTR) of targeted transcript (5). MiRNAs are involved in all biological processes, from proliferation and differentiation to programmed cell death (2).

Several years ago, miRNAs were reported as a potential extracellular biomarkers of various disease conditions, including RA (6-8). They are widely distributed in body fluids (1, 2). Currently the protein-based biomarkers are the most common, however challenge is to find new proteins which will be highly specific and sensitive. The challenge is also the complexity of proteins structure, their posttranslational modifications and often low quantity in serum or plasma. Developing of high-affinity capture solutions is also complicated (9). Polymerase chain reaction (PCR) is a method of high specificity and high sensitivity, which means RNA expression methods, or in the broader sense, the cycle amplification of DNA may be implemented as the tools for searching the new biomarkers of diseases. Moreover, miRNAs are tissue-specific (10), non-invasive and inexpensive (9).

The aim of the present study was to identify miRNAs whose plasma quantity is associated with severity of RA. Based on current literature we selected 8 miRNAs which were related to RA morbidity, however there were insufficient data about their relationships with RA severity. The knowledge about association between miRNAs concentration and disease activity may be useful to diagnose and treat more effectively.

Materials and methods

Patients

A total of 76 subjects, 52 with RA and 24 in a healthy control (HC) group were enrolled. RA patients were selected based on disease activity score 28 (DAS-28). Only patients with high disease activity (DAS-28 >5,1; 58%) and patients in remission (DAS-28 <2.6; 42%) were included in miRNAs analysis. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Bioethics Board at the Medical University in Lublin, protocol number KE-0254/7/2016, and patients provided written informed consent. The characteristics of patients was previously described (11) (DNA methylation analysis section). Due to haemolysis two plasma samples were excluded from evaluation (one from high disease activity group and the second one from remission). RA diagnosis was made according to the 2010 ACR/EULAR or 1987 ACR criteria for classification depending on the time of diagnosis. Infection or severe illness during hospitalisation were the exclusion criteria.

Micro-RNAs quantity analysis

MiRNAs were selected by mean of current literature. We selected 8 miRNAs with well-established concentra-

tion in plasma (miR-26a, miR-125b, miR-20b, miR-22, miR-221, miR-17, miR-93, miR-106b).

Blood samples with EDTA were collected and centrifuged at 2500 rpm for 10 minutes at room temperature to obtain the plasma. After that samples were stored at -80°C until analysis. After thawing samples were centrifuged at 3000 x g for 5 minutes to pellet the cells debris and insoluble components. Plasma miRNAs were extracted using miRCURY RNA Isolation Kit – Biofluids (Exiqon, Vedbaek, Denmark) according to manufacturer's instruction, but the 300 µl of plasma was used. At the beginning of each isolation 5.6 x 10^8 copies of mimic cel-miR-39 (miRNeasy Serum/Plasma Spike-In Control, Qiagen, Germany) and 1.5 µg of carrier RNA (MS2 RNA, Roche, Germany) were added to each sample. After procedure, the miRNAs were stored at -80°C until downstream analysis.

To perform reverse transcription miRNAs to complementary DNA (cDNA) the miRCURY LNA RT Kit was used according to manufacturer's recommendation. After that cDNA was stored at -20°C until PCR. Before PCR cDNA was 10-fold diluted. The reaction was performed using PowerUp SYBR Green master mix (Thermo Fisher Scientific, USA) in the COBAS z480 Real Time PCR System under the thermal cycling conditions given in the mix manual, in 45 amplification cycles, except miR-22, miR-221 – 40 cycles, and with an annealling/elongation step at 60°C, for 1 min. PCR was followed by a melt curve analysis. All samples were evaluated in triplicate. Primers were designed by miRprimer2 software (12). Detailed characterisation of primers and targeted miRNAs were presented in Supplementary Table S7. PCR efficiency for all targets was evaluated as previously described (13, 14). Mimic cel-miR-39-3p was used for data normalisation.

The data were analysed using the Advanced Relative Quantification method (LightCycler 480 SW, version 1.5.1.62 SP2 – UDF v.2.0.0, Roche, Germany), with the maximum second derivative selected as the calculation model. The results were presented as a normalised ratio.

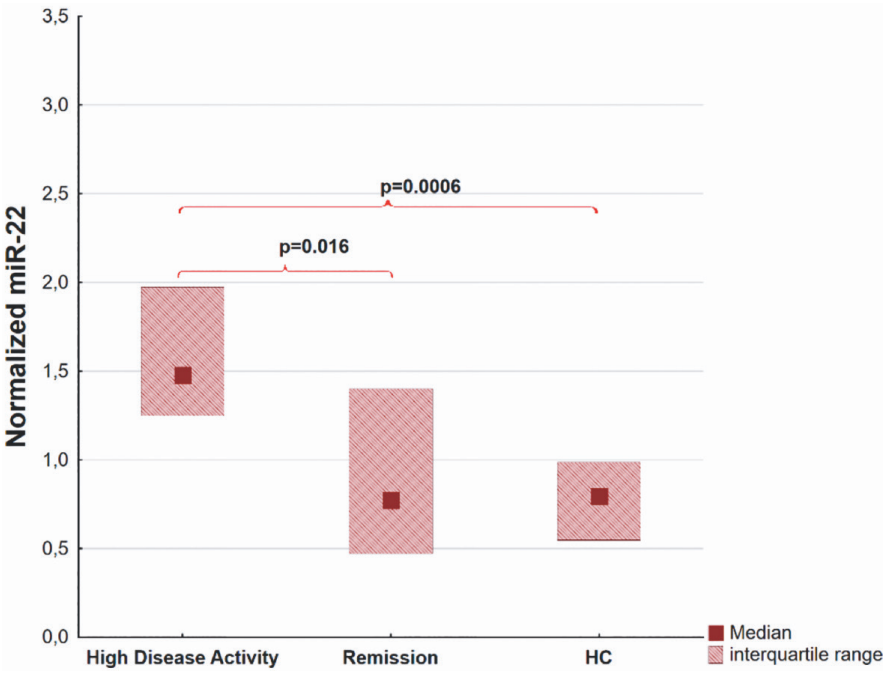

Fig. 1. Differences in the levels of micro-RNA-22 between patients with rheumatoid arthritis divided into disease activity groups and healthy controls. HC: healthy control group.

Statistical analysis

Quantitative values were presented as mean±SD or median (lower – upper quartile). Depending on the distribution, differences between two independent groups were compared by Student's *t*-test or the Mann-Whitney U-test. The Kruskal-Wallis ANOVA and *post-hoc* multiple comparison analyses were used to assess the differences between controls and patients divided into disease activity groups. The relationship between two continuous variables was analysed by Spearman's correlation. The multilinear logistic regression analysis was performed to estimate the impact of miRNAs quantity as markers of RA as well as influence the clinical parameters and miRNAs on DAS-28 as an outcome parameter. A *p*-value less than 0.05 was considered statistically significant. The analysis was performed with STATISTICA v. 13 (Dell Inc. 2016).

Results

The levels of 6 miRNAs (from eight evaluated) were significantly different between patients with RA and HCs (miR26a, $p<0.0001$; miR-125b, $p<0.0001$; miR-20b $p<0.0001$; miR-22, $p=0.005$; miR-17, $p=0.0001$; and miR106b, $p=0.008$. Detailed data are presented in Supplementary Table S1. Also, these miRNAs have shown significant differences between patients divided into disease activity groups and HCs. Results of these analysis are presented in Supplementary Table S2 as well as in the Supplementary Figures S1and 2 (diagrams from A to D and from E to H, respectively). Figure 1 shows the differences in miR-22 levels in regard to patients with high disease activity and remission as well as controls. The multilinear logistic regression analysis using mentioned above 6 miRNAs was performed. As an outcome parameter was used presence of disease – patients with RA *versus* controls. The results showed that miR-22 ($p=0.0003$) and miR-26a ($p=0.049$) may be most useful molecules distinguishing RA patients and HCs. Detailed results were presented in Supplementary Table S3. Subsequently, the correlations between clinical variables: age, disease duration, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), number of swollen joints, number of painful joints, DAS-28, ACPA and RF levels were estimated. Micro-RNA-22 was positively correlated with ESR ($r_s=0.41$), CRP ($r_s=0.49$) and DAS-28 ($r_s=0.33$). Micro-RNA-26a was positively correlated with ESR ($r_s=0.49$),

CRP (r_s=0.41), number of swelling joints (r_s=0.43), number of painful joints (r_s=0.74) and DAS-28 (r_s=0.63). Both miRNAs were not correlated with ACPA and RF autoantibodies level. Detailed results for others miRNAs were given in Supplementary Table S4. All selected miRNAs were also evaluated in patients divided by RF positivity. Only miR-22 levels were different between RF-positive and RF-negative patients with RA (p=0.04, Fig. 2). Micro-RNAs levels in rheumatoid factor-positive and rheumatoid factor-negative patients were presented in Supplementary Table S5. Due to a high positivity of ACPA in the study groups, in both activity subgroups over 90%, the comparison between miRNAs concentration and mentioned antibodies was not performed. The multilinear logistic regression using the clinical parameters and miRNAs concentration as well as DAS-28 as an outcome parameter was performed. As expected, results showed that DAS-28 was related to ESR (p<0.0001) and numbers of swollen (p=0.03) and painful joints (p<0.0001), however was not associated with any miRNAs. Detailed results of the analysis were presented in supplementary Table S6.

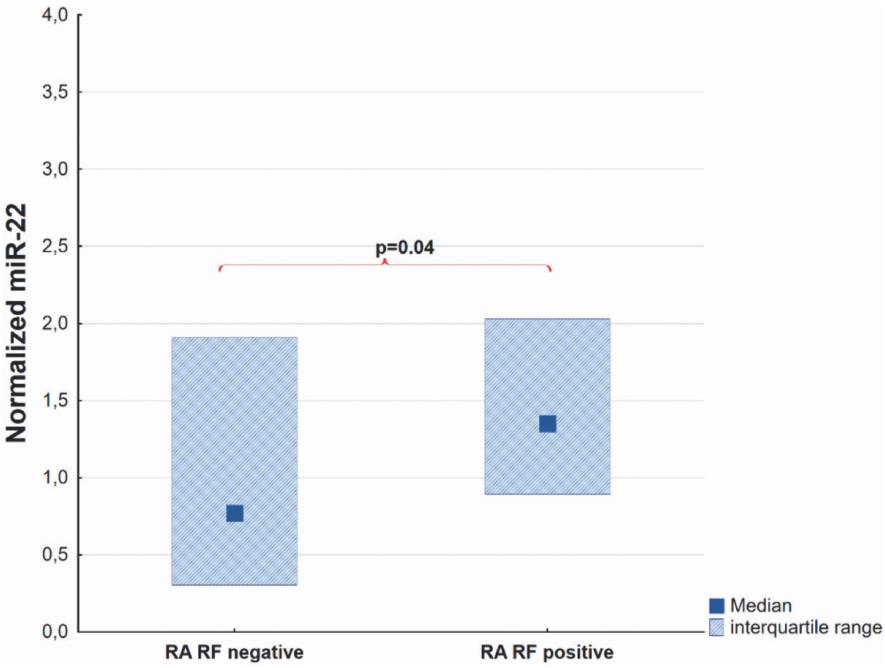

Fig. 2. Differences in the levels of micro-RNA-22 between rheumatoid factor positive and negative patients. RA: rheumatoid arthritis patients; RF: rheumatoid factor.

Discussion

In this study we demonstrated for the first time that plasma concentration of miR-22 may be considered a potential molecular marker associated with disease activity. It has been shown that selected miRNAs: miR-26a, miR-125b, miR-20b, miR-22, miR-17 and miR-106 are associated with RA, especially miR-22 and miR-26a may be useful as a new markers of disease activity.

Previous reports have shown association of miR-22 with autoimmune diseases such as systemic lupus erythematosus (SLE) and RA as well as mental and cardiology diseases and cancers (15-19). MiR-22 is encoded by *C17orf91,* the gene located on chromosome region 17p13. This miRNA is mainly considered as tumour suppressor. In relation to RA, miR-22 stimulates the interleukin 6 (IL-6) overproduction (7). Mentioned miRNA inhibit the expression of the extracellular matrix protein Cyr61 via posttranscriptional level by targeting the 3' UTR of transcript. In the synovial tissue expression of miR-22 is decreased, thus Cyr61 is overexpressed which leads to stimulation of proliferation of fibroblast-like synoviocytes. In turn, fibroblasts are responsible for production of IL-6 and Th17 cells differentiation. This subpopulation of T helper cells plays an important role in the pathogenesis of various autoimmune diseases, including RA (20). Moreover, miR-22 may play a protective role by inhibiting the synovial fibroblasts proliferation and pro-inflammatory effect by inhibiting Sirtuin1 (SIRT1) gene (21).

In the present study the level of miR-22 was higher in patients with RA than in controls. Moreover, quantity of this molecule was higher in patients with the high disease activity than in remission and controls. In the study conducted by Zhang *et al*. (18) the plasma concentration of few miRNAs, including miR-22, was compared between patients with RA and SLE and controls. Decreased level of miR-22 was shown in SLE patients than compared to healthy individuals. Furthermore, patients with RA have also had decreased levels of miR-22. Although no association of the discussed miRNA with SLE activity has been demonstrated, its potential value as a biomarker for distinguishing SLE patients from those with complications of lupus nephropathy has been proven. Contrary to our findings, Zhang *et al*. have showed decreased plasma concentration of miR-22. This may be associated with a few reasons. Primarily, the RA group in mentioned study was not large (n=16) and there was no information about disease duration, the treatment used, current disease activity and autoantibody status, thus it is hard to compare our study with these data. As we showed in the present study, the level of miR-22 is associated with disease activity, however the remission and the controls have had a similar level of evaluated miRNA, which consequently seems logical. Furthermore, present study showed that miR-22 was decreased in RF-negative patients. Taking all this into account, the miRNAs level may be associated with various clinical variables, and carefully analysis of these variables is necessary in the correct assessment of miRNAs concentration in relation to patient's clinical state. Regardless of these findings, the validation of protocols, including standardisation of material collection, miRNAs isolation and quantification is important.

Results presented by Ouboussad *et al*. (19) show potential role of miR-22 as a molecular marker contributing to early diagnosis of the disease. They have presented the important association between miR-22 in serum and the possible progression from systemic immunisation to early stage of RA. The present study showed that the level of miR-22 was different in relation to disease activity in patients with well-established RA. In our study the median disease duration was about 10 years. An aspect worth mentioning is that in both studies, conducted by Ouboussad *et al*. and our study, the concentration level of mentioned miRNA was evaluated in plasma/serum samples, which greatly facilitates its potential use as a molecular marker of disease activity. In patients suffering from ankylosing spondylitis (AS), Perez-Sanchez *et al*. (22) have confirmed the potential role of miR-22 as a biomarker of chronic inflammation. They have shown increased plasma quantity of mentioned miRNA in AS patients compared to controls. Moreover, positive correlation between plasma miR-22 and inflammatory molecules such as CRP, tumour necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-5 (IL-5) was reported. In our research we have confirmed a relationship between miR-22 and CRP and ESR which are commonly used markers of inflammation. Orr *et al*. (23) have shown that CRP may have limited use. In the patients with RA with a normal CRP about 49% had histological signs of inflammation in the synovial tissue as well as about 71% patients in remission (based on DAS-28-CRP) had also tissue inflammation. Furthermore, the sensitivity and specificity of mentioned markers to detect the synovial tissue inflammation was limited. CRP has a sensitivity and specificity of 71% and 56%, respectively. ESR has a sensitivity and specificity of 48% and 64%, respectively. Finally, DAS-28-CRP shows sensitivity of 92% and up to 6% specificity (23). Sokka and Pincus (24) demonstrated in the two cohorts of patients that both CRP and ESR were in normal in 33–42% of cases. Important to emphasise is that they have taken for consideration the first recorded parameter, and the median disease duration were 6 months and 3.3 years, respectively (24). Although, we have found the correlation between studied miRNAs and acute phase reactants as well as DAS-28, but the regression analysis has not shown that DAS-28 is associated with miRNAs concentration. This may indicate that miRNAs may be considered as independent markers related to disease activity.

Perez-Sanchez *et al*. (22) have transfected the lymphocytes by mimic miR-22 and measured the levels of 3 mentioned earlier molecules: TNF-α, IL-1β and IL-5. It turned out that the levels of IL-1β and IL-5 expression were significantly decreased, which may proves the role of miR-22 in the immune response. On the other hand, they previously showed that miR-22 was positively correlated with their plasma concentration, which seems controversial. It is necessary to mention about extracellular concentration may not be correlated with intracellular. The previous studies regarding to another molecule – miR-21 – showed that the plasma concentration may be elevated (25), although concentration in peripheral blood mononuclear cells (PBMC) or $CD4^+$ cells may be reduced (26). In turn, the gene expression is not regulated only by one miRNA and biological effect of plasma miRNA concentration may not be adequate to transcription silencing in cells. Perez-Sanchez *et al*. (22) did not perform miRNAs expression analysis directly from PBMC isolated from patients, thus it is difficult to conclude whether increased concentration of plasma miR-22 *in-vivo* may affect the expression level of the mentioned cytokines, when its intracellular expression level is unknown. Comparing data from both IL-1β and IL-5 serum concentration and miR-22 concentration it seems that further confirmation of inhibitory effect of miR-22 on inflammatory markers is required.

They also have reported association between miR-22 and disease activity. The AS patients were divided into two sub-groups: in an active and inactive phase of disease. Disease activity was evaluated based on structural damage measured by the presence or absence of syndesmophytes. Perez-Sanchez *et al*. (22) have proved that miR-22 shows a higher concentration in patients with an active form of the disease. Data obtained in this study confirmed previous reports that the quantity of this miRNA increases with disease activity and may be used as a potential biomarker of various diseases with altered inflammation. Association between miRNAs and biological treatment in patients with RA was reported by Krintel *et al*. (27). They showed in a group of patients with early RA that low levels in a whole blood of miR-22 in combination with high levels of miR-886-3p is a good predictor of successful adalimumab treatment. According to this study, approximately 30% of RA patients do not respond to biological treatment and a good molecular marker before starting targeted therapy is needed. Micro-RNA-22 seems to be a very valuable molecule from this point of view. Firstly, it is well expressed in plasma, which makes the collection of clinical specimens non-invasive. Secondly, it is expressed in patients in various disease conditions: in the phase of systemic autoimmunity, in the early phase of RA, and – as this work has shown – in patients with established disease additionally dividing them into disease activity.

In our opinion, miR-22 may play a valuable role as a marker associated with RA activity in well-established disease. The median concentration value was higher in high disease activity patients with RA and was similar in remission and HCs. As was mentioned in an introduction the seronegative patients may be misdiagnosed due to lack of specific autoantibodies. As was previously reported, RF shows sensitivity of 71% and specificity of 83%, and false negative results were ranged from 10.6% to 59.3% (28). These data show that further investigation of markers supporting diagnosis is required. We have shown that miR-22 is associated with RF status. Quantity of miR-22 was higher in patients with RF-positive RA patients than RF-negative. MiR-22 seems not to be a useful marker contributing to diagnosis of seronegative RA patients. The median concentration

of miR-22 in RF-negative patients was at similar level as in HCs and remission, thus miR-22 seems not to be helpful at the beginning of diagnosis.

In this study we also found that miR-26a may be the second important molecule which separates RA patients and HCs. We have shown that miR-26a has a lower concentration in patients with RA than HCs as well as may distinguish patients with RA according to their disease severity. Murata *et al*. (1) reported that three miRNAs: miR-24, miR-26a and miR-146 may be considered as plasma biomarkers of RA which differentiate patients and HCs with a high specificity. For miR-26a the specificity was reported as 94.3%. It should be mentioned that elevated plasma levels of miR-26a in RA in comparison to HCs were reported (1, 29). Also increased expression of mentioned miRNA in peripheral blood mononuclear cells and in synovium was reported (30). Niimoto *et al*. have shown that miR-26a was significantly up regulated in the interleukin 17 (IL-17) producing T helper cells. IL-17 is a proinflammatory cytokine and plays a critical role in pathogenesis of RA. This cytokine stimulates overproduction of the other proinflammatory cytokines, such as TNF-α, IL1β and IL-6. IL-17 is also responsible for activating the receptor activator of NF-κB ligand (RANKL). This activation leads to joints destruction (30). In this study we found decreased level of miR-26a. It is not unusual that concentration of some miRNAs were reported contradictory. The most widely studied miRNA in RA – miR-155 – in some studies was reported as downregulated (2, 15) in plasma or serum, but the others shown its overexpression (29). The second example is plasma concentration of miR-146a. Some reports show its decreased concentration (15, 31), other indicated that miR-146a was elevated (29). In our study miR-26a was positively correlated with disease activity, ESR and CRP, as well as with number of painful and swollen joints. Despite these correlations, miR-26a seems to be less valuable as a marker because it has shown the similar trends towards other studied miRNAs (miR-125b, miR-20b, miR-17 and miR-106b) – the lowest level in a remission group, medium level in a high activity group and the highest level in HCs. Therefore, it is not an unambiguous marker associated with the disease severity.

As mentioned above, other studied miRNAs (miR-125b, miR-20b, miR-17 and miR-106b) have shown a more decreased concentration in a remission group that in HCs, which was interesting to us. We decided to check the status of miR-93, which was reported in the part of current literature as having a stable concentration in plasma and serum (32, 33). No significant differences between patients and HCs were found, thus our data were found to be correct. On the other hand, Filková *et al*. (31) have shown that serum expression of miRNAs: miR-16, miR-146a and miR-155, were lower in early RA than established disease. Moreover, miR-146a was significantly decreased in early stage of disease than HCs. It should be noted that patients with early RA have had a DAS-28 score of 2.6 and 2.54 at 3 months and 12 months of beginning of treatment, respectively. The established RA was characterised by DAS-28 score of 4.1. In this study we also observed that some of miRNAs have had a decreased concentration in a remission group (DAS-28 <2.6) and elevated concentration in high activity RA patients (DAS-28 >5.1) and higher in HCs. It is difficult to find the cause of this phenomenon. We propose that it may be associated with long-term treatment and reflect the impact of used medications. If so, such a low miRNAs quantity may indicate the successful treatment independently from the drug used. Further analysis is required to find a solution to this topic. In our study patients with RA were often treated with various groups of medications, thus analysis including impact of treatment is unfounded, especially that the major limitation of our study is relatively small sample size, thus further analysis on a larger cohort is necessary. Another limitation of this study is lack of additional inflammatory control. It is necessary to evaluate the level of specificity of studied miRNAs in relation to RA. However, it is known that miRNAs show a low specificity to one disease, thus use of a single marker to diagnose the disease or its severity may be difficult. Rather miRNAs should be considered as additional tool, independent of the standard parameters, but supporting them. Furthermore, miRNAs are easy and non-invasive to measure in the other compartments *e.g*. in serum, urine or saliva. Biological material may be frozen for further evaluation and most of the assays required a small sample volume with simultaneously high sensitivity and specificity of the PCR method. On the other hand, it is also possible to measure directly the immune cells associated with disease development, *e.g*. by flow cytometry. The biggest challenge is to achieve a sufficient number of events, thus enrichment method may be necessary to obtain significant sensitivity, which will be associated with a long period of data collection. This in turn will be associated with the limited number of samples that can be daily measured. The major limitation of this method is insufficient sample material, especially for the rare subsets of regulatory cells (34, 35).

To conclude, plasma concentration of miR-22 may be considered as a new supporting molecular marker of RA activity. Our findings may be considered as an initial study and further analysis should be made in order to improve or replicate our results.

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