EXTENDED REPORT

Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis

Mária Filková,1 Borbala Aradi,1 Ladislav Šenolt,2 Caroline Ospelt,1 Serena Vettori,1 Heřman Mann,2 Andrew Filer,3 Karim Raza,3 Christopher D Buckley,3 Martyn Snow,4 Jiří Vencovsky,2 Karel Pavelka,2 Beat A Michel,1 Renate E Gay,1 Steffen Gay,1 Astrid Jüngel1

ABSTRACT

Background Identification of parameters for early diagnosis and treatment response would be beneficial for patients with early rheumatoid arthritis (ERA) to prevent ongoing joint damage. miRNAs have features of potential biomarkers, and an altered expression of miRNAs was shown in established rheumatoid arthritis (RA).

Objective To analyse RA associated miRNAs in the sera of patients with ERA to find markers of early disease, clinical activity or predictors of disease outcome.

Methods Total RNA was isolated from whole sera in ERA patients (prior to and after 3 and 12 months of therapy with disease modifying antirheumatic drugs), in patients with established RA and in healthy controls (HC) using phenol–chloroform extraction. Expression of miR-146a, miR-155, miR-223, miR-16, miR-203, miR-132 and miR-124a was analysed by TaqMan Real Time PCR.

Results From all analysed miRNAs, levels of miR-146a, miR-155 and miR-16 were decreased in the sera of ERA patients in comparison with established RA. A change in circulating miR-16 in the first 3 months of therapy was associated with a decrease in DAS28 in long term follow-up in ERA (p=0.002). Levels of circulating miR-223 in treatment naive ERA correlated with C reactive protein (p=0.008), DAS28 (p=0.031) and change in DAS28 after 3 months (p=0.003) and 12 months (p=0.011) of follow-up. However, neither miR-16 nor miR-223 could distinguish ERA from HC.

Conclusions Differential expression of circulating miR-146a, miR-155 and miR-16 in the sera of ERA patients may characterise an early stage of the disease. We suggest miR-223 as a marker of disease activity and miR-16 and miR-223 as possible predictors for disease outcome in ERA.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic, inflammatory, autoimmune disorder with progressive articular damage that may result in severe lifelong disability. A considerable number of RA patients develop severe disability early in the disease.1 Studies have shown a beneficial effect of early treatment on clinical outcome and reduction in joint damage that prevents irreversible joint destruction and disability.2–4 In addition, a delay of a few months from the onset of symptoms to the institution of therapy decreases the ability of the traditional single drug strategy to induce remission in early rheumatoid arthritis (ERA).5–7 Therefore, in order to define patients at earlier stages of the disease who would benefit from early effective intervention, new RA classification criteria were developed.8 However, while some patients profit from initial monotherapy, others fail to demonstrate clinical or radiological responses, even to combination therapy.9 Until now, several predictors of response to disease modifying antirheumatic drugs (DMARD) and biological treatments such as cytokine profiles or gene expression analysis have been described but are either impractical or lack specificity to be adopted in daily clinical practice.9 10 Therefore, it is important to find parameters that identify patients with early arthritis and to find predictors of treatment response in ERA to optimise individual management of early arthritis based on the expected disease course and to prevent over or under treatment.9–10

miRNAs are short single stranded non-coding RNAs involved in the post-transcriptional regulation of gene expression.11 Altered expression of miRNAs has been described under various pathological conditions, including rheumatic and other autoimmune diseases.12 13 We and others reported that miR-146a, miR-155 and miR-203 are overexpressed while miR-124a is suppressed in RA synovial fibroblasts.14–17 Dysregulation of miR-146a, miR-155, miR-223, miR-16 and miR-132 was observed in immune cells derived from patients with RA.14 18–22 As miRNAs are stably present in cell free form in body fluids and circulating miRNAs were shown to have signatures related to tumour classification and disease progression, they are becoming new candidate biomarkers for diagnosis and prognosis in different diseases.23–25 Although expression of several miRNAs has recently been analysed in plasma and synovial fluid of patients with established RA and osteoarthritis,26 there are no reports to date on the important phase of ERA.

The aim of our study was to analyse the profile of selected RA related cells free circulating miRNAs in the sera of patients with ERA prior to and after therapy with DMARD, in established RA and in healthy controls (HC). Using this approach, we hoped to identify potential tools distinguishing early and late phases of the disease, markers of
clinal activity or predictors of disease outcome. In addition, we analysed intracellular expression of these miRNAs in synovial fibroblasts from patients with ERA.

MATERIAL AND METHODS

Collection of sera and characteristics of patients with RA

Sera and clinical details from 34 patients with ERA who fulfilled the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for RA\(^*\) with a symptom duration <8 months were obtained at League Against Rheumatism (EULAR) classification criteria for RA\(^*\) with a symptom duration <8 months were obtained at baseline, and at 3 and 12 months after treatment. Patients with ERA were recruited from the outpatient clinic of the Institute of Rheumatology, Prague. Sera from 28 patients with established treated RA of disease duration 9.28±6.52 years who fulfilled the 1987 revised ACR criteria for the classification of RA\(^*\) were obtained from both the Department of Rheumatology, University Hospital, Zurich, and the Institute of Rheumatology, Prague. Clinical characteristics of the patients and the 16 HC (University Hospital, Zurich) are shown in table 1. Written informed consent was obtained from all participants, and the study was approved by the local ethics committees.

Synovial fibroblasts from patients with inflammatory arthritis

Synovial fibroblasts (SF) were isolated from synovial tissues obtained during ultrasound guided synovial tissue biopsy (University of Birmingham, UK). Synovial tissue samples were taken from patients with untreated ERA (n=7) with a disease duration <13 months who met the 1987 ACR criteria,\(^*\) from patients with knee pain of non-inflammatory origin (n=9) and from patients with resolving arthritis different from RA (n=14). Written informed consent was obtained from each donor, and the study was approved by the local ethics committees.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of patients with early and established rheumatoid arthritis, and healthy controls, whose sera were used for analysis of expression of circulating miRNAs in the present study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early RA</td>
</tr>
<tr>
<td></td>
<td>Sex (F/M)</td>
</tr>
<tr>
<td></td>
<td>25/9</td>
</tr>
<tr>
<td></td>
<td>Age (years)</td>
</tr>
<tr>
<td></td>
<td>Disease duration &lt;8 months</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
</tr>
<tr>
<td></td>
<td>Sulfasalazine</td>
</tr>
<tr>
<td></td>
<td>Leflunomide</td>
</tr>
<tr>
<td></td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td></td>
<td>Biologics</td>
</tr>
<tr>
<td></td>
<td>Leucocytes ×10⁹/mm³</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±SD.
*p<0.05, **p<0.01, ***p<0.001 compared with baseline.
ACPA, anticyclicupdated protein; CRP, C reactive protein; DAS28, disease activity score calculated with ESR; ESR, erythrocyte sedimentation rate; NA, not analysed; RA, rheumatoid arthritis; RF, rheumatoid factor.
were therefore excluded from further analysis (data not shown). ERA, established RA and HC (dCt 5.23±1.25). The levels of miR-16 in established RA were higher than in patients with established RA (dCt 4.93±0.91) than in patients with established RA (dCt 3.85±1.37) significantly decreased in comparison with both baseline (p<0.05) and M3 (p<0.001, figure 2A). In addition, the increase in miR-16 at M3 was verified by an independent method using TaqMan Low Density Arrays cards (TLDAs) (3.768-fold change, see online supplementary table 1). The effect of different treatment modalities on levels of miR-16 is given in the online supplementary table 2.

Higher levels of miR-16 at baseline correlated with a greater improvement in disease activity, represented by a larger decrease in DAS28 from baseline to M3 (r=−0.478, p=0.008) (figure 2B). Similarly, levels of miR-16 at M3 correlated with a change in disease activity ΔDAS28 from M3 to M12 (r=−0.355, p=0.047) (figure 2C). Most importantly, the change in levels of miR-16 from baseline to M3 were negatively associated with the change in DAS28 from M3 to M12 (r=−0.519, p=0.002) (figure 2D) suggesting that the increase in circulating miR-16 within the first 3 months after treatment initiation was followed by the decrease in disease activity in the subsequent 9 months. Interestingly, levels of miR-16 at baseline negatively correlated with levels of anticitrullinated protein antibodies (ACPA) (r=−0.365, p=0.037). However, levels of miR-16 did not correlate with C reactive protein (CRP), erythrocyte sedimentation rate or IgG, IgM or IgA rheumatoid factors (RF) at baseline, M3 or M12 (data not shown).

Although differentially expressed in ERA sera in comparison with established RA sera, levels of circulating miR-16 showed no association with disease activity in established RA (data not shown).

found at significantly lower levels in the sera of patients with ERA (dCt 4.93±0.91) than in patients with established RA (dCt 6.10±1.22, p<0.001) while it was comparable with HC (dCt 5.23±1.25). The levels of miR-16 in established RA were higher than in HC but did not reach statistical significance (figure 1C).

The patterns of miR-132 and miR-223 were not different in ERA, established RA and HC (dCt −4.31±0.90, −3.76±1.07, −4.41±1.23 for miR-132; dCt 5.67±1.04, 6.23±1.45, 5.55±0.77 for miR-223). MiR-124a and miR-203 in sera were present in negligible amounts close to the detection limit and were therefore excluded from further analysis (data not shown).

Change in circulating miR-16 during therapy is associated with decreasing disease activity

Subsequently, the effect of therapy with DMARDs on levels of miRNAs was explored in ERA patients. Also, the association of circulating miRNAs with markers of disease activity in ERA and established RA was evaluated. Follow-up within 3 months (M3) after initiation of therapy appeared to be a very important time point with respect to clinical response and prediction of outcome at 12 months (M12).28 29

Levels of miR-16 increased by 34.31% in 24/34 ERA patients after 3 months of therapy. Although miR-16 was the only miRNA analysed in our study, showing a trend towards higher levels after 3 months of treatment with DMARDs (dCt 4.93±0.91 vs 5.71±1.27), it did not reach statistical significance in the whole group of ERA patients. However, levels of miR-16 at M12 (dCt 3.85±1.37) significantly decreased in comparison with both baseline (p<0.05) and M3 (p<0.001, figure 2A). In addition, the increase in miR-16 at M3 was verified by an independent method using TaqMan Low Density Arrays cards (TLDAs) (3.768-fold change, see online supplementary table 1). The effect of different treatment modalities on levels of miR-16 is given in the online supplementary table 2.

MiR-223 as a marker of disease activity in treatment naive ERA and its association with disease outcome during short and long time follow-up

Levels of miR-223 were found to be decreased by 17.65% in 24/34 ERA patients 3 months after treatment initiation. However, in the group of all ERA patients, the change in levels of miR-223 at M3 in comparison with baseline did not reach statistical significance (dCt 5.67±0.104 vs 5.12±0.91). In fact, after 12 months of treatment, levels of miR-223 (dCt 4.34±0.78) significantly decreased in comparison with levels at baseline (p<0.001) and at M3 (p<0.01) (figure 3A). Further analysis using TLDAs showed no change in MiR-223 at M3 (1.065-fold change) but confirmed the decreased levels of miR-223 at M12 (0.692-fold change, see online supplementary table 1). The effect of different treatment modalities on levels of miR-223 is given in the online supplementary table 2.

In ERA prior to treatment initiation, levels of miR-223 positively correlated with CRP (r=0.519, p=0.008 figure 3B) and with DAS28 (r=0.389, p=0.031) (figure 3C) at baseline. Importantly, levels of miR-223 at baseline correlated with the decrease in DAS28 from baseline to M3 (r=−0.522, p=0.003) (figure 3D) as well as from baseline to M12 (r=−0.460, p=0.011) (figure 3E). Moreover, the greater decrease in levels of miR-223 from baseline to M12, the more improvement in disease activity, represented by a larger decrease in DAS28 from M3 to M12 (r=−0.519, p=0.002) (figure 2D) suggesting that the increase in circulating miR-16 within the first 3 months after treatment initiation was followed by the decrease in disease activity in the subsequent 9 months. Interestingly, levels of miR-16 at baseline negatively correlated with levels of anticitrullinated protein antibodies (ACPA) (r=−0.365, p=0.037). However, levels of miR-16 did not correlate with C reactive protein (CRP), erythrocyte sedimentation rate or IgG, IgM or IgA rheumatoid factors (RF) at baseline, M3 or M12 (data not shown).

Although differentially expressed in ERA sera in comparison with established RA sera, levels of circulating miR-16 showed no association with disease activity in established RA (data not shown).
DAS28 was observed after 12 months of therapy (r=0.483, p=0.006) (figure 3F). In contrast with significant correlations of miR-223 with disease activity in ERA patients before treatment, levels of miR-223 did not correlate with parameters of disease activity (DAS28 or CRP), or levels of ACPA, IgG, IgM or IgA RF at M3 or M12 after initiation of therapy (data not shown).

In established RA, levels of miR-223 showed no correlation with disease activity (data not shown). In addition, neither miR-146a nor miR-155 or miR-132 showed any association with disease activity in patients with ERA at baseline, M3 or M12, or in patients with established RA or any change in expression after therapy in ERA (data not shown).

We found a significant correlation between baseline levels of miR-223 and peripheral leucocyte (PL) count in patients with ERA (r=0.459, p=0.007) (figure 4A). Therefore, we were interested in whether the change in levels of miR-223 after treatment followed the change in PL count. In fact, the larger decrease in miR-223 between baseline and M3 positively correlated with the decrease in PL count between these two time points (r=0.397, p=0.025) (figure 4B). Also, the changes in levels of miR-223 and PL count within the whole period of 12 months correlated significantly (r=0.621, p=0.0001) (figure 4C). This suggests that the decrease in miR-223 in the sera of ERA patients after therapy is very likely attributable to the change in the number of PLs.

Expression of miRNAs in synovial fibroblasts from patients with ERA

Based on different levels of miRNAs in ERA reported here, we anticipated dysregulation of miRNAs in SF from patients with ERA. Therefore, expression of miR-146a, miR-155, miR-203, miR-124a, miR-223, miR-16 and miR-132 was analysed in isolated SF cultured in vitro.

In contrast with differences in levels of miRNAs observed in sera, there were no significant differences in expression of miRNAs analysed in our study among SF from patients with ERA, resolving arthritis of different disease entities as an example of non-RA arthritis and controls with non-inflammatory arthralgia (see online supplementary figure 2).

**DISCUSSION**

RA is a chronic inflammatory disease characterised by progressive joint destruction. We and others have shown that altered expression of miRNAs in immune and resident cells involved in the pathogenesis of RA contributes to maintenance of pathognomonic features typical of RA. For example, miR-155 and miR-146a are induced in response to inflammatory stimuli and are overexpressed in RA SF in comparison with osteoarthritis SF. MiR-155 acts as a positive regulator and miR-146a as a negative regulator of inflammation in RA, as shown in vivo. MiR-223 was shown to be overexpressed in synovial tissue of RA patients and, importantly, silencing of miR-223 reduced disease severity in experimental arthritis.

The evidence of high stability of miRNAs in body fluids due to incorporation into microvesicles or stabilisation in complexes with RNA binding proteins (Argonaute 2, high density lipoproteins, nucleophosmin 1) and their easy accessibility make miRNA...
ideal biomarkers. Moreover, changes in the number of miRNAs originating from blood cells or tissues and their specific profiles in influenced by pathophysiological conditions, such as sepsis, have been described previously. Based on these data, we anticipated that levels of circulating miRNAs in sera would be different in patients with ERA and established RA.

We report here for the first time reduced levels of miR-146a, miR-155 and miR-16 in the sera of patients with ERA in comparison with patients with established RA. Levels of these miRNAs were comparable between patients with established RA and HC, similar to the data published by Murata et al, except for miR-132 that was shown to be lower in RA than in HC. Although intracellular expression of miR-146a and miR-155 was shown to be induced by the proinflammatory milieu in vitro, it is possible that cell-free circulating miRNAs in sera do not necessarily reflect levels in the intracellular compartment. In fact, release of miRNAs may be selective due to the existence of a cellular selection mechanism for miRNA release and therefore the extracellular and cellular miRNA profiles may differ. Secretory cell-free miRNAs were shown to transfer inhibitory signals to recipient cells. Although the functional role and impact of circulating miRNAs in living organisms remains currently unknown, we speculate that in ERA, miR-146a and miR-155 may be extensively taken up by recipient cells where they exert their regulatory activity. We propose that low levels of miR-146a, miR-155 and miR-16 in ERA may represent the early phase of RA. We hypothesise that progression of disease severity, disease duration, effect of treatment or other yet unknown factors can modulate levels of circulating miRNAs in established RA.

MiR-16 was reported to be overexpressed in peripheral blood mononuclear cells of RA patients with active disease in comparison with RA patients with low disease activity or with HC. We showed here that the higher levels of miR-16 in the sera of treatment naïve patients with ERA correlated with better improvement in disease activity during the 3 months of follow-up. This is important because most clinical responses in ERA are seen in the first 3 months of therapy, as shown in table 1. In addition, change in levels of circulating miR-16 within the first 3 months after initiation of therapy is associated with disease outcome in the following 9 months.

Figure 3 Change in levels of circulating miR-223 in the sera of patients with early rheumatoid arthritis (ERA) from baseline (M0) to 3 (M3) and 12 (M12) months after treatment with disease modifying antirheumatic drugs (A). Levels of circulating miR-223 (dCt) at baseline (M0) in the sera of ERA patients correlated with the levels of CRP (B) and disease activity (DAS28) (C) at M0. Levels of circulating miR-223 at M0 correlated with the change in DAS28 (ΔDAS28) from M0 to M3 (D) as well as M12 (E). Change in levels of miR-223 from M0 to M12 correlated with ΔDAS28 from M0 to M12 (F). dCt was calculated as follows: Ct (let−7a) − Ct (miR-223) and therefore higher dCt values represent higher levels. The decrease in ΔDAS28 is indicated by a full arrow.
MiR-223 was recently demonstrated to be upregulated in peripheral T cells from patients with early as well as established RA compared with HC, but no correlation of miR-223 in these cells with DAS28, CRP, RF or ACPA was found in these patients. In addition, no differences in expression of miR-223 in T cells were observed in untreated RA patients and those treated with glucocorticoids. Although not differentially expressed in the sera of ERA and RA patients, we suggest circulating cell free miR-223 as a marker of disease activity in treatment naïve patients with ERA based on significant associations with CRP and DAS28. At the same time, higher baseline levels of circulating miR-223 were associated with better improvement in disease activity in ERA after the therapeutic intervention. We suggest that levels of miR-16 and miR-223 may characterise patients with good/worse response to therapy.

In the study, we showed the changes in levels of miR-16 and miR-223 over time after initiation of therapy. We suggest that the decrease in miR-223 in ERA sera after therapy may reflect altered expression in blood cells or solid tissues. Apart from the contribution of inflammatory cells abundantly present in the hyperplastic RA synovium, SF are believed to actively drive joint inflammation and destruction. Although we observed different levels of miRNAs in ERA sera, we did not see any differences in SF from patients with ERA cultivated in vitro, possibly due to type II statistical error. As shown previously, fibroblast-like synoviocytes, mononuclear cells and synovial tissues secrete miRNAs in distinct patterns. A differential profile of cytokines influencing the microenvironment required for persistent RA has been observed in the synovial fluids of patients with early synovitis which developed later into RA. Also, distinct expression of miRNAs in synovial tissues in established RA, as a result of chronic exposure of SF to the inflammatory milieu accompanying RA, has been reported. Therefore, our data obtained in vitro in isolated SF do not necessarily reflect the situation in synovial tissue. The association of miRNAs with specific immunohistochemical findings in synovial tissues at the early phase and how cell free miRNAs originating from a joint compartment contribute to the spectrum and levels of circulating miRNAs in blood serum/plasma remain to be investigated.

In conclusion, we have shown here for the first time low expression of miR-146, miR-155 and miR-16 in the sera of patients with early in comparison with established RA. Although further studies in larger patient cohorts are needed, our data support circulating miR-223 as a marker of disease activity in patients with treatment naïve ERA. Moreover, monitoring levels of miR-16 and miR-223 may become a useful tool to predict disease outcome in patients with ERA.

References


Acknowledgements The authors acknowledge Jitka Směkalová for technical assistance.

Funding This work was supported by IMI BTCure, IAR Epalinges, EU-TEAM, project No 023728 for conceptual development of research organisation by the Ministry of Health, Czech Republic, IGA project No NT14498 and SVV project No 264511.

Competing interests None.

Ethics approval The study was approved by the ethics commission of Zurich, Kantonalen Ethikkommision Zürich), the ethics committee at the Institute of Rheumatology, Charles University, Prague, Czech Republic and the ethics committee at University of Birmingham, UK.

Provenance and peer review Not commissioned; externally peer reviewed.

Accepted Online This is an Open Access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 3.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/3.0/.

References

Basic and translational research


Supplementary material

Material and methods

Collection of sera

Patients with early rheumatoid arthritis (ERA), established RA and healthy controls were recruited from the outpatient clinic of the Institute of Rheumatology in Prague and the Department of Rheumatology at the University Hospital in Zurich. ERA patients fulfilled the 2010 ACR/EULAR classification criteria for RA, patients with established RA met the 1987 revised ACR criteria for the classification of RA.[8,27] Written informed consents were obtained from all participants, and the study was approved by the local ethics committees. The blood was withdrawn in the morning after overnight starvation. Samples were taken in plastic tubes containing spray-coated silica. Samples were handled according standardized laboratory procedures and were processed within 4 hours after withdrawal. All samples were stored at -80°C until use and experienced no freeze-thawing cycles before use.

Collection of synovial tissues and cell cultures

Synovial fibroblasts (SF) were isolated from synovial tissues obtained during ultrasound-guided synovial tissue biopsy (University of Birmingham, UK). Synovial tissue samples were taken from patients with untreated ERA who met the 1987 revised ACR criteria for the classification of RA [27] at the time of biopsy (n=7) with disease duration <13 months. Study included SF from patients with a knee pain of non-inflammatory origin (n=9) and patients with resolving arthritis different from RA (reactive arthritis n=5, parvovirus n=3, pseudogout n=1, unclassified n=5) who had synovitis of at least 1 joint and a symptom duration of ≤3 months. Resolving arthritis was diagnosed after 18 months follow up if there was no evidence
of joint related soft tissue swelling on examination and the patient had not received DMARDs or glucocorticoids within the previous 3 months. Written informed consent was obtained from each donor, and the study was approved by the local ethics committee.

SF were grown from a minimum of 8 tissue biopsy specimens per joint to account for heterogeneity and were cultured as described previously.[14,15] Briefly, cells were grown in full Dulbecco’s minimum essential medium (Gibco-Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin/streptomycin, 0.2% Fungizone, and 10 mM HEPES (all Gibco-Invitrogen).

Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2. All SF between passages 4 and 8 were used for RNA isolation.

**RNA isolation**

Isolation of RNA from sera aimed for further analysis using single assays (see below in Reverse transcription, TaqMan Real Time PCR paragraph) was performed as follows: sera (500 µl) were homogenized with Trizol LS reagent (500 µl, Invitrogen, Basel, Switzerland) and incubated for 5 minutes at room temperature. The samples were cleared by centrifugation at 12,000 × g for 10 minutes at 4°C. The supernatant containing RNA was then processed 3 times by acid phenol-chloroform (Ambion, Life Technologies, Paisley, UK) extraction and aqueous phase was separated by centrifugation at 12,000 × g for 5 minutes at 4°C. RNA was precipitated by adding RNase-free glycogen (10 µg, Roche Diagnostics, Mannheim, Germany) and 100% isopropanol, incubated for 10 minutes at room temperature with subsequent centrifugation at 12,000 × g for 10 minutes at 4°C. The pellet was washed with 75% ethanol, spun at 7500 x g for 5 minutes at 4°C and air dried. RNA was dissolved in RNase-free water by incubating at 58 °C for 10 minutes.
For selected experiments as explained further, samples were spiked in with synthetic C. elegans miR-39 miRNA mimic (cel-39, 25 fmol) (Qiagen, Hilden, Germany) after denaturation with Trizol LS.

Isolation of RNA from sera aimed for further analysis using Taqman Low Density Array Cards (see below in Reverse transcription, TaqMan Real Time PCR paragraph) was performed using commercially available miRCURY RNA isolation kit – Biofluids (Exiqon Vedbaek, Denmark) from 200 µl of sera according manufacturer´s instructions.

Total RNA from all SF was isolated with mirVana™ miRNA Isolation Kit (Ambion, Life Technologies) and RNA from PBMC using miRNeasy Mini Kit (Qiagen) as recommended by the manufacturer.

Quality control of RNA samples was performed using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and Bioanalyzer with the Small RNA Kit and the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA).

**Reverse transcription, TaqMan Real Time PCR**

Two-step protocol was used for quantification of miRNAs using single assays. For reverse transcription fixed amount 12.5 ng of total RNA was reverse-transcribed using TaqMan® MicroRNA Reverse Transcription Kit with a miRNA-specific primer, followed by real-time PCR with TaqMan® probes, TaqMan Universal PCR Master Mix using a 7500 real-time PCR system (all Applied Biosystems- Life Technologies Foster City, CA, USA) as described previously.[15] The number of cycles was set to 40. The dCt method was used for relative quantification of the differences in miRNA expression in sera or SF samples. The expression of let-7a was used as an endogenous control. Value of dCt was calculated as follows: Ct (let-7a) - Ct (miRNA of interest).
For analysis by Taqman Low Density Array Cards for microRNAs, equal amounts of RNA isolated with miRCURY RNA isolation kit (as mentioned above) were pooled from 5 sera samples obtained from patients with ERA. The pool was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT Primers. cDNAs were preamplified with 2x TaqMan PreAmp Master Mix and Megaplex PreAmp Primers (all Applied Biosystems-Life Technologies). Expression of miRNAs was measured with Taqman Low Density Array Cards for microRNAs, Human Pool A (Applied Biosystems-Life Technologies). All steps were performed according to the manufacturer’s instructions. Data were analyzed with the Expression Suite Software (Applied Biosystems-Life Technologies), using global normalization. ddCt method was used to calculate fold-change.

**Statistical analysis**

Data are expressed as the mean±SD if not indicated otherwise. The Mann-Whitney U test or Wilcoxon signed rank test were used where appropriate for comparisons between two variables. The Kruskal-Wallis test, along with Dunn's multiple comparison tests, was used for comparisons among more than two variables. Spearman correlation coefficient was used to correlate any two variables. P values less than 0.05 were considered statistically significant. The analysis and the graphs were performed using GraphPad Prism 5 (version 5.02; GraphPad Software, La Jolla, CA, USA).
Results

Reproducibility of RNA isolation from sera and miRNA analysis, selection of endogenous control

In order to validate the reproducibility of the work flow (from RNA isolation, reverse transcription to TaqMan Real Time PCR), several experiments were performed as follows:

Serum was obtained from 2 healthy donors and 500 µl aliquots were frozen at -80°C until use. RNA was isolated from 3 aliquots from donor 1 and 4 aliquots from donor 2 in day 1 and one aliquot from each donor in day 2 and 3 (Suppl Fig 1a). Reverse transcription and TaqMan Real Time PCR for let-7a (used further as an endogenous control) and miR-223 (one of the highly expressed miRNAs in sera) as well as dCt calculation were performed as mentioned in Material and methods. The differences lower than 0.6 cycles were considered reproducible. Expression of miR-223 presented as dCt was analyzed in donor 1 as follows: day (1) -5.9495, -5.7210 and -5.5365 (dCt mean±SD: -5.736±0.2069); day (2) -5.571; day (3) -5.386. Expression of miR-223 in donor 2 on day (1) -5.1895, -5.3285, -5.3460 and -5.4030 (dCt mean±SD: -5.317±0.09060); day (2) -5.386; day (3) -5.496 confirmed the intra- and inter-assay reproducibility of our method (Suppl. fig. 1a).

In additional set of experiments, 5 samples (2 aliquots from 2 donors, 1 aliquot from 3rd donor) were spiked in with synthetic cel-39 as described above. As an outcome, the expression of spiked-in control cel-39 and the expression of naturally occurring miR-16 were measured by TaqMan Real Time PCR. The minimal variations in Ct for cel-39 among all samples (16.41±0.36) and identical Ct for miR-16 in corresponding aliquots confirm consistent recovery and good reproducibility of the method (Suppl. fig. 1b).
Performing RNA dilution series (Suppl. fig. 1c) we observed a linear range of the system showing no signs of inhibition by carry-over inhibitory compounds interfering with Real-Time PCR reaction.

Since there are no validated reference genes that are used for normalization of miRNA levels in sera or plasma, the use of appropriate endogenous control is recommended to be tested for experiments. Several miRNAs control candidates were tested for their suitability before setting up the actual miRNA expression analysis. Expression of miR-103, miR-191, miR-423, miR-425 and let-7a, considered by Exiqon (www.exiqon.com) as candidate reference genes for serum/plasma PCR panel, was analyzed in randomly selected 6 sera from patients with established RA, ERA and HC. The differences in Ct mean lower than 1 cycle between groups were taken into account. Ct for let-7a showed the highest expression and was of lowest variations amongst miRNAs tested (Suppl fig. 1d). Finally, no differences in let-7a Ct values were observed in sera of all patients with established RA (29.79±1.150), healthy controls (29.68±1.593) or patients with ERA at baseline (30.63±1.497) or after 3 months follow up (30.35±1.262) included in our study. These results suggest that let-7a is expressed in sera at a consistent level and is suitable for using as an endogenous control to normalize sampling and RT-PCR variations in our study.
Supplementary tables:

Supplementary table 1. Along with analysis of miRNAs in serum of ERA patients over time using single assays, an independent experiment was performed to confirm the data. In contrast to RNA isolation aimed for analysis using single assay, RNA isolation for this experiment was performed using a commercial kit. Reverse transcription and preamplification were performed before the final measurement with Taqman Low Density Arrays as described in Material and Methods. The table shows the expression of miR-16 and miR-223 in 5 sera samples obtained from ERA patients at baseline and 3 and 12 months after the initiation of therapy using the global normalization approach. Fold change was calculated using ddCt method referring to baseline levels.

<table>
<thead>
<tr>
<th>Follow up at:</th>
<th>Expression Suite - Global normalization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dCt mean</td>
</tr>
<tr>
<td>miR-16</td>
<td>miR-16</td>
</tr>
<tr>
<td>baseline</td>
<td>-7.273</td>
</tr>
<tr>
<td>3 months</td>
<td>-9.187</td>
</tr>
<tr>
<td>12 months</td>
<td>-7.936</td>
</tr>
</tbody>
</table>

Supplementary table 2. The effect of different treatment modalities on levels of miR-16 and miR-223 after 3 (M3) and 12 (M12) months therapy with DMARDs in patients with ERA in comparison with baseline (M0). The Kruskal-Wallis test, along with Dunn's multiple comparison tests, was used to compare all 3 groups over time. NS; not significant.

<table>
<thead>
<tr>
<th>Treatment modalities</th>
<th>n</th>
<th>Δ dCt miR-223</th>
<th>Δ dCt miR-16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M3-M0</td>
<td>M12-M0</td>
<td>M12-M3</td>
</tr>
<tr>
<td>MTX+glucocorticoids</td>
<td>25</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>other DMARDs+glucocorticoids</td>
<td>5</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>all DMARDs+glucocorticoids</td>
<td>30</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>glucocorticoids only</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX only</td>
<td>3</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>all DMARDs only</td>
<td>4</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>all treatment modalities</td>
<td>34</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Legend to Supplementary figures

Supplementary figure 1. Expression of circulating miR-223 in multiple aliquots of sera obtained from 2 donors that were processed in 3 different days shows a good reproducibility of the work-flow (a). Spike-in control with synthetic C. elegans miR-39 miRNA mimic (cel-39) was added during RNA isolation in 5 samples (1-2 aliquots) originating from 3 donors. The Ct of cel-39 shows comparable recovery of RNA. Along with identical Ct for miR-16 in the aliquots from 1 donor, the experiment confirms a good reproducibility of the work-flow (b). RNA dilution series resulting to linear range of the system shows no signs of inhibition by carry-over inhibitory compounds interfering with Real-Time PCR reaction (c). Comparison of expression of miR-103, miR-191, miR-423, miR-425 and let-7a in randomly selected 6 sera from patients with established rheumatoid arthritis (RA), early rheumatoid arthritis (ERA) and healthy controls (HC). Let-7a showed the lowest Ct and limited variation among samples. Therefore let-7a, a naturally occurring miRNA in sera reflecting therefore unique characteristics of samples, was selected as an endogenous normalization control.

Supplementary figure 2. Analysis of miRNA in synovial fibroblasts obtained from patients with early rheumatoid arthritis (ERA), resolving arthritis of different inflammatory origin and from patients with a knee pain of non-inflammatory origin. No differences in expression of miR-146a (a), miR-155 (b) and miR-223 (c) were observed among samples. dCt was calculated as follows: Ct (let-7a)- Ct (miRNA of interest) and therefore higher dCt values represent higher levels.