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 The blood was withdrawn in the morning after overnight starvation.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 m*M* L-glutamine, 50 IU/ml penicillin/streptomycin, 0.2% Fungizone, and 10 m*M* 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, spinned 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 preamlified 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 aliqouts 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.

	Expression Suite - Global normalization					
Follow up at:	dCt mean miR-16	ddCt miR-16	Fold change miR-16	dCt mean miR-223	ddCt miR-223	Fold change miR-223
baseline	-7,273	0	1	-11,462	0	1
3 months	-9,187	-1,914	3,768	-11,553	-0,091	1,065
12 months	-7,936	-0,663	1,583	-10,93	0,532	0,692

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.

		Δ dCt miR-223			**Δ dCt miR-16**		
Treatment modalities	**n**	M3-M0	M12-M0	M12-M3	M3-M0	M12-M0	M12-M3
MTX+glucocorticoids	25	NS	p<0.001	p<0.01	p<0.05	p<0.05	p<0.001
other DMARDs+glucocorticoids	5	NS	NS	NS	NS	NS	p<0.05
all DMARDs+glucocorticoids	**30**	NS	p<0.001	p<0.01	p<0.05	p<0.05	p<0.001
glucorticoids only	**0**						
MTX only	3	NS	NS	NS	NS	NS	NS
all DMARDs only	**4**	NS	NS	NS	NS	NS	NS
all treatment modalities	**34**						

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.