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# Circulating serum miR-223-3p and miR-16-5p as possible biomarkers of early rheumatoid arthritis

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## Summary

**Small non-coding RNAs have emerged as possible biomarkers for various diseases including autoimmune diseases. A number of studies have demonstrated that the expression of specific microRNAs (miRNAs) is dysregulated in rheumatoid arthritis (RA). So far, all studies on miRNAs in RA patients have been performed using either microarray or reverse transcription–quantitative polymerase chain reaction (RT–qPCR) analyses. Compared to RT–qPCR and microarray analyses, next-generation sequencing (NGS) allows the genome-wide analysis of small RNAs and the differentiation between miRNAs that differ by a single nucleotide. The application of NGS to the analysis of small RNAs circulating in sera of RA patients has not been reported. This study provides a global overview of the circulating small RNAs in the sera of RA patients and healthy subjects and identifies differences between these groups using NGS. Several classes of small RNAs, including hY RNA-derived fragments, tRNA-derived fragments and miRNAs, were determined. Differentially expressed individual small RNAs were verified by RT-qPCR. The levels of two miRNAs, miR-223-3p and miR-16-5p, were significantly lower in the sera from early RA patients than in those from established RA patients and healthy controls. In contrast, the serum level of miR-16-5p was higher in patients with established RA than in healthy control samples. These miRNAs may not only serve as biomarkers, but may also shed more light on the pathophysiology of RA.**

**Keywords:** circulating small RNA, miRNAs, next generation sequencing, rheumatoid arthritis

## Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder that primarily affects the synovial tissue, leading to destruction of articular cartilage and bone. The precise cause of RA is still unknown, although both genetic and environmental factors have been shown to play a role. Serological parameters, e.g. anti-citrullinated protein antibodies (ACPA), are important for diagnosing RA. Despite the excellent diagnostic value of ACPA and, albeit to a lesser extent, of rheumatoid factor, a substantial fraction of early RA (eRA) patients lack these serological markers. Moreover, these autoantibodies are not suitable to monitor treatment response. Identification of additional biomarkers for early RA diagnosis, for the prediction of disease outcome and for the monitoring of treatment response is among the most challenging issues in RA research.

Circulating nucleic acids have emerged as possible biomarkers of various diseases, including autoimmune diseases. Various types of small RNAs have been identified recently in body fluids, the best documented of which are microRNAs (miRNAs). The small RNAs differ in their size, structure, function and biogenesis. The vast majority (above 90%) of circulating miRNA is assembled in complexes with proteins that stabilize them [1,2].

A number of studies have demonstrated that expression of specific miRNAs is dysregulated in RA. The levels of

  

specific miRNA in plasma, serum, blood cells and fibroblasts showed correlations with the disease stage and activity, suggesting that they might serve as blood-based biomarkers useful for diagnostic and therapeutic applications [3–6]. Both increased and decreased levels of miRNAs have been reported in RA, and in some cases the results are inconsistent [7].

Three techniques have been used to characterize miRNAs profiles; reverse transcription–quantitative polymerease chain reaction (RT–qPCR), microarray hybridization and next-generation sequencing (NGS). All published studies on miRNAs in RA patients have been performed by either microarray hybridization or RT–qPCR. Compared to these techniques, NGS has several advantages, such as the global analysis of circulating (small) RNAs, including discovery of novel miRNAs, the ability to distinguish between miRNAs that differ by a single nucleotide (isomiRs), and it is more sensitive to rare small RNAs. Moreover, it provides absolute counts of small RNA abundance.

The objective of this study was to obtain a comprehensive overview of the abuncance of small RNAs in the sera of RA patients and healthy subjects, as well as differences between these groups.

## Patients and methods

### Patient samples

Three different sets of samples were used for this study. Set 1 consisted of four serum samples from healthy controls (CTR) and four serum samples from eRA patients and was used for NGS analysis. Set 2 consisted of 31 eRA samples, 15 established RA (estRA) samples and 30 CTR samples, and set 3 of freshly collected blood serum samples of six CTR subjects, three eRA and five estRA patients. Sets 2 and 3 were used to validate the NGS data with RT–qPCR analysis. The eRA and estRA patient sera were collected at the Erasmus Medical Center in Rotterdam and at the Radboud University Medical Center in Nijmegen. eRA patients were defined as patients who had arthritis in at least one joint with symptoms of < 1 year. Serum samples from CTR subjects were collected at the Sanquin Blood Bank in Nijmegen or at the Radboud University Medical Center. Demographic and clinical characteristics of the study participants are summarized in Supporting information, Table S1. All RA patients fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria [8] for the classification of RA. Patient sera were used in accordance with the code of conduct of research with human material in the Netherlands. All subjects gave informed consent and the study protocol was approved by local medical ethics committees.

### RNA isolation, library preparation and deep sequencing

Small RNA was isolated from sera using the miRCURY RNA isolation kit (Biofluids; Exiqon, Vedbaek, Denmark), according to the manufacturer's manual. A 100-μl aliquot of each serum sample was used for RNA extraction. The TruSeq Small RNA sample preparation kit (Illumina, San Diego, CA, USA) was used to generate sequence libraries. The barcoded small RNA-derived cDNA libraries from four eRA patients and from four CTR subjects were pooled and the quality and quantity of the cDNAs were determined by a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Sequencing of 51 cycles (single end read) was performed on a HiSeq2000 system (Illumina) using the 50 base pairs (bp) fragment sequencing protocol using a service provider (Imagif, CNRS, Paris, France).

### Bioinformatics analyses

The raw Illumina reads were first demultiplexed and fastq files were generated. Both steps were performed using CASAVA version 1.8.2 software. Adapter trimming was conducted using Cutadapt-1.3 to produce reads in FastQ format. FastQC and FASTX tools were applied to check the read quality. Sequences with quality lower than phred = 30 were discarded from the analyses. Reads shorter than 18 nucleotides (nt) and with fewer than 10 counts were also discarded. A primary alignment was performed using Bowtie 2 against the human genome (hg38). Up to two mismatches were allowed during the alignment. Reference library sequences corresponding to mature human miRNAs, tRNAs and hY RNAs were downloaded from miRBase (www.mirbase.org; release 21), the genomic tRNA database (https://gtrnadb.ucsc.edu/) and GenBank (https://www.ncbi.nlm.nih.gov/nuccore/), respectively. Raw sequencing data are available on demand.

### RT–qPCR

To validate the NGS data, the abundance of specific small RNAs in sera was analysed using the miR-Q, RT–qPCR method [9]. Small RNA was purified from sera using the miRCURY RNA isolation kit (Biofluids; Exiqon). The RevertAid M-MuLV reverse transcriptase (ThermoFisher Scientific, Fremont, CA, USA) was employed to convert miRNA into cDNA, using primers with 3′-ends complementary to the terminal six nucleotides at the 3′-end of the small RNA of interest. The primer sequences are listed in Supporting information, Table S2. Subsequently, a qPCR was performed by using GoTaq©qPCR Master Mix (Promega, Madison, WI, USA) in combination with specific forward primers containing at least 16 nucleotides corresponding to the 5′-end of the RNA of interest. All

reactions were performed in duplicate, in parallel with no-template controls. Two different methods for normalization were applied. The small RNA levels were normalized either based upon the serum volume used for RNA isolation, as equal volumes and the same isolation procedure were used for all samples, or the 5′ end of hY4 RNA fragment was used as endogenous control, as all three patient sets showed stable levels in sera. LinRegPCR software (version 2015.3) was used to determine amplification efficiency for each of the small RNA samples tested [10].

### Statistical analysis

The edgeR algorithm (www.bioconductor.org/packages/3.3/bioc/html/edgeR.html version 3.12.0) [11] was applied to detect differential expression (DE) and to perform multi-dimensional scaling (MDS) analysis. miRNAs were normalized by the trimmed mean of M-values (TMM) normalization method in edgeR. To account for multiple testing and to control for the number of false positives, we performed Benjamini–Hochberg *P*-value correction and the false discovery rate (FDR) was adjusted. Small RNAs with FDR < 0·05 and $\log_2$FC > 1 were considered as expressed differentially. Significance across more than two groups was assessed using analysis of variance (ANOVA) (Kruskal–Wallis test). All statistical calculations were performed using GraphPad Prism 5 software. All *P*-values were two-sided and *P*-values < 0·05 were considered statistically significant.

## Results

### Sample statistics and serum small RNA classification

To compare the profiles of small RNAs in sera of eRA patients and CTR, cDNA libraries from four samples of both groups (training set) were generated and sequenced using Illumina HiSeq 2000 with eight multiplexed samples in one sequencing lane. After the adapter trimming step, 6 669 112 high-quality reads were obtained for all eight libraries together; range = 0·34–2·0 million for CTR and range = 0·3–1·2 million for the eRA samples, respectively. RNAs of 18–50 nucleotides long were selected for further analyses.

An analysis of the size distribution of the small RNAs revealed two main peaks, both for total number of reads and for the reads mapped to the human genome (hg38; Supporting information, Fig. S1). The highest read counts corresponded to RNA molecules of 30–32 nucleotides in length, while the second peak represented molecules of approximately 22 nts. The proportion of reads mapped to the human genome was significantly lower for eRA samples. In total, 84% of the CTR reads (Supporting information, Fig. S1a) and 41% of the eRA reads (Supporting information, Fig. S1b) could be mapped to the human genome.

Mapping of reads revealed that the majority of the human reads corresponded to three classes of small RNAs; hY RNAs, tRNAs and miRNAs (Supporting information, Fig. S2). Most of the human 30-32 nts reads aligned to hY RNAs and tRNAs (Supporting information, Fig. S2a,b), whereas the 21–23 nts reads represented mainly miRNAs (Supporting information, Fig. S2c). The NGS results for individual samples are summarized in Fig. 1 and Supporting information, Table S3. These results show that the relative abundance of hY RNA- and tRNA-derived molecules appeared to be reversed in the eRA and CTR samples, tRNAs being more abundant in the eRA samples and hY RNA being more abundant in the CTR samples. The non-human reads, which include reads mapped to non-human databases or reads which do not match to any sequence database, showed a less size-restricted pattern, although the most abundant non-human reads corresponded to molecules of 22–23 and 50 nts (Supporting information Fig,. S2d). The latter category may represent RNA fragments longer than 50 nts or failure of adapter removal.

Multi-dimensional scaling (MDS) analysis was applied to examine the samples for outliers and their relationship. In MDS plots, dimension 1 separates the RA samples from CTR samples, while dimension 2 corresponds to patient number. MDS analysis showed that CTR and RA samples were well grouped together for reads aligned to the human genome (Supporting information, Fig. S3a) and to miRNAs (Supporting Information, Fig. S3c) and to a lesser extent for tRNA derived fragments (Supporting information, Fig. S3b). In general, the RA samples appear more heterogeneous than the CTR samples.

### hY RNA-derived fragments

The most abundant type of circulating small RNAs detected in sera of all samples was hY RNA. Y RNAs constitute a family of highly conserved small non-coding RNAs, and in human cells four distinct Y RNAs (84–112 nts), hY1, hY3, hY4 and hY5 RNA have been found [12]. Fragments of hY4 RNA were the most abundant hY RNA in all samples (Fig. 2a). Most hY RNA-derived fragments are 30–32 nts long and correspond to both 5′- and 3′-ends of the hY RNAs. However, 5′-end fragments were much more abundant in comparison with 3′-end fragments. Note that due to the size restriction of RNAs selected for sequencing (50 nts), our data do not provide information on the presence of full-length hY RNAs. A comparison of CTR and eRA samples demonstrated lower read counts for hY4 and higher read counts for hY1 and

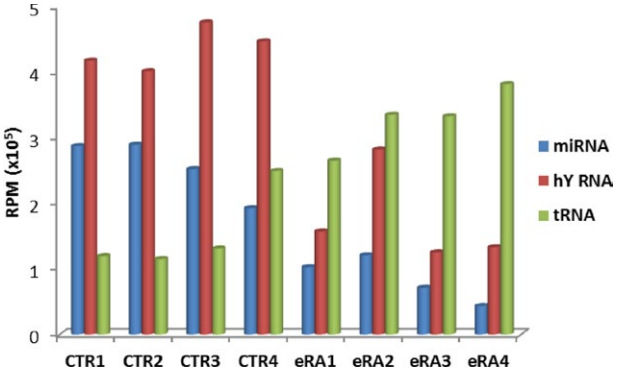


**Fig. 1.** Small RNA abundance in sera of early rheumatoid arthritis (eRA) patients and control (CTR) individuals. The number of reads for miRNAs and fragments of hY RNAs and transfer RNAs (tRNAs) relative to reads mapped to the human genome is illustrated graphically for four CTR and four eRA samples. RPM = reads per million.

hY5 RNA fragments in the eRA samples, but the differences were not significant.

### tRNA-derived fragments

The second class of abundant small RNAs in sera was tRNA, more specifically fragments derived from tRNAs (tRFs). Most of the tRFs are 29–33 nts in length and correspond to the 5′ ends of various tRNAs with cleavage sites located preferentially at the anti-codon loops. Fragments corresponding to the internal parts and the 3′-ends of tRNAs were also found, but with a much lower frequency than fragments derived from the 5′-ends. The most abundant circulating tRNA fragments were derived from tRNA-Gly-(C/G)CC (average 67%), tRNA-Val-(A/C)AC (average 13%) and tRNA-Glu-CTC (average 6·6%, Fig. 2b, Supporting information, Table S4). The raw read frequencies for each tRF did not correlate with the number of tRNA genes per isotype (data not shown).

To determine whether tRFs were expressed differentially in sera of eRA patients and CTR, edgeR differential expression analysis was applied. The results showed that there was no significant difference between the levels of tRFs in eRA and CTR sera (FRD > 0·05; Supporting information, Table S5).

### miRNA content

The third class of small RNAs for which a relatively large number of reads was obtained is miRNAs. An alignment to the miRBase database (version 21; containing 2588 human miRNAs) identified reads for 605 miRNAs throughout all samples together (Supporting information, Table S6). In each individual sample the number of miRNA reads varied from one to 426 883. Of the identified miRNAs, 82 miRNAs were detected in all samples. The 20 most abundant miRNAs identified in the eRA and CTR samples are listed in Table 1; miR-486-5p, miR-92a-3p, miR-16-5p and miR-22-3p were the most prominent. All these miRNAs are known to be expressed in blood cells, especially in erythrocytes [13–15].

A comparison of the levels of miRNAs in eRA and CTR sera by edgeR analysis demonstrated that six differentially expressed miRNAs passed the Benjamini–Hochberg correction; three were found at higher levels (miR-10a, miR-10b and miR-320a) in eRA and the other

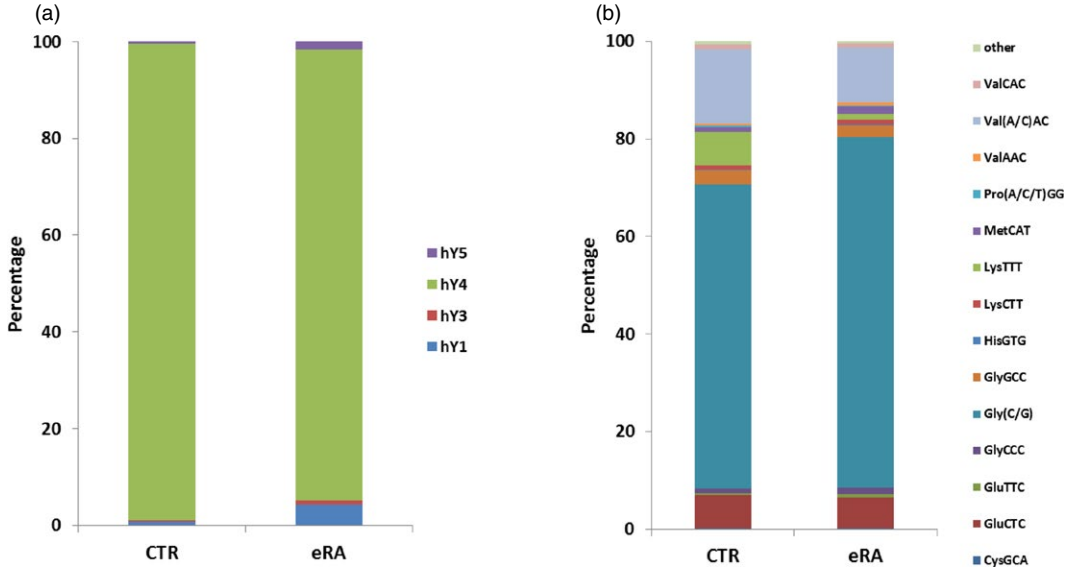


**Fig. 2.** Relative abundance of distinct hY RNA- and transfer RNA (tRNA)-derived fragments in early rheumatoid arthritis (eRA) and control (CTR) sera. The next-generation sequencing (NGS) data were used to calculate the relative abundance of fragments from individual hY RNAs (a) or tRNAs (b) in sera from eRA patients and CTR individuals. Percentage of reads aligned to each type denotes the mean value for four serum samples.

**Table 1.** Top 20 most abundant miRNA in sera of rheumatoid arthritis (RA) patients and control (CTR) individuals

| Human miRNAs | CTR sera* (%) | RA sera* (%) | Possible source of miRNA† |
|---|---|---|---|
| hsa-miR-486-5p | 54 ± 9 | 46 ± 12 | RBC [16] |
| hsa-miR-92a-3p | 14 ± 2 | 13 ± 3 | RBC, platelets, T cells, B cells, RASF [16–18] |
| hsa-miR-16-5p | 4·8 ± 2·2 | 2·1 ± 1·4 | RBC, platelets, granulocytes, leucocytes [17–18] |
| hsa-miR-22-3p | 2·3 ± 0·7 | 3·8 ± 1·5 | Not known |
| hsa-miR-451a | 2·3 ± 0·3 | 1·4 ± 0·6 | RBC [17–18] |
| hsa-miR-486-3p | 2·2 ± 0·3 | 1·3 ± 1·5 | Granulocytes [19] |
| hsa-miR-191-5p | 2·1 ± 0·4 | 2·6 ± 2·6 | Neutrophils, monocytes [20] |
| hsa-miR-26a-5p | 1·4 ± 0·7 | 2·1 ± 1·7 | Platelets, T cells, B cells [17] |
| hsa-miR-423-5p | 1·4 ± 0·8 | 1·8 ± 0·8 | Not known |
| hsa-miR-25-3p | 0·9 ± 0·1 | 0·40 ± 0·04 | RBC, granulocytes [17] |
| hsa-miR-181a-5p | 0·7 ± 0·2 | 1·0 ± 0·3 | Neutrophils, RASF [18,21] |
| hsa-miR-30e-5p | 0·7 ± 0·3 | 0·4 ± 0·1 | RASF [21] |
| hsa-miR-3184-3p | 0·6 ± 0·5 | 2·1 ± 1·5 | Not known |
| hsa-let-7f-5p | 0·6 ± 0·3 | 1·3 ± 1·4 | RBC, platelets, granulocytes [17] |
| hsa-miR-126-5p | 0·5 ± 0·2 | 1·5 ± 1·2 | Platelets, RASF [18,22] |
| hsa-miR-30d-5p | 0·5 ± 0·2 | 0·4 ± 0·2 | Not known |
| hsa-miR-223-3p | 0·5 ± 0·1 | 0·2 ± 0·2 | Granulocytes, platelets [17,23] |
| hsa-miR-10b-5p | 0·49 ± 0·07 | 2·9 ± 1·3 | RASF [18] |
| hsa-miR-423-3p | 0·4 ± 0·1 | 0·3 ± 0·1 | Not known |
| hsa-miR-10a-5p | 0·32 ± 0·05 | 1·7 ± 1·1 | Not known |

*Percentage of total number of miRNA reads.
†RBC, red blood cells; RASF, rheumatoid arthritis synovial fibroblasts.

**Table 2.** miRNAs differentially expressed in eRA patients' sera vs control (CTR) samples

| | $Log_2FC$* | $Log_2CPM$† | *P*-value | FDR‡ |
|---|---|---|---|---|
| hsa-miR-10b-5p | 2·02 | 14·01 | 6·3E–05 | 0·0011 |
| hsa-miR-10a-5p | 1·83 | 13·16 | 7·0E–05 | 0·0011 |
| hsa-miR-320a | 1·90 | 11·16 | 0·00036 | 0·0038 |
| hsa-miR-223-3p | –2·03 | 11·58 | 0·00049 | 0·0039 |
| hsa-miR-25-3p | –1·30 | 13·02 | 0·0056 | 0·03 |
| hsa-miR-16-5p | –1·48 | 15·44 | 0·023 | 0·03 |

*$Log_2FC$ is $log^2$ fold change; positive values indicate elevated and negative values reduced levels in eRA samples, respectively.
†$Log_2CPM$: $log^2$ read count per million;
‡FDR = false discovery rate.

three at reduced levels (miR-223-3p, miR-25-3p and miR-16-5p) in eRA sera in comparison with CTR samples (Table 2; $log_2FC > 1$, FDR < 0·05).

### Validation of NGS data using RT–qPCR

To validate the NGS data, the 5′-end of hY4 RNA and the six differentially expressed miRNAs were selected for analysis with independent patient (eRA, $n = 31$ and estRA, $n = 15$) and control samples ($n = 30$; set 2). The levels of these RNAs were determined by RT–qPCR, using the miR-Q approach, which has been developed for small miRNA-sized RNA molecules [9]. For four of the miRNAs (miR-10a, miR-10b, miR-320a and miR-25-3p) the Ct values appeared to be too low for reliable conclusions and thus the altered levels of these miRNAs in eRA could not be confirmed. In agreement with the NGS data, the relative levels of the 5′-end of hY4 RNA molecules did not show differences between CTR and eRA samples. The levels of miR-223-3p and miR-16-5p were found to be significantly lower in eRA sera in comparison with CTR and estRA samples (Fig. 3a), confirming our NGS data. Although miR-223-3p levels did not differ between estRA and CTR samples, the level of miR-16-5p was significantly higher in estRA compared with CTR samples ($P = 0·014$). The levels of these miRNAs did not correlate with age, disease activity score 28 in joints (DAS28) and C-reactive protein (CRP) levels, except for the ESR, which appeared to correlate with serum miR-223-3p levels in eRA ($P = 0·034$).

### Influence of pre-analytical variability on serum samples

Previous studies indicated that there is a general systematic influence of the storage conditions and/or specimen processing procedures on small RNA patterns of biofluids and that the specific effect of the storage conditions has to be verified for each small RNA separately [13–15]. To determine whether a pre-analytical step such as freezing/thawing affected the outcome of small RNA measurements in serum samples, small RNAs were isolated immediately after blood withdrawal (set 3) and the levels of hY4, miR-223-3p and miR-16-5p were determined. Similar to the observations made with the frozen samples, lower levels of miR-223-3p and miR-16-5p were observed in eRA

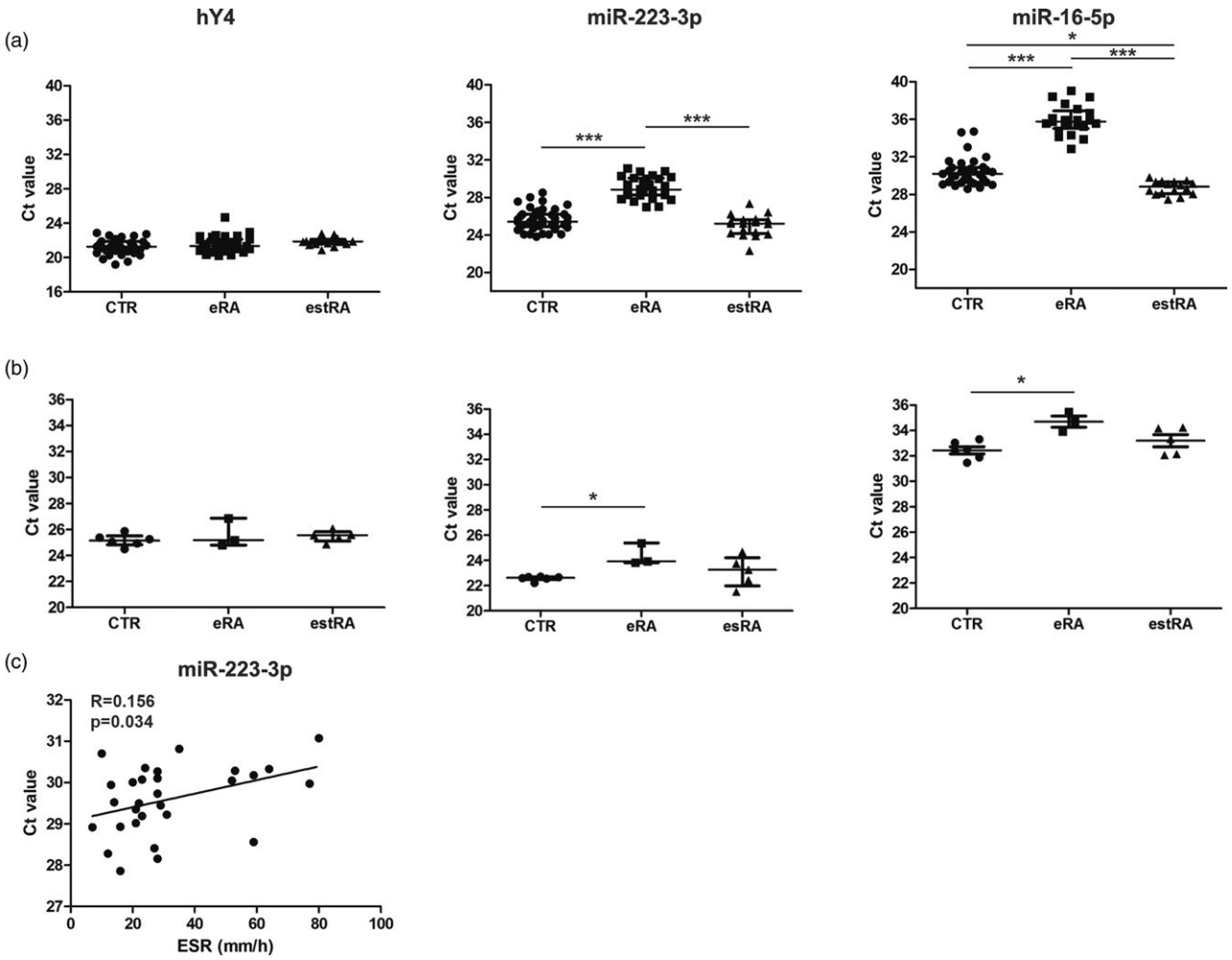


**Fig. 3.** Reverse transcription–quantitative polymerase chain reaction (RT–qPCR) analysis of the 5′-end of hY4 RNA, miR-223-3p and miR-16-5p in rheumatoid arthritis (RA) and control (CTR) sera. Small RNAs were isolated from frozen sera and the presence of the 5′-end fragment of hY4 RNA, miR-223-3p and miR-16-5p was determined by the miR-Q approach (a). miR-Q analyses of the same small RNAs in fresh sera from eRA and estRA patients and from CTR individuals (b). Spearman's correlation between serum miR-223-3p levels in eRA patients and ESR (c). Spearman's coefficient (r) is shown along with its significance (P-value). The Mann–Whitney two-sample tests were performed to examine the differences between RA and CTR (*$P < 0{\cdot}05$; ***$P < 0{\cdot}001$); n.s. = non-significant.

compared to CTR samples (Fig. 3b; $P = 0.04$ and $P = 0.06$ for miR-223-3p and miR-16-5p, respectively). Moreover, there was no significant difference for the 5′-end of hY4 RNA, miR-223-3p and miR-16-5p between CTR and estRA samples (Fig. 3b).

## Discussion

The results of this study provide a global overview of circulating small RNAs in the sera of RA patients. To our knowledge, this is the first study in which the profiles of circulating small RNAs in sera of RA patients were analysed and compared with those of healthy controls using the NGS approach. We found a high degree of diversity among the circulating small RNAs in all tested samples, although the profiles were dominated by fragments derived from hY4 RNA, tRNA-Gly, tRNA-Glu and tRNA-Val, and by miR-486-5p, which together comprised 51 and 71% of all reads mapped to hg38 for eRA and controls, respectively. The abundance of these RNAs in serum/plasma is similar to observations made in independent studies [24,25]. However, no significant differences in the levels of these most abundant small RNAs were found between eRA and healthy control samples. In contrast, for two less abundant miRNAs, miR-223-3p and miR-16-5p both NGS and RT–qPCR data revealed decreased levels in sera of eRA patients. Both miRNAs have been reported previously to be associated with RA. In one study the miR-16-5p level was decreased in the sera of naive RA patients in comparison with estRA, while

it was comparable with that in samples of healthy subjects [26]. Our data demonstrated significantly lower levels of miR-16-5p in the sera of patients with eRA in comparison with CTR and estRA samples in two separate cohorts of frozen and fresh samples. The differences in treatment could be one possible explanation for discrepancy between our results and others. In our study eRA patients in both cohorts were treated with either methotrexate (MTX), prednisone or both. Another potential explanation is the quantification method used to measure miRNA levels. It has been shown previously that quantification of serum miRNAs is influenced significantly by different normalization approaches; *let*-7a was used as a stable reference miRNA for normalization [27]. Indeed, *let-7a* is among the most stable reference genes, although Niimoto *et al.* [28] reported that *let-7a* was up-regulated significantly in the interleukin (IL)-17 producing $CD4^+$ T cells from RA patients. We have used two different approaches to normalize the data; the small RNA levels were normalized based either upon the serum volume used for RNA isolation or the 5′ end of the hY4 RNA fragment was used as endogenous control.

In another study, elevated levels of miR-16-5p were found in peripheral blood mononuclear cells from RA patients in comparison with healthy controls, and the levels correlated with active disease in RA patients [4]. The concentration of miR-16-5p in RA synovial fluid was significantly higher than that in osteoarthritis (OA) synovial fluid [29]. The differences between the biological materials analysed might, at least, in part explain the discrepancy.

Interestingly, it has been shown previously that chloramidine (Cl-amidine), a pan-protein arginine deiminase (PAD) inhibitor, up-regulates miR-16-5p expression in mice [30]. In agreement with this, Cl-amidine treatment decreased the expression of the miR-16-5p targets, such as cyclins D1, E1 and the cell proliferation marker Ki67, in epithelial cells. As an increase in PAD enzyme activity in sera of RA patients compared to controls has been reported [31], it is tempting to speculate that PAD activation during disease development leads to a decrease of miRNA-16-5p expression in blood and/or synovial fluid cells and increased cell proliferation rates, e.g. during chronic immune activation and pannus formation. It remains to be established whether variation in PAD activation affects miR-16-5p expression in blood cells or synovium from RA patients.

A number of studies also demonstrated an association of miR-223-3p with RA. Anti-tumour necrosis factor (TNF)-α/disease-modifying anti-rheumatic drug (DMARD) combination therapy of RA patients resulted in significant up-regulation of serum miRNAs, including both miR-223-3p and miR-16-5p. The changes observed in miR-223-3p and miR-16-5p expression correlated significantly with the changes observed for clinical parameters, such as DAS28, and for inflammatory parameters, such as CRP or ESR [32]. In the case of frozen samples, we observed no difference for the miR-223-3p level and a higher level of miRNA-16-5p in sera from estRA patients, who were treated with DMARDs, in comparison with CTR samples. The miR-16-5p levels were similar for estRA and CTR when fresh blood was used to isolate small RNAs. These data indicate that changes in miRNA levels should be interpreted with care if the samples of the different groups are not collected and stored by the same procedures.

miR-223-3p is expressed more highly in the synovium from of RA patients than in osteoarthritis synovium due to the increased number of miR-223-3p-positive cells [33]. Higher miR-223-3p levels were also observed in T cells from RA patients [34,35] and in microvesicles from RA synovial fluid [36]. In contrast to our results, the concentrations of miR-223-3p were reported to be increased significantly in plasma of RA patients in another study [37]. This discrepancy might be explained by the fact that we analysed serum samples. It has been shown recently that plasma samples are commonly contaminated by platelets and a single freeze/thaw cycle of plasma increases the number of platelet-derived microparticles dramatically, contaminates the extracellular miRNA pool and affects the levels of miRNA detection [38]. Serum samples become depleted of platelets during the clotting process. MiR223-3p is expressed in cells of the myeloid lineage and its levels are relatively high in polymorphonuclear neutrophils. Therefore, these cells can be an important source of circulating miR-223-3p [17]. The analysis of leucocyte/platelet markers, such as CD45 and ITGA2B, might shed more light on this possibility [22].

Although there were no significant differences between the relative quantities of the individual hY RNA and tRNA fragments, our NGS data revealed a higher proportion of reads mapped to tRNA in eRA samples and higher frequency of reads mapped to hY RNA in the CTR samples. These differences might be explained by either preferential release or higher turnover rates of certain types of circulating small RNAs. Similar differences in serum abundance of tRNA- and Y RNA-derived small RNAs have been observed in head and neck squamous cells carcinoma and breast cancer patients when compared to healthy individuals [39,40]. tRNA-derived small RNAs are involved in inhibition of apoptosis and promotion of survival of injured cells [41]. Y RNA-derived small RNAs influence DNA replication, cell proliferation, stress response and apoptosis [42]. Further studies are required to shed more light on this possibility and to reveal

potential (patho)physiological effects of the circulating fragments of these RNAs.

We also observed a higher proportion of non-human reads in eRA samples compared with healthy individuals. DNA from a wide variety of bacteria has been detected previously in the synovium and serum of RA patients and was shown to be associated with different forms of arthritis [43–45]. We detected the presence of circulating small RNAs derived from different bacterial species. Small RNAs from fungi and yeasts were also found. Systematic, prospective studies will be required to verify these data.

This study has one notable limitation. To identify small RNA biomarker candidates, we used a small sample size for the NGS analyses. As a consequence, NGS results need to be validated by an independent, less laborious method and a larger sample size. Indeed, only a fraction of the candidates identified by NGS were confirmed by RT–qPCR.

In conclusion, the results of our study suggest that circulating miR-223-3p and miR-16-5p can be used as a biomarker for eRA and provide further support for the association of miR-16-5p and miR-223-3p with RA. Previously, miR-223 was proposed as a marker of disease activity and both miR-16 and miR-223 as possible predictors of disease outcome in eRA [26]. Nevertheless, the clinical value of these potential biomarkers still needs to be established. In this respect, it is also important to note that miR-16 was linked to oncosuppression and was identified as a promising biomarker in diagnosing cancers such as gastric, breast and prostate cancers [17,22,38]. As a consequence, the interpretation of quantitative data on these molecules in a clinical setting might be somewhat complex.

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## Disclosure

The authors declare no conflicts of interest.

## Author contributions

All authors were involved in drafting of the article or revising it critically and all authors approved the final version to be published. M. D. has full access to all of the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis. Study concept and design: M. D. and G. P.; acquisition of data: M. D., J. B. and G. P.; analysis and interpretation of data: M. D., J. B., R. T. and G. P.

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## Supporting Information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Length distribution of reads mapped to hg38 and total reads of small RNA isolated from sera of control (CTR) and early rheumatoid arthritis (eRA) patients. (a) Length distribution of reads obtained from four serum samples of CTR subjects. (b) Length distribution of reads obtained from four serum samples of eRA patients. The y-axis shows the number of reads for a given small RNA length (in nucleotides, nt). Bars represent mean values. Different colours of bars denote total reads (blue) and reads which aligned to hg38 (red).

**Fig. S2.** Size distribution of different classes of small RNAs. Length distribution of reads obtained from serum samples of control (CTR) (upper graphs) and early rheumatoid arthritis (eRA) (lower graphs) samples. (a) hY RNA-derived molecules; (b) transfer RNA (tRNA)-derived molecules; (c) miRNAs; (d) non-human reads.

**Fig. S3.** Multi-dimensional scaling (MDS) plot of abundance levels of small RNAs in sera of early rheumatoid arthritis (eRA) patients and control (CTR) subjects. MDS analysis of the sequence data clusters the samples based on similarities; subjects with similar small RNA expression are represented by points close on the other. The leading log-fold-change (logFC) is the average of the largest absolute log-fold changes between each pair of RNA samples. (a) Small RNAs aligned to hg38 genome. (b) Transfer RNA (tRNA)-derived small RNA. (c) miRNAs. The filtering criteria are read counts > 10 and two mismatches.

**Table S1.** Demographic and clinical characteristics of the patients with rheumatoid arthritis and healthy subjects

**Table S2.** Complete list of primers used in the study

**Table S3.** List of sequence reads identified in sera of early rheumatoid arthritis (eRA) patients and control (CTR) subjects

**Table S4.** Percentage of different transfer RNA (tRNA) derived fragment (tRFs) in sera of rheumatoid arthritis (RA) patients and controls (CTR)

**Table S5.** The abundance of tRNA-derived fragments in sera of early rheumatoid arthritis (eRA) patients versus control (CTR) samples

**Table S6.** miRNAs identified by next-generation sequencing (NGS). miRNA raw read counts and normilised read counts (per million reas) are shown for each sample