

Digital PCR-Based Assessment of Piwi-RNA PIR35982 as a Supporting Biomarker in Rheumatoid Arthritis

Marek Cieřła¹, Dorota Darmochwał-Kolarz², Hubert Kubis¹, Bogdan Kolarz³

¹Laboratory of Diagnostic and Clinical Epigenetics, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland; ²Department of Obstetrics and Gynecology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland; ³Department of Rheumatology, Faculty of Medicine, University of Rzeszow, Rzeszow, Poland

Correspondence: Marek Cieřła, Laboratory of Diagnostic and Clinical Epigenetics, Faculty of Medicine, University of Rzeszow, Kopisto 2a St, Rzeszow, 35-959, Poland, Tel +48 178516851, Email mcieřla@ur.edu.pl

Background: Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder with an etiology that remains incompletely understood. Its development is influenced by both environmental and molecular factors, with growing attention to epigenetic mechanisms. Among these, PIWI-interacting RNAs (PIRs) have emerged as molecules of interest. PIRs are small, single-stranded noncoding RNAs, and play a crucial role in maintaining genome stability in germline cells; however, they were also found in somatic cells.

Purpose: The aim of this study was to identify PIRs whose expression may be associated with the severity of RA.

Patients and Methods: A total of 57 individuals were enrolled in the study, including 37 patients with RA and 20 healthy controls (HCs). Quantitative real-time polymerase chain reaction (qPCR) and digital polymerase chain reaction (dPCR) were used to evaluate the plasma concentrations and peripheral blood mononuclear cells (PBMCs) expression levels of selected PIRs.

Results: In PBMCs, PIR27124 showed elevated expression in RA compared to HCs ($p=0.04$). Additionally, both PIR27731 and PIR35982 showed increased expression in rheumatoid factor (RF)-negative RA patients relative to RF-positive patients (p -values 0.045 for both). In plasma, PIR35982 showed a decreased concentration in RA compared to HCs ($p=0.001$). Furthermore, both qPCR and dPCR confirmed that RF-negative RA patients had decreased plasma levels of PIR35982 compared to HCs ($p=0.01$ and $p=0.02$, respectively).

Conclusion: PIR35982 can be considered a potential molecular marker associated with RA and may be useful for identifying RF-negative patients. Moreover, dPCR represents a promising tool for the discovery and evaluation of novel biomarkers, such as PIRs.

Keywords: digital PCR, epigenetic, molecular marker, piwi-RNA, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a complex, chronic inflammatory disease of autoimmune origin, the etiology of which remains incompletely understood. The disease affects approximately 0.5% of the population worldwide.^{1,2} Disease development is a multifactorial process, involving a combination of genetic predispositions, epigenetic mechanisms, environmental factors, and dysregulation of the immune system.³ RA is characterized by destructive synovitis that leads to cartilage degradation and bone erosion. Cardinal clinical manifestations include joint pain, swelling, stiffness, and tenderness that often resulting in functional impairment, substantial morbidity, and increased risk of premature mortality.⁴ Early diagnosis and treatment are essential for effective disease management and for improving the quality of life in patients with RA.⁵ However, determining disease activity and exacerbations is important to assess the effectiveness of treatment.^{3,6}

In recent years, increasing attention has been directed toward epigenetic factors involved in the pathogenesis of RA. Epigenetic mechanisms do not alter the genetic information encoded in DNA but can reversibly modulate gene expression. Therefore, they may constitute modifiable factors influencing the course of disease.⁷ The main epigenetic mechanisms regulating gene expression include DNA methylation, modifications of histone proteins that affect chromatin structure and transcriptional activity, and the action of interfering non-coding RNAs. It is estimated that only approximately 1% of the transcriptome is

associated with protein-coding genes.^{8,9} The remaining part is noncoding RNAs, which, apart from ribosomal RNAs and transfer RNAs involved in translation, constitute a group of regulatory molecules that remain incompletely understood.¹⁰ These non-coding RNAs include microRNA (miR), small nuclear RNA, small nucleolar RNA, P-element-induced wimpy testis-interacting RNA (piwi-RNA, PIRs), as well as longer molecules including circular RNAs and long non-coding RNAs.¹¹ PIRs are a class of small, single-stranded non-coding RNA molecules, typically ranging from 26 to 32 nucleotides in length. PIRs play a crucial role in maintaining genome stability, primarily through the silencing of transposable elements in germline cells, which is essential to ensure proper gametogenesis and fertility. Although initially discovered in germline cells, PIRs have also been shown to regulate gene expression in somatic cells. Their presence has been confirmed extracellularly in exosomes and other biofluids such as plasma.¹² Additionally, PIRs have a 2'-O-methyl modification at their 3' end, which confers resistance to nuclease activity. As a result, PIRs can be considered potentially stable biomarker candidates.¹³

PIRs show limited conservation and considerable variability between species. Their maturation, unlike that of miRs and small interfering RNAs, is independent of the DICER complex and, in somatic cells, is carried out mainly according to phasing processing pathways. Approximately 90% of PIRs are encoded in piwi clusters located primarily in intragenic regions. They have the ability to bind to PIWI proteins, which are a subclass of Argonaute proteins, and form the PIR-induced silencer complex (piRISC).^{14,15} PIRs are involved in the regulation of various biological processes, including transposon silencing by transcriptional repression or post-transcriptional degradation, regulation of messenger RNA (mRNA) through mRNA decay or mRNA turnover and translation, epigenetic regulation by DNA methylation and chromatin remodeling via histone modification, as well as transgenerational inheritance.^{11,15} PIRs are associated with the pathogenesis of various diseases, including tumorigenesis and autoimmune diseases such as RA, systemic lupus erythematosus (SLE), and multiple sclerosis (MS).¹³ However, the relationship between PIRs and the development or activity of RA remains poorly understood.

PIRs were selected from the current literature.^{12,16–18} Five PIR molecules – PIR27731, PIR35982, PIR27400, PIR27124, and PIR823 – were selected as potential markers for RA. PIR35982 was reported as a potential indicator of treatment efficacy in antirheumatic drug therapy.¹⁷ Previous studies have shown that PIR27124 is upregulated in RA, and its level may be associated with disease activity.¹⁸ PIR823 showed increased expression in RA compared to osteoarthritis (OA).¹⁶ PIR27400 and PIR27731 were selected due to their high expression levels in biological samples.^{12,18}

Digital polymerase chain reaction (dPCR) enables the quantification of nucleic acid amplification products without the need for a standard curve, which is often essential in quantitative polymerase chain reaction (qPCR). This eliminates the variability and bias associated with standard curve generation and mismatches in amplification efficiency. Moreover, the dPCR exhibits greater resistance to the presence of reaction inhibitors compared to qPCR. As a result, it allows quantification of nucleic acid concentration with greater precision and reproducibility. This makes dPCR particularly well-suited for evaluating gene expression or quantifying the concentration of target molecules in extracellular materials such as plasma, serum, urine, or other biofluids, where these transcripts are often present in low copy numbers.¹⁹

The primary objective of this study was to investigate the relationship between selected PIRs and disease activity. The secondary objective was to evaluate the feasibility of using the dPCR method as a technique to assess the expression of the selected molecules.

Materials and Methods

Patients

A total of 57 individuals were included in the study, 37 RA patients, aged 57.8±7.1 years, 76% women and 20 healthy controls (HCs), aged 54.8±6.8, 75% women. The diagnosis of RA was established according to the 2010 ACR/EULAR classification criteria.⁶ The exclusion criteria were as follows: a history of other autoimmune diseases (including autoimmune thyroiditis), other inflammatory conditions, overlap with other connective tissue diseases, severe coexisting medical conditions (active infection, malignancy, severe heart failure, end-stage renal disease), or any other serious illness during hospitalization. The HC group consisted of patients hospitalized for non-inflammatory, non-autoimmune conditions. The study population consisted of consecutive patients admitted to the hospital, without stratification or selection based on age or sex.

The Disease Activity Score of 28 joints (DAS28), based on the erythrocyte sedimentation rate (ESR), was used to assess disease severity. Based on DAS28, RA patients were divided into two groups: a high disease activity group (DAS28>5.1; n=22, 38.6%) and a low disease activity group, which included both patients with low disease activity and those in remission (DAS28 ≤3.2; n=15, 26.3%), as well as HCs (n=20, 35.1%). The clinical variables included in the characteristics were obtained from medical records. Rheumatoid factor (RF; Rheumatoid Factor IgG ELISA kit, Demeditec Diagnostics, Kiel, Germany) and anti-citrullinated protein antibodies (ACPA; EIA CCP IgG, TestLine Clinical Diagnostics, Brno, Czech Republic) were evaluated by measuring serum absorbance values using enzyme-linked immunosorbent assay and BioTek 800 TS absorbance reader (Agilent, Santa Clara, CA, USA). The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethics Committee of the University of Rzeszow, (protocol number 9/11/2020). All participants provided written informed consent. Detailed characteristics of RA patients and HCs are presented in Table 1. Two 9 mL tubes of whole blood with ethylenediaminetetraacetic acid (EDTA) and one 4.9 mL serum tube were collected. One EDTA tube was centrifuged at 3600 rpm for 10 minutes at room temperature to obtain the plasma; the second was used to isolate peripheral blood mononuclear cells (PBMCs). Plasma and serum were stored at −80 °C until further analysis.

RNA Extraction

The whole blood samples were diluted with phosphate-buffered saline (PBS) in a 2:1 ratio, and then added to Gradisol reagent (Aqua-Med, Lodz, Poland). The samples were centrifuged at 2100 rpm for 20 minutes. PBMCs were collected and washed with PBS. After centrifugation, the cell pellets were dissolved in Extracol RNA (EURx, Gdansk, Poland) and frozen at −80 °C for further analysis. Total RNA from PBMCs was extracted using the DNA/RNA Extracol Kit (EURx, Gdansk, Poland), according to the manufacturer's instructions. The extracted RNA was stored at −80 °C until molecular analysis. Plasma total RNA was extracted using the NucleoSpin RNA Set for NucleoZOL (Machery-Nagel, Düren, Germany). Briefly, 200 µL of plasma was mixed with 500 µL of NucleoZOL reagent, and RNA isolation and purification were performed according to the manufacturer's instructions. The extracted RNA was stored at −80 °C.

PIRs Expression Analysis by qPCR

One hundred nanograms of RNA were reverse transcribed using the miRCURY LNA RT Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. The resulting complementary DNA (cDNA) was stored at −20 °C until further analysis. Prior to qPCR, the cDNA samples were diluted eightfold. The qPCR reaction was performed using

Table 1 Characteristics of the Subjects Included in the Study

Characteristics	RA, n=37	HCs, n=20	p-value
Age, years	57.8±7.1	54.8±6.8	0.13
Females, n (%)	28 (75.7)	15 (75)	0.79
Duration of the disease, years	11.5 [6–20]	n/a	n/a
RF positive, n (%)	27 (73)	1 (5)	<0.0001
ACPA positive, n (%)	29 (78.4)	0	<0.0001
ESR, mm/h	18 [11–41]	8.5 [6–13.5]	0.007
DAS28	5.38 [2.88–5.99]	n/a	n/a
Swollen joints	3 [0–6]	n/a	n/a
Tender joints	7 [2–14]	n/a	n/a

Notes: Data are presented as mean ± SD; median [lower – upper quartile] or number (%).

Abbreviations: ACPA, anticitrullinated protein antibodies; DAS28, disease activity score 28; ESR, erythrocyte sedimentation rate; HCs, healthy controls; n/a, not applicable; RA, patients with rheumatoid arthritis; RF, rheumatoid factor. Significant differences are bolded.

Fast SG qPCR Master Mix (EURx, Gdansk, Poland) on a QuantStudio5 real-time instrument (Thermo Fisher Scientific, CA, USA). Thermocycling conditions for cDNA obtained from PBMCs followed the manufacturer's recommendations, with 40 amplification cycles for PIR27331, PIR35982, PIR27400, and 45 cycles for PIR823 and PIR27124. The annealing/elongation step was carried out at 60°C for 30 seconds. For plasma-derived cDNA, thermocycling was also performed according to the manufacturer's instructions, with 45 amplification cycles for both PIR27331 and PIR35982, and the same annealing/elongation conditions (60 °C, 30 seconds). Each qPCR reaction was followed by melting curve analysis. All samples were evaluated in three technical replicates. Primers were designed with miRprimer2 software.²⁰ Detailed characteristics of the primers are provided in [Supplementary Table 1](#). A calibrator, prepared from 10 randomly selected samples, was used to normalize inter-plate variability. Two reference genes were used for normalization of PBMC-derived cDNA: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 small nuclear 1 (RNU6-1). Due to the lack of a validated reference gene for PIRs in plasma, miR-425-5p (miR-425), considered a stable molecule in plasma, was used for normalization. The relative quantification method was used to calculate the expression/concentration levels. Data were analyzed using QuantStudio5™ Design & Analysis Software v1.5.2 (Thermo Fisher Scientific, CA, USA), and results are presented as normalized ratio.

Absolute Quantification of PIRs from Plasma by dPCR

Based on the plasma qPCR results, PIR35982 was selected for further analysis. The analysis included 37 samples from the RA group and 14 samples from HC group. Due to limitations in RNA yield, a smaller number of HC samples were analyzed. Only samples with the highest concentration of the tested molecule, as determined by qPCR, were selected. Reverse transcription was performed as described above. dPCR (QIAcuity One, Qiagen) was used to perform PIRs absolute quantification. Reactions were carried out on 26K 24-well nanoplates (Cat. No. 250001, Qiagen, Hilden, Germany) using the QIAcuity EG PCR Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The same primer sequences were used in dPCR as in qPCR. Thermocycling conditions followed the manufacturer's protocol, with 45 amplification cycles and with an annealing / elongation step at 60°C for 30 seconds. A previously prepared calibrator sample was used to normalize interplate variability. MiR-425 was used as the reference gene. Absolute quantification was applied to calculate expression levels. Data were analyzed using the QIAcuity software suite (Qiagen, Hilden, Germany). The results are presented as: relative gene expression, direct target concentration (given as mean concentration, copies/μL), absolute copy number relative to miR-425 (given as absolute copy number) absolute copy number relative to miR-425 normalized to absolute copy number in the calibrator sample (given as absolute copy number / CAL).

Statistical Analysis

Qualitative variables are given as numbers with percentage and were evaluated using contingency tables with a χ^2 test, including Yates correction. Depending on the distribution, assessed using the Shapiro–Wilk *W*-test, quantitative values were presented as mean standard $\pm$ deviation (SD) or median with [interquartile range, IQR]. Associations between two independent groups were evaluated using the Student's *t*-test or the Mann–Whitney *U*-test. For comparisons involving more than two groups, the Kruskal–Wallis ANOVA followed by post hoc multiple comparison analysis was applied. Correlations between two continuous variables were analyzed using Spearman correlation coefficient or Pearson's linear correlation. A *p*-value <0.05 was considered statistically significant. All analyses were performed using STATISTICA Version 13 (Dell Inc. 2016).

Results

PIRs Expression in PBMCs

Of the five selected PIRs, only PIR823 demonstrated low amplification, rendering it unsuitable for accurate quantification by qPCR. Consequently, the results obtained for this target were not subjected to statistical analysis. PIR27124 showed elevated expression in RA patients compared to the HC group (median [interquartile range]); 1.79 [1.13–3.27] vs 0.94 [0.44–2.39], respectively, *p* = 0.04. There were no differences between the patients stratified by disease activity according to DAS28-ESR (high disease activity and low disease activity) and HCs. In RA patients, both PIR27731 and PIR35982

were negatively correlated with ESR ($r_s = -0.47$ and $r_s = -0.33$, respectively). Detailed correlations with clinical variables are presented in [Supplementary Table 2](#). Furthermore, both PIR27731 and PIR35982 showed increased expression in RF-negative patients compared to RF-positive patients: PIR27731 3.31 [1.22–16.56] vs 1.25 [0.64–1.84], and PIR35982 3.37 [2.3–6.34] vs 1.66 [0.99–3.37], both p-values are 0.045, respectively. These differences are presented in [Figure 1](#) and in [Supplementary Table 3](#).

However, when comparing three groups - RA patients with positive RF, RA patients with negative RF and, HCs - only PIR27124 showed elevated expression in RF-negative patients compared to HCs: 2.59 [1.79–3.27] vs 0.94 [0.44–2.39], $p=0.045$, respectively. Details are presented in [Figure 2](#) and in [Supplementary Table 4](#).

PIRs Expression in Plasma

Based on the results obtained in the PBMCs, two PIRs (PIR27731 and PIR35982) with the highest expression levels were selected for quantification in plasma. The expression level was determined as delta Ct values between the target transcript and the reference gene.

Only PIR35982 showed a decreased concentration in RA compared to HCs (0.4 [0.27–0.61] vs 0.74 [0.54–0.97], $p=0.001$). PIR27731 showed a positive correlation with the number of swollen joints (SJN; $r_s = 0.34$), Patient's Global Assessment of Disease Activity measured on a Visual Analogue Scale (VAS PGA; $r_s = 0.35$) and Physician's Global Assessment of Disease Activity measured on a Visual Analogue Scale (VAS PhGA; $r_s = 0.36$). The concentration level of PIR35982 did not show a significant correlation with the clinical variables ESR, VAS PGA, VAS PhGA, SJN, tender

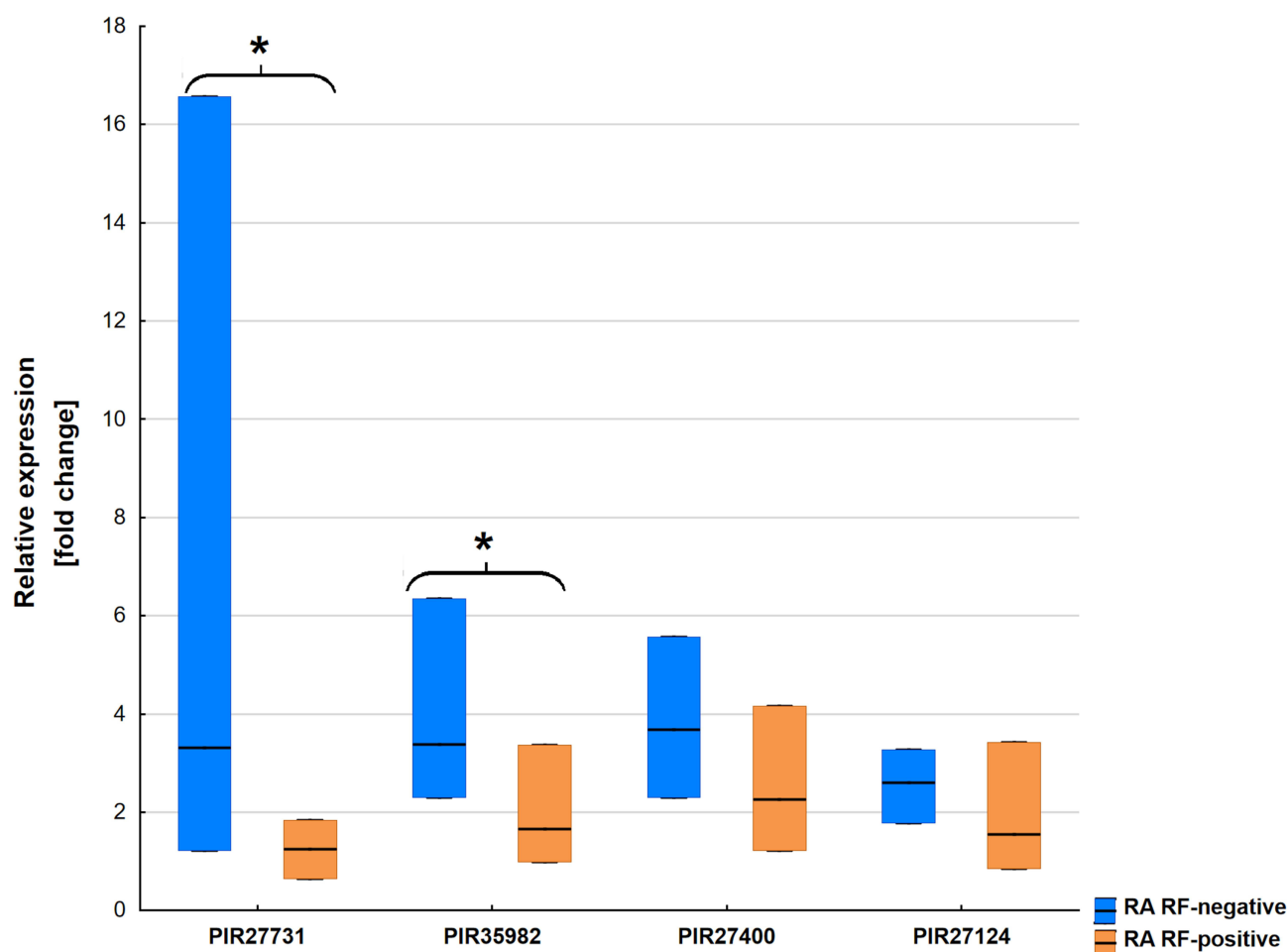


Figure 1 Differences in the expression level in peripheral blood mononuclear cells of the studied piwi-RNAs between RA patients divided according to the presence of rheumatoid factor. Boxes represent the interquartile range, and the median is indicated by a line. *P-value <0.05.

Abbreviations: RA, patients with rheumatoid arthritis; PIR, piwi-RNA; RF, rheumatoid factor.

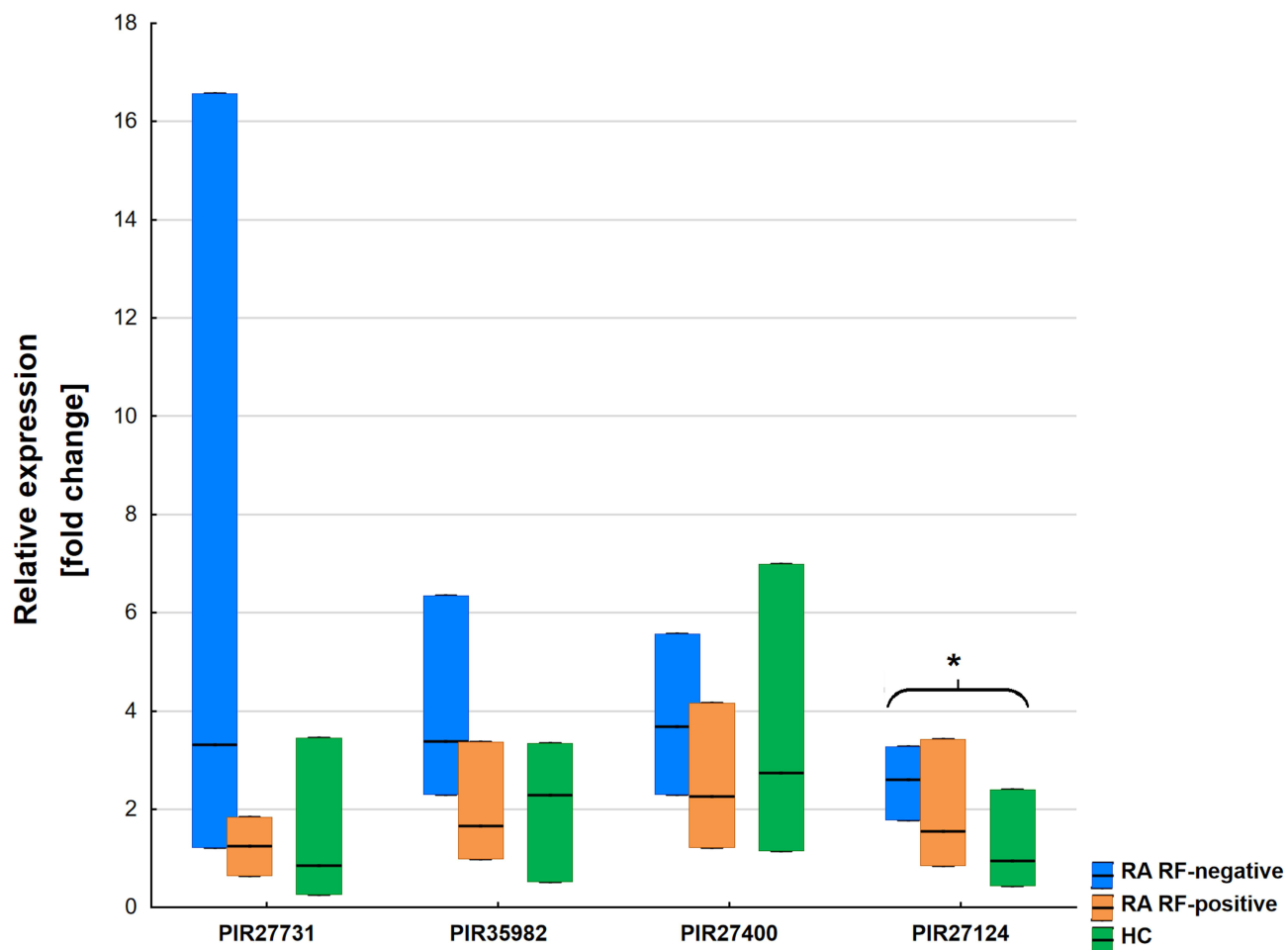


Figure 2 Differences in the expression level in peripheral blood mononuclear cells of the studied PIRs between RA patients divided according to the presence of rheumatoid factor and healthy control group. Boxes represent the interquartile range, and the median is indicated by a line. * P-value <0.05.

Abbreviations: HC, healthy control group; RA, patients with rheumatoid arthritis; PIR, piwi-RNA; RF, rheumatoid factor.

joint number (TJN), DAS28, Simplified Disease Activity Index (SDAI) and Clinical Disease Activity Index (CDAI). Detailed correlations with clinical variables are presented in [Supplementary Table 2](#). PIR27731 was decreased in patients with low disease activity compared to HCs (0.46 [0.33–0.63] vs 0.73 [0.54–1.21], $p=0.047$). PIR35982 showed decreased concentration in patients with high disease activity compared to HCs (0.36 [0.24–0.64] vs 0.74 [0.54–0.97], $p=0.006$), as well as in those with low disease activity compared to HCs (0.42 [0.33–0.58] vs 0.74 [0.54–0.97], $p=0.04$). Details are presented in [Figure 3](#). Patients with RA, when stratified by RF status did not show significant differences in the concentration of both molecules. RF-positive RA patients showed decreased PIR35982 concentration in relation to HCs (0.41 [0.28–0.64] vs 0.74 [0.54–0.97], $p=0.01$, accordingly). A similar association was observed in RF-negative RA patients compared to HCs (0.33 [0.24–0.52] vs 0.74 [0.54–0.97], $p=0.01$, accordingly).

Validation of PIR35982 Expression in Plasma by dPCR

Thirty-seven plasma samples from RA patients and 14 samples from HC group were validated using dPCR. The smaller number of samples in the HC group was due to the limited amount of material collected from the patients, as well as the primary aim of the study which was not to compare RA patients with HCs, but to validate a selected molecular marker in relation to disease severity. PIR35982 was selected for validation due to its higher mean expression in plasma samples and more pronounced differences in expression between RA patients and HCs. The analysis confirmed that PIR35982 was reduced in RA patients compared to HCs, as previously observed in qPCR; details are presented in [Table 2](#). A significant correlation was observed between the results from plasma samples using qPCR and those obtained using

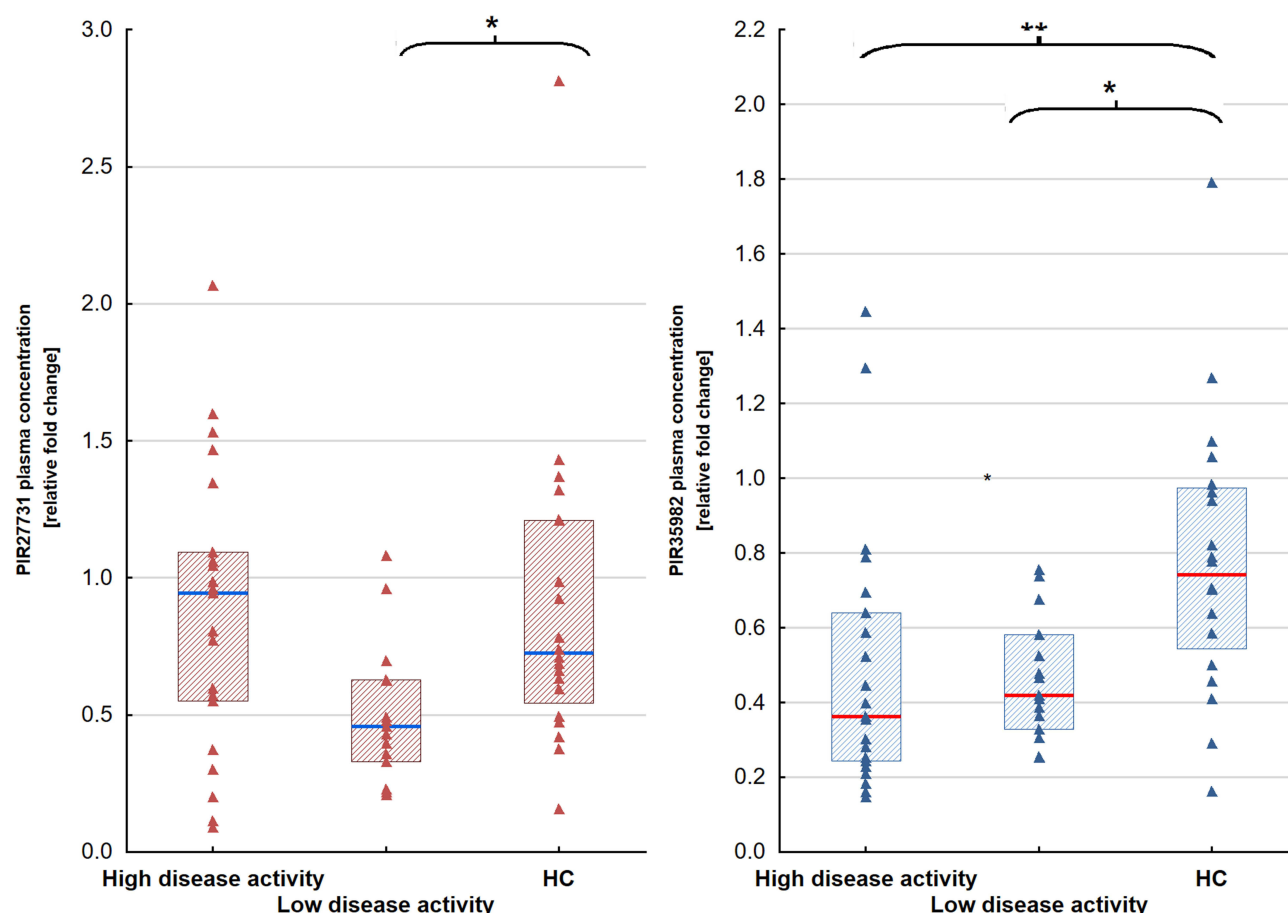


Figure 3 Plasma piwi-RNA expression in patients with rheumatoid arthritis divided by disease severity based on DAS28-ESR and healthy controls. ** P-value < 0.01; * P value < 0.05.

Abbreviations: HC, healthy controls group; PIR, piwi-RNA.

dPCR. (Tables 3 and 4). Similarly to the results obtained by qPCR, there was no significant correlation between the dPCR results and clinical variables (ESR, VAS PGA, VAS PhGA, SJN, TJN, DAS28, SDAI, and CDAI). Details are presented in [Supplementary Table 5](#).

When individuals were stratified into 3 groups – high disease activity, low disease activity, and HCs – and the concentrations of PIRs were assessed using dPCR, only the absolute copy number parameter demonstrated statistically significant differences between the groups. Patients with RA with low disease activity showed a reduced absolute copy number of PIR35982 in plasma compared to HCs, 0.39 [0.31–0.55] vs 0.68 [0.43–0.83], $p=0.028$. Details are presented in [Figure 4](#). The results related to other dPCR parameters are presented in [Supplementary Table 6](#). There were no significant

Table 2 Differences in Plasma PIR35982 Concentrations Between Patients with RA and Healthy Controls Obtained by Digital PCR

dPCR Parameter*	RA Patients, n=37	HCs, n=14	p-value
Expression of the PIR35982 dPCR gene expression [fold change]	−2.04 [−2.7] – [−1.46]	−1.33 [−1.64] – [−1.4]	0.026
PIR35982 dPCR mean concentration, [copies/μL]	1.67 [0.55–5.02]	4.47 [2.29–6.51]	0.042
Absolute copy number	0.39 [0.26–0.56]	0.68 [0.43–0.83]	0.006
PIR35982 absolute copy number / CAL	0.49 [0.33–0.67]	0.76 [0.61–1.43]	0.016

Notes: Data are given by median [interquartile range].

Abbreviations: CAL, calibrator sample; dPCR, digital polymerase chain reaction, for others, please refer to [Table 1](#). Significant p-values are in bold.

Table 3 Sperman Rank Correlation Between the Results Obtained by Quantitative PCR and Digital PCR in Patients with Rheumatoid Arthritis

PIR35982 Expression or Concentration	dPCR Gene Expression – Plasma [fold change]	dPCR Mean Concentration in Plasma, [Copies/ μ L]	Absolute Copy Number in Plasma	Absolute Copy Number / CAL in Plasma
qPCR - PBMCs expression	–0.043862	–0.112613	0.013751	0.022523
qPCR - plasma concentration	0.621573	0.282129	0.613939	0.629513

Notes: Significant correlations were bolded.

Abbreviations: PBMCs, peripheral blood mononuclear cells; for others, refer to Table 1 and Table 2.

Table 4 Sperman Rank Correlation Between the Results Obtained by Quantitative PCR and Digital PCR in Samples From All Individuals Enrolled in the dPCR Analysis (Patients with Rheumatoid Arthritis and Healthy Controls)

Expression or Concentration of PIR35982	dPCR Gene Expression in Plasma [Fold Change]	dPCR Mean Concentration in Plasma, [Copies/ μ L]	Absolute Copy Number in Plasma	Absolute Copy Number / CAL in Plasma
qPCR - PBMCs expression	0.034254	–0.156018	0.039186	0.082443
qPCR - plasma concentration	0.693800	0.294523	0.747905	0.709871

Notes: Data are presented as median [interquartile range]. Significant correlations are bolded.

Abbreviations: qPCR; quantitative polymerase chain reaction; others refer to previous tables.

differences in the level of the molecule tested between RA patients with RF-positive and RF-negative status (evaluated only among RA patient samples).

Additionally, the association between three groups was evaluated: RA with RF-positive, RA with RF-negative, and HCs. The mean plasma concentration of PIR35982 was reduced in RA patients with negative RF compared to HCs ($p=0.01$). Detailed results are presented in Table 5 and Figure 5. One measurement was excluded from the calculation due to an extreme outlier value (please refer to Figure 5).

Discussion

In this study, we demonstrated for the first time that circulating plasma PIR35982 may serve as a potential biomarker for the diagnosis of RA and could be particularly useful in identifying RF-negative patients. Additionally, the results obtained using qPCR were validated by dPCR, highlighting its significant potential as a diagnostic technology.

In this study, we evaluated the expression of PIRs in both PBMCs and plasma, however, further validation of results was conducted only in plasma samples. Whole blood and plasma collection is a noninvasive procedure, and the material is easily accessible. This allows for repeatable measurements, which is important when assessing changes in the concentration of the tested marker in the same patient in the case of repeated measurements.²¹ Plasma is more homogeneous in terms of molecular expression profile compared to whole blood, which contains several subpopulations of nucleated cells, or tissue, which may also be heterogeneous. Moreover, variations in nucleic acid concentrations within biofluids may indicate widespread physiological changes in the body. This may be useful for diseases that affect multiple organs or have systemic manifestations. On the other hand, the evaluation of gene expression in body tissues renders the expression tests locally specific and limited to the analyzed tissue. Furthermore, PIR has a 2'-O-methylation modification at the 3' end of the molecule, which enhances its resistance to degradation by nucleases.¹³

The study conducted by Freedman et al¹² showed that PIRs are frequently present in human plasma. Based on the RNA-seq results, 97 PIR molecules were selected and validated using high-throughput reverse transcription qPCR involving 2763 samples. In more than 70% of the tested samples, 9 PIR molecules were detected, and half of the participants showed the presence of at least 20 PIR molecules. This may indicate that PIR molecules exhibit considerable individual variability in expression and occurrence. Another study by Huang et al²² showed that PIRs are also present in

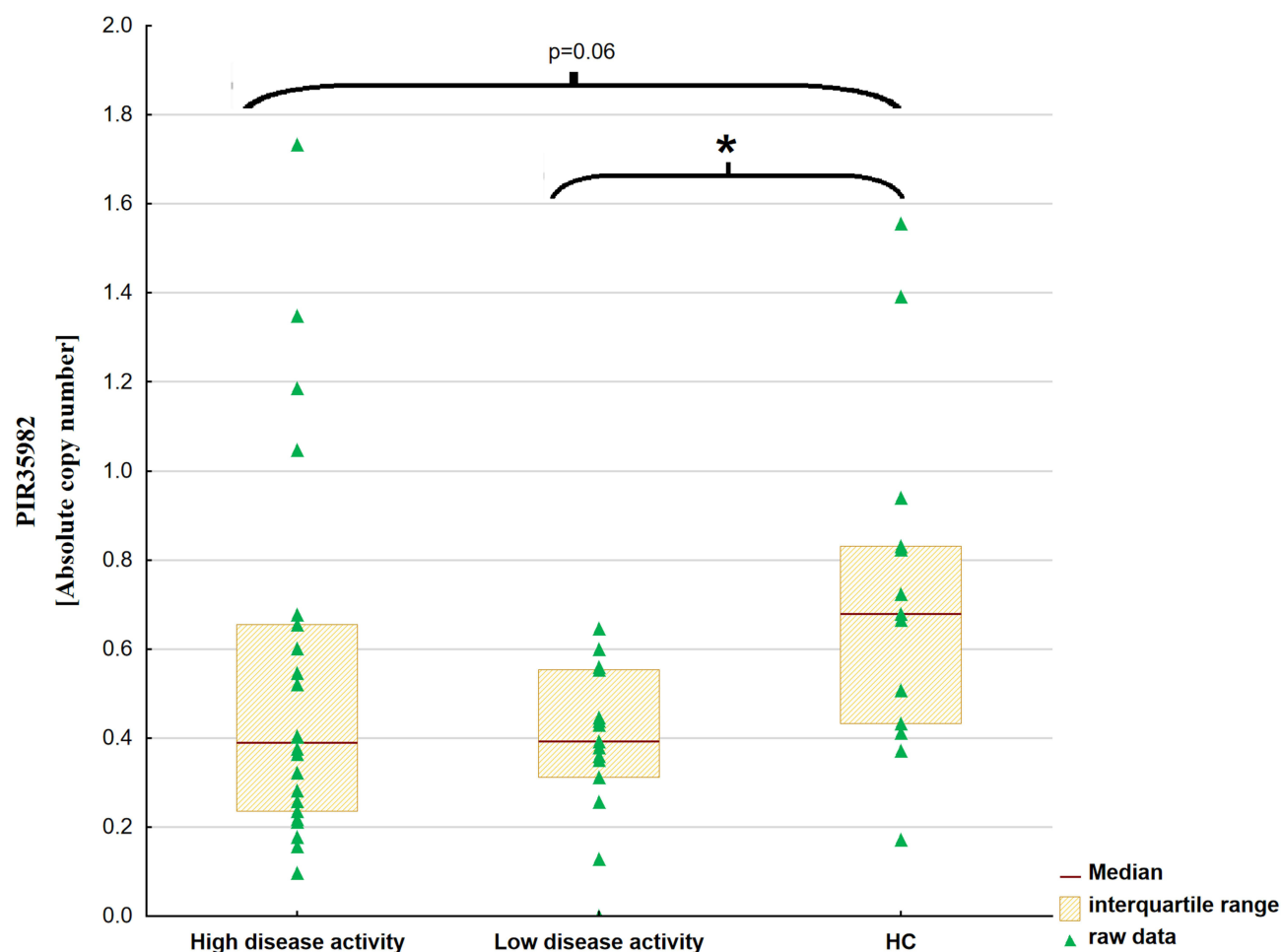


Figure 4 Plasma piwi-RNA PIR35982 concentration of digital PCR in patients with rheumatoid arthritis divided by severity of the disease according to DAS28-ESR and healthy controls. * P value <0.05.

Abbreviation: HC, healthy controls group.

human plasma-derived exosomes; however, these molecules constitute approximately 1.3% of non-coding RNA. Therefore, determining PIR levels can be a challenge, which may be addressed using the dPCR technique.

The main advantage of the dPCR technique is its ability to detect the absolute copy number, allowing it to successfully replace the relative qPCR method, which requires the use of a housekeeping gene or a set of genes. Selecting an appropriate reference gene is often tissue-specific and poses a challenge in heterogeneous tissues in terms of cell type. In the absence of suitable endogenous reference genes another method of expression normalization can be used. Spike-in control genes, commonly added during the nucleic acid extraction stage, can be implemented as a reference

Table 5 Differences in Plasma Concentrations of Piwi-RNA 35982 Obtained by Digital PCR Between Patients with Rheumatoid Arthritis Divided by Rheumatoid Factor Positivity and Healthy Controls

dPCR Parameter	RA RF Negative, n = 9	RA RF-Positive, n=27	HCs, n=14	p-value	Differences between Individual Groups
dPCR gene expression [fold change]	-2.13 [(-2.32) – (-1.78)]	-2.0491 [(-2.99) – (-1.26)]	-1.33 [-1.64–1.4]	0.06	n/a
dPCR mean concentration, [copies/ μ L]	0.91 [0.33–1.32]	2.21 [0.56–5.96]	4.47 [2.29–6.51]	0.013	RA RF-negative vs HCs; p=0.01
Absolute copy number	0.4 [0.26–0.44]	0.38 [0.24–0.6]	0.68 [0.43–0.83]	0.014	RF RF positive vs HCs, p=0.02
Absolute copy number / CAL	0.49 [0.43–0.58]	0.47[0.33–0.7]	0.76 [0.61–1.43]	0.038	RF positive for RF vs HCs, p=0.049

Notes: Data are given by median [interquartile range]. Significant p-values are in bold.

Abbreviations: dPCR, digital polymerase chain reaction; HCs, healthy controls; RA, patients with rheumatoid arthritis; RF, rheumatoid factor.

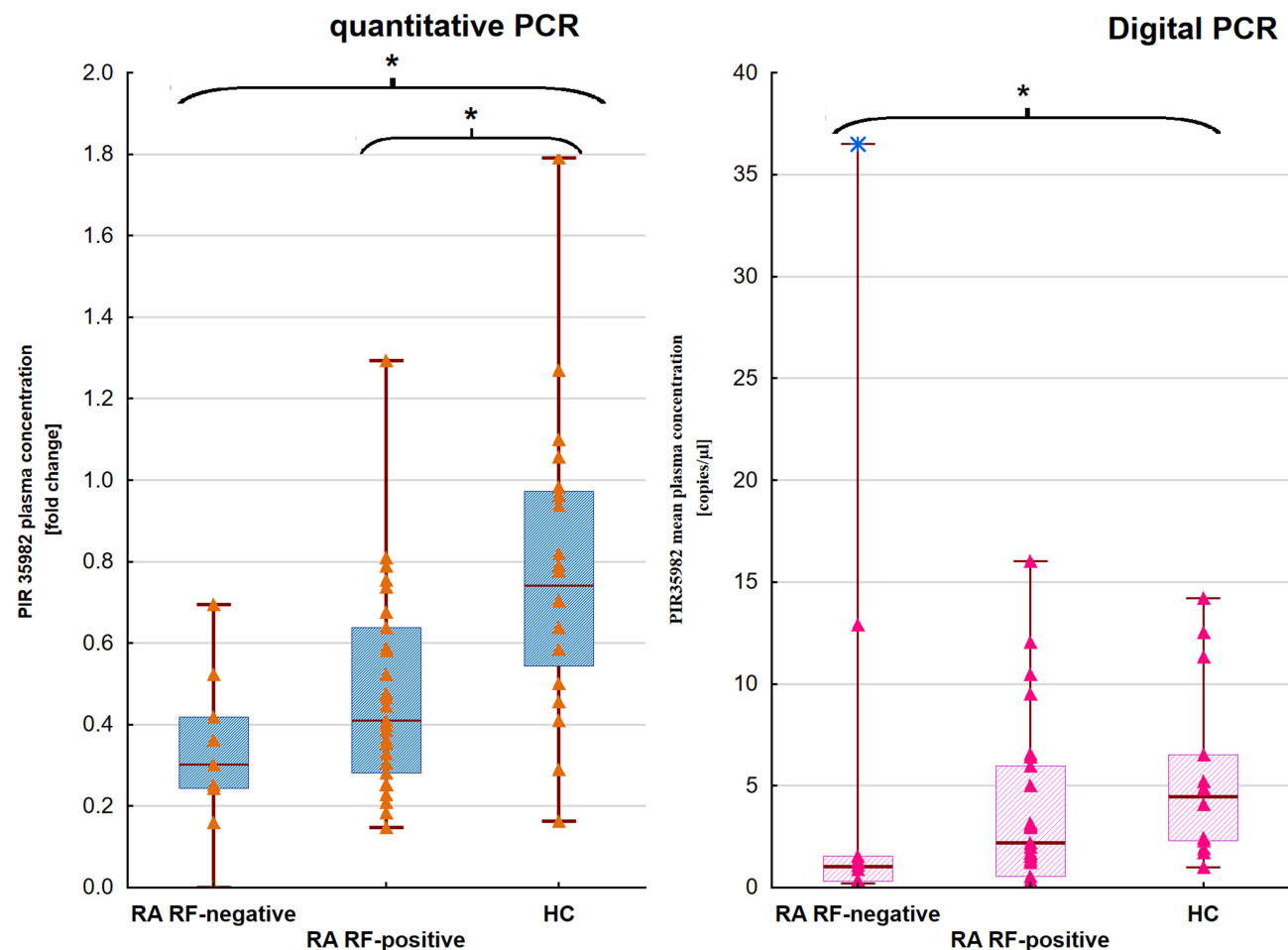


Figure 5 Differences in piwi-RNA PIR35982 plasma concentrations obtained by quantitative PCR and digital PCR. Markings on the charts: box - median - line, interquartile range - box, whisker - percentile range 1%-99%, triangles - raw data, blue asterisk - extreme data, black asterisk - significant differences. * P-value < 0.05.

Abbreviations: HC, healthy control group; RA, patients with rheumatoid arthritis; PIR, piwi-RNA; RF, rheumatoid factor.

point. A good example of the lack of consensus in the selection of an appropriate reference gene is quantification of miRs by qPCR. For instance, previous studies have shown that miR-16 is frequently used as a reference gene in plasma.²³ On the other hand, the study by Filková et al²⁴ showed that miR-16 was decreased in patients with early rheumatoid arthritis compared to HCs and increased in established rheumatoid arthritis compared to controls. The study conducted by McDonald et al²⁵ focused on the effect of preanalytical and analytical variables on circulating miRs quantification. They showed that spike-in controls used for normalization (cel-miR-39, mean of cel-miR-39 and cel-miR-54, and mean of cel-miR-39, cel-miR-54 and cel-miR-238) reduced the interassay variability of miRs expression more effectively than endogenous miR-16. Spike-in controls also have limitations, such as the lack of full representation of the physiological conditions in which a given organism or tissue exists. Another problem is the correct addition of spike-ins to tissues with high cellular complexity, as well as ensuring a consistent amount across all samples.^{26,27} Finally, the use of different endogenous reference genes makes it difficult to compare the results of the studies with each other. Implementation of the dPCR method with direct counting of the number of copies of the tested molecule, addresses this problem. We obtained a satisfactory correlation of the results from both qPCR and dPCR methods, although further research is necessary to optimize and confirm these findings.

There is limited information in the literature on PIR35982 in relation to autoimmune diseases. The study by Foers et al¹⁷ indicated that PIR35982 may be a potential biomarker of treatment efficacy for triple disease-modifying antirheumatic drug (DMARD) therapy. They showed that its serum concentration was lower in responders with early RA compared to non-responders. Furthermore, a slightly decreased concentration of PIR35982 was reported in patients with positive RF compared to

RF-negative patients. In contrast to these findings, in this study, the concentration of PIR35982 in plasma was decreased in RF-negative patients compared to RF-positive RA and HCs. Furthermore, Foers et al reported a negative correlation between PIR35982 and laboratory parameters such as C-reactive protein (CRP) and ESR. Our study did not confirm these observations. This variability between studies may be attributed to several factors. The first is the overall duration of the disease development. In our study it was longer (median over 11 years), thus well-established RA was compared to early RA (duration of disease <12 months). The second reason may be an overall disease activity, as reflected by the DAS28-ESR score. In our study, the difference was approximately 5.2 (median) versus 5.7/5.6 in responders/non-responders, respectively, in the study conducted by Fores et al. At first glance, this difference may not appear substantial; however, our study group was heterogeneous and consisted of patients with high and low disease activity, including patients in remission. The final inconsistency concerns the type of treatment. In this study, patients were treated with various types of drugs, in contrast to triple DMARD therapy. Despite these differences, it seems important that the tested molecule may play a role as a potential biomarker in RA.

From a clinical point of view, it is important to better identify seronegative RA patients, especially those who are double negative for ACPA and RF. In this study, we found that PIR35982 may be a potential diagnostic marker of RA as well as a potential molecule that can support the identification of RF-negative RA patients. We found that patients with RA have a decreased plasma concentration of PIR35982, particularly those who are RF-negative. Furthermore, these observations were confirmed in PBMCs, where the expression level in patients was also different (elevated) compared to HCs. It is not unusual to observe a negative correlation in expression between these two biological materials. Both miRs involved in the pathogenesis of RA, miR-155 and miR-132, are downregulated in plasma but overexpressed in PBMCs.²⁸ Classical serological markers, such as RF or ACPA, have limitations in identifying RA patients.^{29,30} This highlights the need for additional supportive diagnostic markers. Previous studies have demonstrated that PIRs may serve as potential biomarkers associated with the development of RA. A study by Pleřilov et al¹⁶ demonstrated the potential role of PIRs and PIWI-like proteins in the pathogenesis of RA. Using next-generation sequencing, they investigated the expression profile of short noncoding RNAs, including PIRs, and compared their expression in synovial fibroblasts between RA and OA patients. They found no significant differences in the expression of these molecules between these groups; however, they showed that some PIRs, such as PIR823, PIR4153, and PIR16659, exhibit differential regulation in RA compared to OA. An important observation regarding PIRs is that about 300 molecules have been identified in synovial fibroblasts from RA and synovial fibroblasts from OA at similar expression levels, suggesting that they are quite common and may represent a valuable source of information about the clinical condition of patients. On the other hand, a significant limitation of using PIRs as diagnostic markers may be their uneven expression and low abundance in the studied material. Pleřilov et al showed that the top four PIRs (PIR16735, PIR18570, PIR17724 and PIR20388) cover approximately 25% of all reads related to this class of molecules. This suggests that although PIR molecules are quite common in biological material, most of them have a relatively low level of expression and the use of modern molecular techniques such as dPCR may facilitate their evaluation and potentially diagnostic use. In this study we evaluated the expression level of PIR823; however, it was expressed at a very low level, and the standard qPCR technique was unable to accurately estimate its expression. The use of the more precise dPCR method could potentially resolve this issue, although further confirmation is required.

The study by Ren et al¹⁸ showed the diagnostic potential of PIRs as supportive markers derived from peripheral blood leukocytes. They showed that PIR expression in combination with classic serological markers (ACPA, RF) or inflammatory parameters (CRP), increases diagnostic precision. Specifically, they found that PIR27620 and PIR27124 were upregulated in RA compared to HCs, which we also confirmed in our study using PBMCs. These molecules were characterized by sensitivities of 79% and 81.5%, respectively, and specificities of 69% and 59.5%, respectively. These values were lower than those for ACPA (sensitivity/specificity about 0.93/1, accordingly) and RF (approximately 0.91/1) and CRP (approximately 0.94/0.74). However, the use of a combination of these two PIRs with one of the aforementioned standard laboratory parameters increased either the sensitivity or specificity in each case. Interestingly, in our study and that of Ren et al, PIR27124 levels were approximately twofold higher, despite differences in disease duration between the patient groups. In our study, the median disease duration was over 11 years, whereas in the study by Ren et al, it was over 7 months. Patients in both studies were characterized by similar disease activity. Furthermore, Ren et al noted that despite the lack of correlation with disease activity expressed in DAS28-CRP, PIR27124 showed decreased expression in patients with high disease activity and disease duration exceeding one year. In both our study and that of Ren et al, high disease activity was defined in the same way (DAS28>5.1); however, we did not observe an association

between PIR27124 expression and disease activity. To better understand the potential use of PIRs as markers of disease exacerbation these findings require further confirmation.

This study is preliminary and indicates the potential for using poorly understood PIR molecules, and should encourage further research in this area. It is difficult to find information about these molecules in the literature. Literally in single publications, their role in the pathogenesis of other autoimmune diseases, such as SLE or MS, has been indicated.¹³ Additional difficulty is the lack of available bioinformatic tools or databases that will allow us to determine the mutual interactions of these molecules and genes responsible for the pathogenesis of human diseases, especially in autoimmune diseases. Our study has several limitations. First, the small number of patients included. The results obtained in this study should be validated in a larger cohort, as well as in other autoimmune diseases such as SLE, MS, or Sjögren's syndrome, and in patients with pre-RA. Molecules included in this study were selected based on existing literature. It seems reasonable to conduct a broader screening of PIRs using high-throughput techniques such as RNA-seq.

Conclusion

In conclusion, PIR35982 may serve as a potential marker of disease activity or support the diagnosis of RA, particularly in the identification of RF-negative patients, especially when combined with absolute molecule quantification using the dPCR technique.

Disclosure

The authors report no conflicts of interest in this work. Part of the data included in this publication (analysis of PIRs expression in PBMCs using qPCR) was presented as a poster abstract with oral presentation at the XXV Congress of the Polish Rheumatology Society. The poster's abstract was published in *Reumatologia* 2024;62 (Suppl 1):74-75 (DOI: 10.5114/reum/193271).

References

1. Aletaha D, Smolen JS. Diagnosis and management of rheumatoid arthritis: a review. *JAMA*. 2018;320:1360–1372. doi:10.1001/jama.2018.13103
2. Almutairi KB, Nossent JC, Preen DB, Keen HI, Inderjeeth CA. The prevalence of rheumatoid arthritis: a systematic review of population-based studies. *J Rheumatol*. 2021;48:669–676. doi:10.3899/jrheum.200367
3. Deane KD, Holers VM. Rheumatoid arthritis pathogenesis, prediction, and prevention: an emerging paradigm shift. *Arthritis Rheumatol*. 2021;73:181–193. doi:10.1002/art.41417
4. Guo Q, Wang Y, Xu D, et al. Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. *Bone Res*. 2018;6:15. doi:10.1038/s41413-018-0016-9
5. Smolen JS, Aletaha D. Rheumatoid arthritis therapy reappraisal: strategies, opportunities and challenges. *Nat Rev Rheumatol*. 2015;11:276–289. doi:10.1038/nrrheum.2015.8
6. Aletaha D, Neogi T, Silman AJ, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum*. 2010;62:2569–2581. doi:10.1002/art.27584
7. Buch MH, Eyre S, McGonagle D. Persistent inflammatory and non-inflammatory mechanisms in refractory rheumatoid arthritis. *Nat Rev Rheumatol*. 2021;17:17–33. doi:10.1038/s41584-020-00541-7
8. Loganathan T, Doss C, P G. Non-coding RNAs in human health and disease: potential function as biomarkers and therapeutic targets. *Funct Integr Genomics*. 2023;23:33. doi:10.1007/s10142-022-00947-4
9. Richard Boland C. Non-coding RNA: it's not junk. *Dig Dis Sci*. 2017;62:1107–1109. doi:10.1007/s10620-017-4506-1
10. Poliseno L, Lanza M, Pandolfi PP. Coding, or non-coding, that is the question. *Cell Res*. 2024;34:609–629. doi:10.1038/s41422-024-00975-8
11. Taverna S, Masucci A, Cammarata G. PIWI-RNAs small noncoding RNAs with smart functions: potential theranostic applications in cancer. *Cancers*. 2023;15:3912. doi:10.3390/cancers15153912
12. Freedman JE, Gerstein M, Mick E, et al. Diverse human extracellular RNAs are widely detected in human plasma. *Nat Commun*. 2016;7.
13. Jiang M, Hong X, Gao Y, et al. piRNA associates with immune diseases. *Cell Commun Signaling*. 2024;22. doi:10.1186/s12964-024-01724-5
14. Peng JC, Lin H. Beyond transposons: the epigenetic and somatic functions of the Piwi-piRNA mechanism. *Curr Opin Cell Biol*. 2013;25:190–194. doi:10.1016/j.ceb.2013.01.010
15. Zhang Q, Zhu Y, Cao X, et al. The epigenetic regulatory mechanism of PIWI/piRNAs in human cancers. *Mol Cancer*. 2023;22. doi:10.1186/s12943-023-01749-3
16. Pleřilov L, Neidhart M, Russo G, et al. Expression and regulation of PIWI-proteins and PIWI-interacting RNAs in rheumatoid arthritis. *PLoS One*. 2016;11:e0166920. doi:10.1371/journal.pone.0166920
17. Foers AD, Garnham AL, Smyth GK, et al. Circulating small noncoding RNA biomarkers of response to triple disease-modifying antirheumatic drug therapy in white women with early rheumatoid arthritis. *J Rheumatol*. 2020;47:1746–1751. doi:10.3899/jrheum.191012
18. Ren R, Tan H, Huang Z, Wang Y, Yang B. Differential expression and correlation of immunoregulation related piRNA in rheumatoid arthritis. *Front Immunol*. 2023;14. doi:10.3389/fimmu.2023.1175924

19. Taylor SC, Laperriere G, Germain H. Droplet Digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. *Sci Rep.* 2017;7:2409. doi:10.1038/s41598-017-02217-x
20. Busk PK. A tool for design of primers for microRNA-specific quantitative RT-qPCR. *BMC Bioinf.* 2014;15. doi:10.1186/1471-2105-15-29
21. Murata K, Furu M, Yoshitomi H, et al. Comprehensive microRNA analysis identifies miR-24 and miR-125a-5p as plasma biomarkers for rheumatoid arthritis. *PLoS One.* 2013;8:e69118. doi:10.1371/journal.pone.0069118
22. Huang X, Yuan T, Tschannen M, et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics.* 2013;14(1):319. doi:10.1186/1471-2164-14-319
23. Donati S, Ciuffi S, Brandi ML. Human circulating miRNAs real-time qRT-PCR-based analysis: an overview of endogenous reference genes used for data normalization. *Int J Mol Sci.* 2019;20:4353. doi:10.3390/ijms20184353
24. Filková M, Aradi B, Šenolt L, et al. Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis. *Ann Rheum Dis.* 2014;73:1898–1904. doi:10.1136/annrheumdis-2012-202815
25. McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimmich A. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem.* 2011;57:833–840. doi:10.1373/clinchem.2010.157198
26. Bower NI, Moser RJ, Hill JR, Lehnert SA. Universal reference method for real-time PCR gene expression analysis of preimplantation embryos. *Biotechniques.* 2007;42:199–206. doi:10.2144/000112314
27. Wang Y, Zhang H, Wang Z, et al. Therapeutic effect of nerve growth factor on cerebral infarction in dogs using the hemisphere anomalous volume ratio of diffusion-weighted magnetic resonance imaging. *Neural Regen Res.* 2012;7:1873–1880. doi:10.3969/j.issn.1673-5374.2012.24.005
28. Moran-Moguel MC, Del Rio SP, Mayorquin-Galvan EE, Zavala-Cerna MG. Rheumatoid arthritis and miRNAs: a critical review through a functional view. *J Immunol Res.* 2018;2018:1–16. doi:10.1155/2018/2474529
29. Swart A, Burlingame RW, Gürtler I, Mahler M. Third generation anti-citrullinated peptide antibody assay is a sensitive marker in rheumatoid factor negative rheumatoid arthritis. *Clin Chim Acta.* 2012;414:266–272. doi:10.1016/j.cca.2012.09.015
30. Motta F, Bizzaro N, Giavarina D, et al. Rheumatoid factor isotypes in rheumatoid arthritis diagnosis and prognosis: a systematic review and meta-analysis. *RMD Open.* 2023;9:e002817. doi:10.1136/rmdopen-2022-002817

Journal of Inflammation Research

Publish your work in this journal

The Journal of Inflammation Research is an international, peer-reviewed open-access journal that welcomes laboratory and clinical findings on the molecular basis, cell biology and pharmacology of inflammation including original research, reviews, symposium reports, hypothesis formation and commentaries on: acute/chronic inflammation; mediators of inflammation; cellular processes; molecular mechanisms; pharmacology and novel anti-inflammatory drugs; clinical conditions involving inflammation. The manuscript management system is completely online and includes a very quick and fair peer-review system. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/journal-of-inflammation-research-journal>

Dovepress
Taylor & Francis Group