

Serum MicroRNA Signature as a Diagnostic and Therapeutic Marker in Patients with Psoriatic Arthritis

Sarah M. Wade¹ , Trudy McGarry¹, Siobhan C. Wade², Ursula Fearon¹, and Douglas J. Veale³

ABSTRACT. *Objective.* MicroRNA (miRNA) are small endogenous regulatory RNA molecules that have emerged as potential therapeutic targets and biomarkers in autoimmunity. Here, we investigated serum miRNA levels in patients with psoriatic arthritis (PsA) and further assessed a serum miRNA signature in therapeutic responder versus nonresponder PsA patients.

Methods. Serum samples were collected from healthy controls (HC; n = 20) and PsA patients (n = 31), and clinical demographics were obtained. To examine circulatory miRNA in serum from HC and PsA patients, a focused immunology miRNA panel was analyzed utilizing a miRNA Fireplex assay (FirePlex Bioworks Inc.). MiRNA expression was further assessed in responders versus nonresponders according to the European League Against Rheumatism response criteria.

Results. Six miRNA (miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, miR-26a-5p, and miR-21-5p) were significantly higher in PsA compared to HC (all $P < 0.05$), with high specificity and sensitivity determined by receiver-operating characteristic curve analysis. Analysis of responder versus nonresponders demonstrated higher baseline levels of miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, and miR-26a-5p were associated with therapeutic response.

Conclusion. This study identified a 6-serum microRNA signature that could be attractive candidates as non-invasive markers for PsA and may help to elucidate the disease pathogenesis.

Key Indexing Terms: autoimmune diseases, inflammation, psoriatic arthritis

Small noncoding RNA called microRNA (miRNA) that function as posttranscriptional regulators of target genes have become increasingly recognized as key regulators in the development of autoimmune disease¹. In addition to being capable of modulating numerous autoimmunity-related genes^{2,3,4}, several studies have also reported the potential utilization of circulating miRNA [serum, plasma, and peripheral blood mononuclear cells (PBMC)] as predictive noninvasive biomarkers of therapeutic response^{5,6}.

As a complex, chronic, multifaceted, and progressive auto-inflammatory disease, psoriatic arthritis (PsA) would greatly benefit from markers of early diagnosis and therapeutic intervention to effectively suppress inflammation and control disease

progression^{7,8}. The multifaceted presentation of synovial inflammation, joint destruction, enthesitis, axial disease, and skin manifestations often makes diagnosis difficult, with as many as 15–21% of patients remaining undiagnosed in dermatology clinics⁹. Diagnostic delays as short as 6 months might result in elevated bone erosion rates and significant deterioration in long-term physical function¹⁰. While the treatment of PsA has greatly improved in the last decade¹¹, up to 47% of patients show evidence of bone erosions within the first 2 years of initial diagnosis, despite the use of conventional disease-modifying agents¹².

MiRNA have been shown to be altered in the synovial tissue and PBMC of patients with PsA^{13,14}, supporting miRNA as possible diagnostic and prognostic biomarkers for PsA. However, no studies have evaluated serum miRNA profiles in early PsA patients compared to healthy controls (HC) or assessed the ability of baseline miRNA expression to stratify patient outcomes to treatment. Therefore, the present study was designed to evaluate the expression profile of circulating miRNA, specifically focusing on a defined immunology miRNA panel, in patients with PsA to identify miRNA associated with the disease and with response to therapy.

MATERIALS AND METHODS

Patient recruitment. Thirty-one patients with PsA were recruited from the outpatient clinics at the Centre for Arthritis and Rheumatic Diseases, St Vincent's University Hospital, Dublin. All patients fulfilled the Classification for Psoriatic Arthritis criteria (CASPAR) criteria for the classification of PsA¹⁵. All patients were naïve to treatment and had active inflammation in one or both knees at the time of assessment. The control

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group consisted of 20 healthy individuals who were negative for autoimmune diseases.

Baseline clinical and laboratory assessments included C-reactive protein (CRP), erythrocytes sedimentation rates (ESR), swollen joint count (SJC), and tender joint count (TJC).

A group of 31 patients with follow-up clinical data were classified as good, moderate, and nonresponders to treatment according to the European League Against Rheumatism (EULAR) response criteria as previously described^{16,17,18,19}. Assessments were made independent of treatment type [disease-modifying antirheumatic drugs (DMARD)] or time (3 mos, 6 mos, and 9 mos). Macroscopic synovitis and vascularity were quantified under direct visualization at arthroscopy using a well-established visual analog scale (VAS) 1–100 mm as previously described²⁰.

All research was performed in accordance with the Declaration of Helsinki, and approval for this study was granted by the St. Vincent's University Hospital Medical Research and Ethics Committee (RHEV7_09/16). All patients gave fully informed written consent prior to inclusion.

FirePlex serum miRNA expression analysis. Peripheral blood was collected in anticoagulant-free tubes for obtaining serum. All bloods were processed within 1 h of collection. Samples were centrifuged at 2000 g for 10 min at room temperature. Isolated serums were transferred to RNase- and DNase-free tubes and stored at –80°C until required for further analysis. Rather than performing an exploratory study examining 100 s of miRNA, we performed a focused immunology panel, designed based on a detailed analysis of published miRNA data by the Abcam Fireplex miRNA Biofluid assay (FirePlex Bioworks Inc.), as previously described²¹. Briefly, a focused panel of 68 miRNA were analyzed in 20 µL of serum by the Abcam Fireplex miRNA Biofluid assay. Target miRNA were hybridized to miRNA target sequences on hydrogel FirePlex particles (35 µL) at picomolar sensitivity and high specificity following incubation at 37°C for 60 min with shaking. Bound miRNA were labeled with fluorescent adaptor molecules (75 µL of Labelling Buffer) and incubated at room temperature for 60 min with shaking. Eluted miRNA/adaptor complexes were amplified using single-step universal primer RT amplification consisting of 27 cycles of PCR amplification followed by 6 cycles of asymmetric amplification. Amplified products were rehybridized and incubated with 75 µL of Reporting Buffer at room temperature for 15 min with shaking. Following the addition of 175 µL of Run Buffer, miRNA particles were quantitatively detected on an EMD Millipore Guava 8HT flow cytometer and analyzed by the FirePlex Analysis Workbench (FirePlex Bioworks). Red blood cell contamination was assessed by miRNA hemolysis makers: hsa-miR-451a and hsa-miR-486-5p²². Samples were also corrected for background signals determined by positive, negative, and blank control wells. The GeNorm normalization strategy was used for normalization²³. Heatmaps were generated in FirePlex Analysis Workbench and hierarchical clustering was performed with weighted and Ward linkage. Six statistically significant miRNA (Bonferroni-corrected, $P \leq 0.02$) were chosen for further analysis.

Statistical analysis. The statistical significance of intergroup differences was determined by Kruskal-Wallis 1-way ANOVA test comparing the median values among all groups. Datasets for pair-wise analysis were assessed using the D'Agostino-Pearson, and/or Shapiro-Wilk normality test to confirm Gaussian distribution, followed by *t* tests to determine statistical significance. Unsupervised 2-way hierarchical clustering algorithm based on Ward linkage and weighted linkage correlation metric was performed based on the normalized expression profiles from the top 20 of the most variable miRNA across all samples. The predicted probability of being a good, moderate, or nonresponder was used to construct receiver-operating characteristic (ROC) curves. Area under the ROC curve (AUC) with 95% CI, sensitivity, specificity, and likelihood ratios were calculated to determine the accuracy index for evaluating the predictive performance of the selected miRNA. Spearman rank correlation coefficient was used to evaluate the association between miRNA levels and clinical variables.

RESULTS

Distinct miRNA expression profile in PsA patient serum compared to HC. To evaluate the relationship of serum miRNA expression profiles in early PsA patients and HC, we profiled miRNA signatures in 31 early PsA patients naïve to treatment and 20 HC subjects (Figure 1). The baseline characteristics of the patients are provided in Supplementary Table 1 (available with the online version of this article). Using the high throughput Firefly Bioworks miRNA-profiling technology, we focused on a 68-miRNA immunology panel based on their association with immune dysfunction (Supplementary Table 2). Unsupervised hierarchical clustering of the 20 most differentially expressed miRNA between PsA and HC serum showed independent clustering of PsA patient and HC groups (Figure 2), suggesting distinct miRNA serum profiles in PsA patients compared to HC, with only 3 of the 31 PsA patients incorrectly clustered within the HC cluster. In this profile, 14 miRNA showed elevated expression, while 6 miRNA were decreased compared to HC (Supplementary Table 3). Among the identified miRNA, 6 miRNA were significantly overexpressed in PsA compared to HC and were further analyzed: miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, miR-26a-5p (all $P < 0.001$) and miR-21-5p ($P < 0.01$; Figure 3). ROC analysis was performed to determine the sensitivity and specificity of these miRNA to discriminate between PsA and HC (Figure 4). Levels of miR-130a-3p and miR-26a-5p showed strong statistical separation between PsA and controls with an AUC of 0.863 and 0.892, respectively. Multivariate analysis for the strongest performing miRNA and the complete 6 miRNA signature did not improve the predictive value as compared to the individual miRNA (Supplementary Figure 1). Further, to ensure that the differential expression of miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, miR-26a-5p, and miR-21-5p observed in PsA serum is owing to disease-specific associations, miRNA serum expression was compared to the nonspecific markers of inflammation, ESR, and CRP (Supplementary Figures 2 and 3). Among the 6 differentially expressed miRNA, only miR-130a-3p and miR-146a-5p expression levels correlated with CRP levels ($P = 0.004$, $r = 0.52$ and $P = 0.03$, $r = 0.41$, respectively), with no significant correlation observed between these differentially expressed miRNA and the ESR.

Baseline serum miRNA expression predict treatment response. Treatment response was determined according to the EULAR response criteria as previously described^{16,17,18,19}, independent of type or duration of medication. Baseline characteristics of the study population separated according to their follow-up treatment response status are presented in Supplementary Table 4 (available with the online version of this article). We found that demographic variables (age, sex), clinical scores (SJC, TJC, VAS), and markers of synovial inflammation (synovitis, vascularity) were not significantly different across good/moderate and nonresponder PsA patients at baseline. In contrast to this, we demonstrated that baseline serum expression of miR-130a-3p ($P < 0.01$), miR-221-3p, miR-146a-5p, miR-151a-5p, and

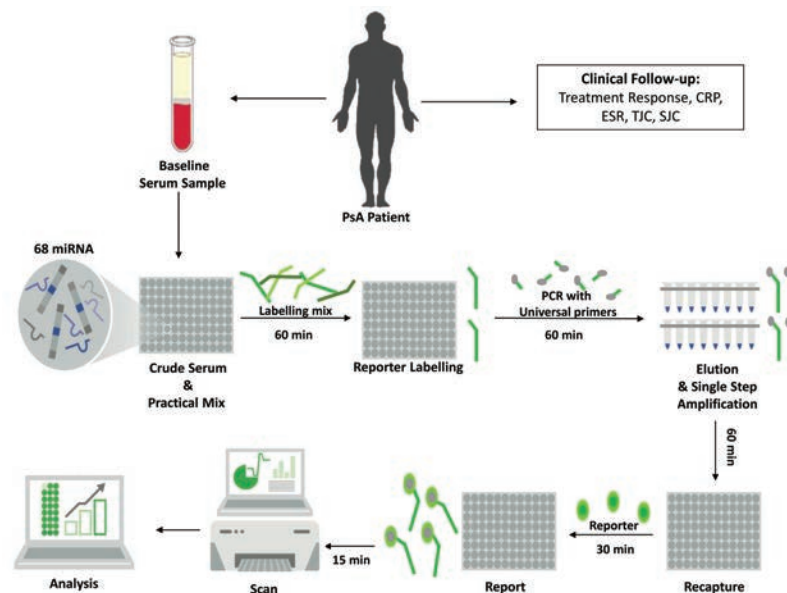


Figure 1. Process of PsA patient serum miRNA analysis. PsA patients naïve to treatment were recruited at baseline and their clinical progression followed. Fireplex Biofluid miRNA assays (FirePlex Bioworks Inc.) with 68 immune-associated miRNA were used to identify candidate diagnostic biomarkers in a quick and easy step-wise protocol. Baseline miRNA levels were retrospectively stratified to identify potential serum miRNA biomarkers of treatment response in early disease. CRP: C-reactive protein; DAS28: Disease Activity Score in 28 joints; ESR: erythrocyte sedimentation rate; miRNA: microRNA; PsA: psoriatic arthritis; SJC: swollen joint count; TJC: tender joint count. Image has been modified with permission from the FirePlex miRNA assay technical note. Abcam, Inc. hereby authorizes The Journal of Rheumatology Co. Ltd to publish the attached image [Figure 1 in Fearon publication] in *The Journal of Rheumatology*. This image may be modified by The Journal of Rheumatology Co. Ltd and used for subsequent publication upon written mutual agreement between Abcam, Inc. and The Journal of Rheumatology Co. Ltd.

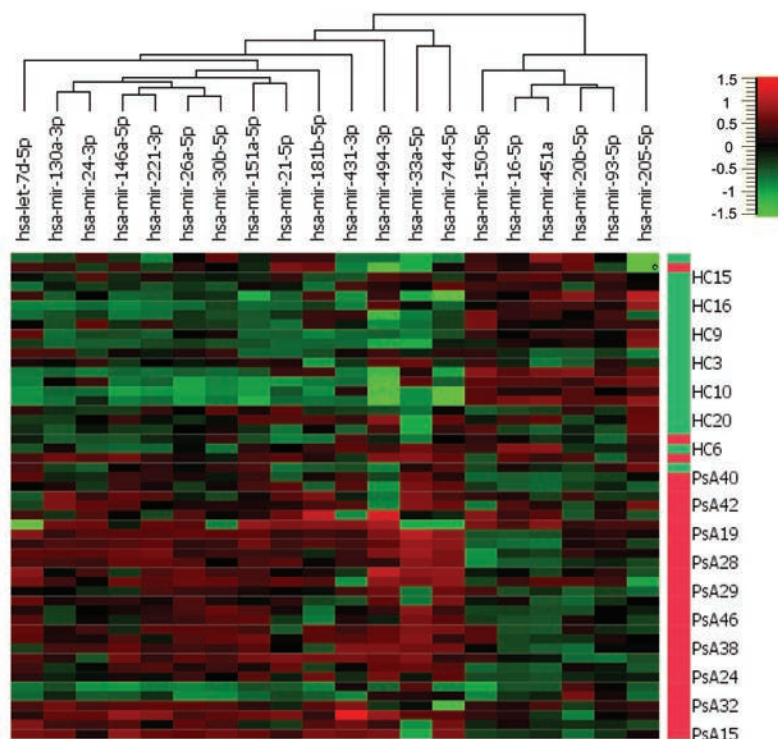


Figure 2. Hierarchical clustering analysis of miRNA expression for the top 20 differentially expressed miRNA in early PsA. Heatmap of individual serum miRNA expression in 31 patients with PsA and 20 HC. Each column represents single miRNA and each row represents individual samples. The color key indicates the degree of differential expression: green = lower expression; black = no change, and red = higher expression. Two major clusters were identified by unsupervised 2-way hierarchical miRNA (weighted linkage) and sample (Ward linkage) clustering analysis. HC: healthy controls; miRNA: microRNA; PsA: psoriatic arthritis.

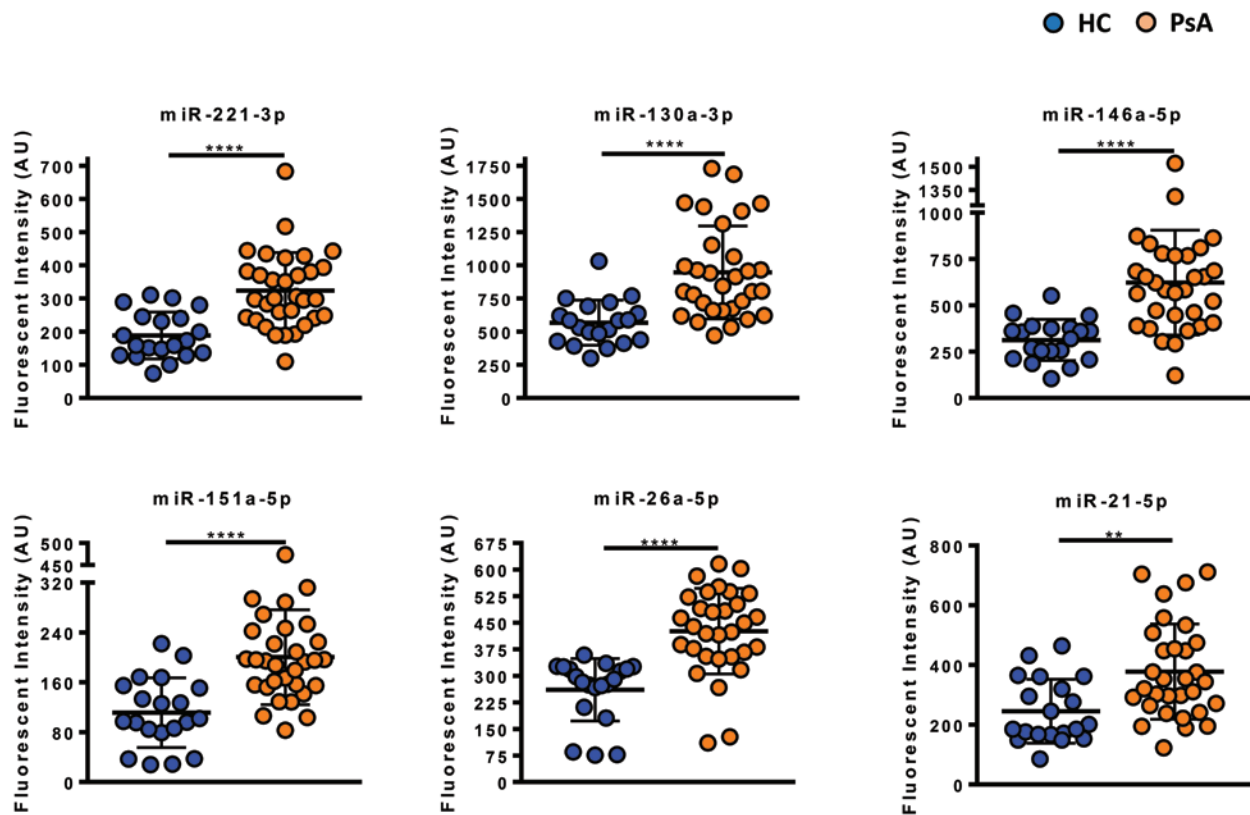


Figure 3. The expression of the 6 most differentially expressed miRNA in the serum from patients with early PsA compared to healthy controls. Relative expression of a group of 6 serum mRNA in PsA patients (n = 31) versus HC subjects (n = 20). *P* values were determined using *t* test when comparing the differences between 2 groups. **P* < 0.05, significantly different to comparator. AU: arbitrary units; HC: healthy controls; miRNA: microRNA; PsA: psoriatic arthritis.

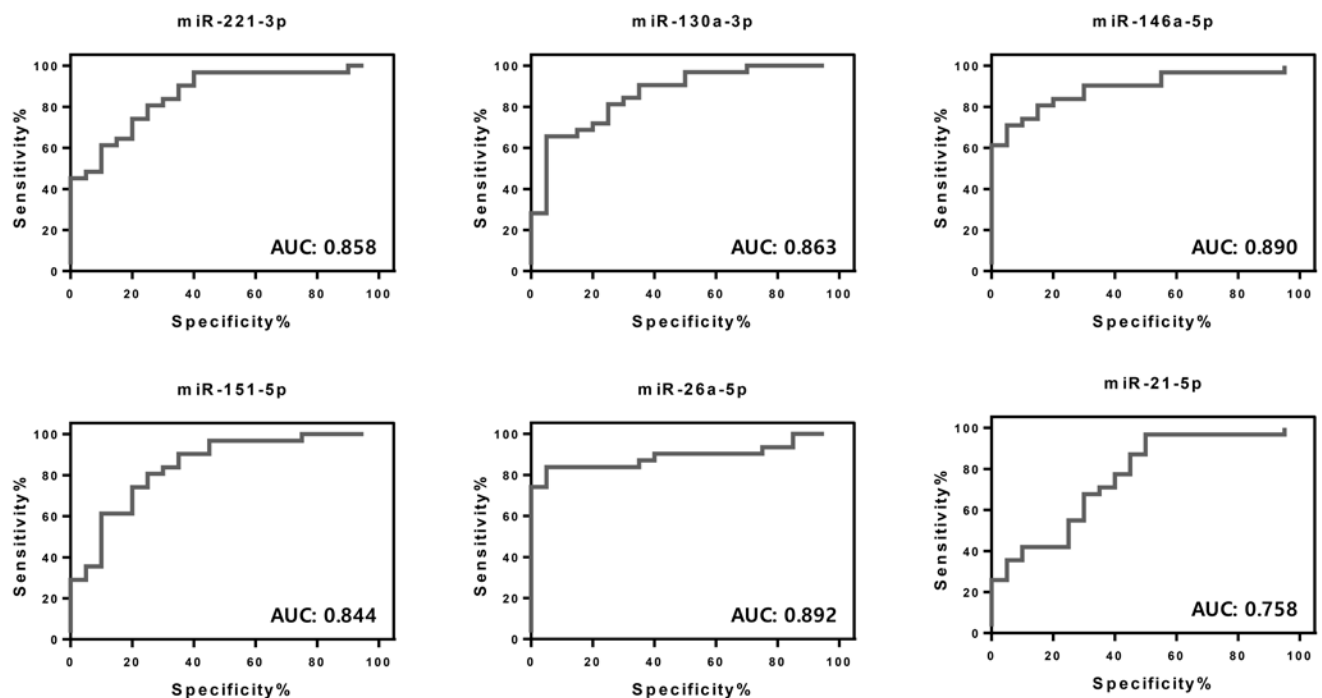


Figure 4. ROC curve analysis for PsA diagnosis. ROC curves of PsA patients (n = 31) based on miRNA expression in serum compared to healthy control subjects (n = 20). AUC was calculated with 95% CI. AUC: area under the curve; PsA: psoriatic arthritis; ROC: receiver-operating characteristic.

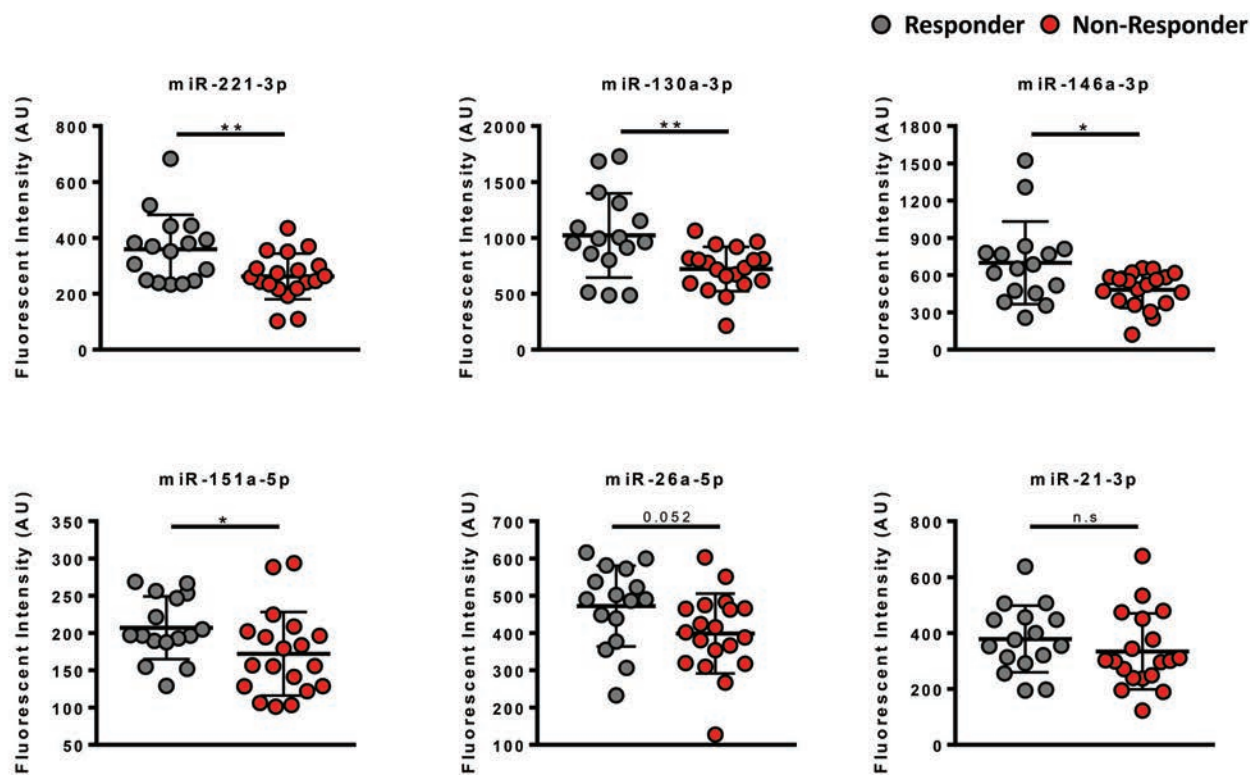


Figure 5. Relative expression of baseline serum miRNA in PsA patients with good, moderate, and nonresponse to treatment. Relative expression of a group of 6 baseline serum miRNA plotted as a function of treatment response. Nonresponding patients ($n = 19$) tended to have lower baseline serum miRNA levels than in those with a good to moderate response ($n = 16$). P values were determined using t test when comparing the differences between 2 groups. * $P < 0.05$, significantly different to comparator. AU: arbitrary units; miRNA: microRNA; PsA: psoriatic arthritis; n.s.: not significant.

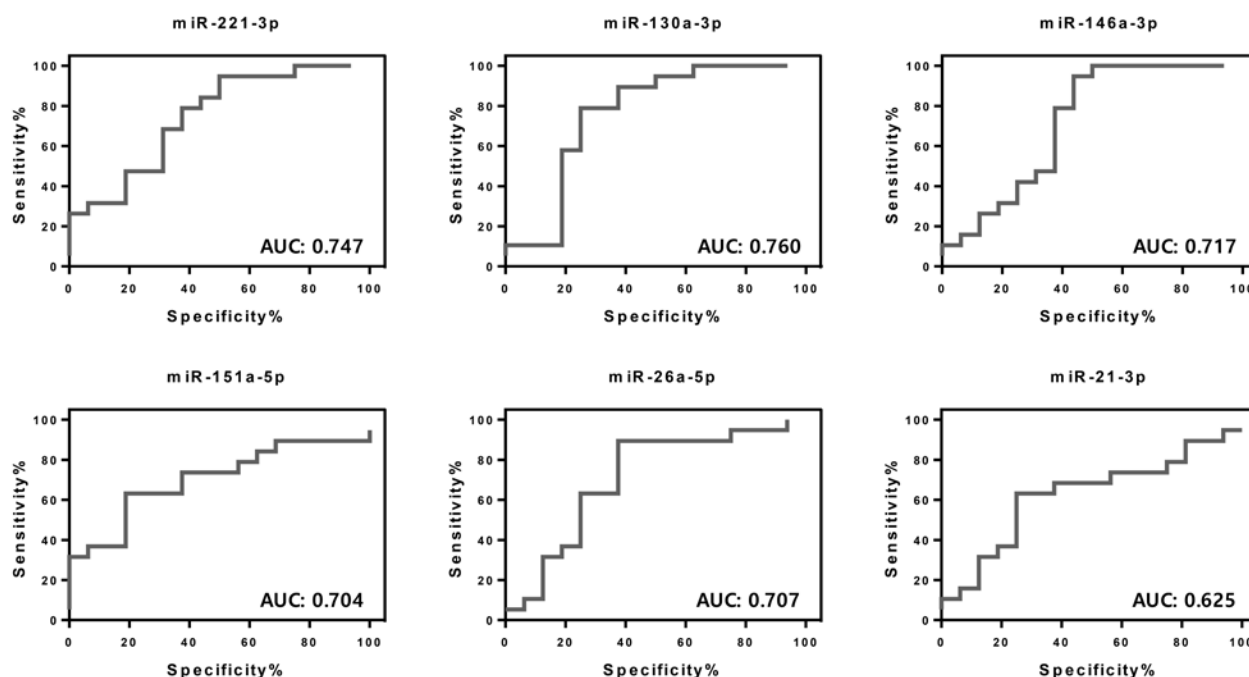


Figure 6. ROC curve analysis for baseline serum miRNA as a predictor of treatment response. AUC estimation for serum miRNA in responders ($n = 16$) compared to nonresponders ($n = 19$). AUC was calculated with 95% CI. AUC: area under the curve; ROC: receiver-operating characteristic.

miR-26a-5p (all $P < 0.05$) discriminated PsA responders and nonresponders to treatment, with a good therapeutic response associated with a higher baseline miRNA expression (Figure 5). This discrimination was found to be independent of disease duration because miRNA levels did not correlate with disease duration. Among the patients analyzed, 49% were receiving DMARD treatment while 51% were being treated with biologic agents. Further analysis demonstrated that any significant difference in baseline expression of miR-130a-3p, miR-221-3p, miR-146a-5p, miR-151a-5p, and miR-26a-5p and the response rates did not differ between the 2 treatment classes.

To further support these observations, ROC analysis was performed to evaluate the performance of baseline serum miRNA expression to discriminate patient treatment responses. The best performing miRNA for separation between good and moderate/nonresponder treatment outcomes were miR-221-3p, miR-130a-3p, and miR-146a-3p, with AUC values of 0.747, 0.760, and 0.717, respectively (Figure 6). Multivariate analysis did not improve the prognostic value of this 6-miRNA signature in distinguishing patients according to treatment response (Supplementary Figure 4, available with the online version of this article).

DISCUSSION

The identification of noninvasive, convenient, and reliable biomarkers for the early diagnosis and prediction of treatment response outcomes in autoimmune disease is an area of intensive investigation. In the present study, we identified serum-derived miRNA as potential biomarkers that can support the early diagnosis of PsA patients and retrospectively stratify patients as good/moderate and nonresponders to treatment. The levels of miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, miR-26a-5p, and miR-21-5p were differentially expressed in PsA patients when compared to HC subjects, with higher levels observed in patients with higher disease activity. Lower baseline levels of miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, and miR-26a-5p, but not miR-21-5p, were observed in PsA with a poor therapeutic response compared to good and/or moderate responders. Among them, miR-221-3p, miR-130a-3p, and miR-146a-3p exhibited the highest accuracy in the stratification of patient response.

A number of clinical trials have previously assessed circulating miRNA as biomarkers of disease diagnosis and treatment response in a number of diseases, including cancer and type 2 diabetes^{24,25,26}. More recent efforts to identify serum-based biomarkers have increased as advances in technology have occurred. In addition to their stability across repeated freeze-thaw cycles²⁷, the ability of serum miRNA to correlate with disease activity scores are also feasible. Therefore, we hypothesized that serum miRNA could be utilized as potential biomarkers of both PsA diagnosis and treatment response. In this study, we identified for the first time to our knowledge several differentially expressed serum-derived miRNA in PsA patients compared to HC subjects. Hierarchical clustering revealed a distinguished

2-cluster miRNA heatmap profile, with most of the HC subjects clustering together in cluster 1 and PsA patients separating out into cluster 2, supporting clear miRNA expression differences between PsA and control serum. By focusing on miRNA with a significant fold change of > 1.5 and previous associations with immunological functionalities, we identified 9 miRNA of special interest: miR-130a-3p, miR-146a-5p, miR-221-3p, miR-744-5p, miR-33a-5p, miR-151-5p, miR-26a-5p, miR-494-3p, and miR-21-5p. Interestingly, elevated levels of these miRNA were also found in early PsA patients with low disease activity, suggesting the potential value of these miRNA as biomarkers of early disease diagnosis. Many of these miRNA are also elevated in the circulation of patients with other inflammatory-driven autoimmune diseases, such as ulcerative colitis, rheumatoid arthritis (RA), and multiple sclerosis^{28,29,30}, but have not been previously reported as a serum miRNA biomarker for PsA diagnosis. Consistent with a previous miRNA expression profile in the plasma and peripheral blood of early PsA patients¹³, serum-based miR-21-5p was significantly increased in early PsA patients compared to HC. Previous studies have revealed that elevated miR-21-5p is involved in psoriasis-related inflammation and modulates keratinocyte proliferation, represses T cell apoptosis, and promotes angiogenesis mechanisms^{31,32,33}. A 2014 study demonstrated that inhibition of miR-21 ameliorated dermal thickness in patient-derived psoriatic skin xenografts and psoriasis models³⁴, which indicate that miR-21-5p plays a pivotal role in psoriasis development and progression. However, further experiments are required to elucidate the pathogenic mechanism of action of miR-21-5p in PsA pathogenesis. In support of the observations made in the present study, recent reports demonstrate elevated expression of miR-146a-5p in CD14+ monocytes from patients with PsA compared to both psoriasis patients and HC³⁵, and that a single-nucleotide polymorphism in the *miR-146a* gene was associated with the progression of PsA³⁶, an association that appears to be functional in patients with PsA but not RA³⁷. Further, in terms of therapeutic response, elevated serum levels of miR-130a-3p in patients with ovarian cancer have been associated with therapeutic resistance to cisplatin³⁸, which is consistent with other reports in glioblastoma patients and their response to a temazolamide treatment regimen⁶. Similarly, serum expression of miR-26a was significantly decreased in patients with psoriasis following effective treatment with the antitumor necrosis factor- α (TNF- α) agent, etanercept³⁹. While our findings are similar to these external validation studies, larger multicenter studies will be required to validate our findings. As the majority of PsA patients develop psoriasis first, future studies to evaluate the association between this 6-miRNA signature and psoriasis may also further understanding of their pathogenic role in disease progression.

Another novel aspect of this work is the utilization of the more clinically amenable FirePlex Biofluids miRNA assay. Previous protocols that were employed to detect circulating miRNA in patients with inflammatory arthritis relied on laborious isolation techniques, high sample volume to obtain sufficient miRNA,

or pooled patient samples^{13,40,41,42}. These approaches exhibit high yield variability among commercial isolation kits, require rigorous posthoc confirmation analysis, and fail to provide individual patient information⁴³. Therefore, to date, none of these approaches have led to clinical use. In contrast to this, the FirePlex platform allows for miRNA detection in just 20 µL of crude nonisolated serum that can be measured by conventional flow cytometry machines and allows for statistical analysis on individual patient miRNA levels to stratify disease groups and potentially inform clinical decisions.

Effective management in the early stages of disease will slow progression and have significant implications for improving patient quality of life, yet it remains a great challenge to identify “at-risk” or “high-priority” patients by currently available routine clinical variables. Here, we identify a set of 6 miRNA (miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, miR-26a-5p, and miR-21-5p) that distinguished good/moderate and nonresponders in early PsA, independent of treatment type or duration. Interestingly, serum miRNA better stratified patients according to treatment response compared to routine variables of disease activity. In particular, our study revealed that higher baseline levels of miR-130-3p, miR-146a-3p, and miR-221-3p were associated with more optimal treatment responses. Consistent with these observations, miR-221-3p expression has been previously associated with patient prognosis and sensitivity to therapy in cancer^{44,45}. Moreover, miR-130a-3p was reported to modulate TNF-related, apoptosis-inducing ligand sensitivity in nonsmall cell lung cancer through MET-mediated regulation of miR-221/222⁴⁶. These data support the potential ability of these miRNA to improve the identification of treatment responders in early disease. However, these findings require confirmation in larger study populations. Future studies might also analyze the usefulness of serum miRNA as pharmacodynamic biomarkers to monitor response to treatment. Further, identification of the cellular source of these circulating miRNA could offer deeper insights into the functional roles of these miRNA in the pathogenesis of PsA and treatment response.

In conclusion, serum-based expression of miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, miR-26a-5p, and miR-21-5p discriminated PsA patients with early disease from HC and successfully stratified treatment response groups from baseline levels, supporting serum miRNA as potential diagnostic and prognostic markers in PsA.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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