Serum microRNA signature as a diagnostic and therapeutic marker in patients with psoriatic arthritis

Sarah M. Wade[1,2], Trudy McGarry[1,2], Siobhan C. Wade[1], Ursula Fearon[1,2], Douglas J. Veale[2*].

[1] Molecular Rheumatology, School of Medicine, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland.

[2] The Centre for Arthritis and Rheumatic Disease, St. Vincent's University Hospital, and University College Dublin, Dublin 4, Ireland.

All correspondence to:

Prof Douglas Veale,

The Centre for Arthritis and Rheumatic Disease, St. Vincent's University Hospital, and University College Dublin, Dublin 4, Ireland.

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Email: douglas.veale@ucd.ie

Phone: 00 353 1 896 2659

Running title: Serum miRNA in PsA

Objective: MicroRNAs (miRNAs) are small endogenous regulatory RNA molecules, which have emerged as potential therapeutic targets and biomarkers in autoimmunity. Here, we investigated serum miRNA levels in Psoriatic arthritis (PsA) patients and further assessed a serum miRNA signature in therapeutic responder versus non-responder PsA patients.

Methods: Serum samples were collected from healthy controls (n=20) and PsA (n=31) and clinical demographics obtained. To examine circulatory miRNA in serum from HC and PsA patients a focussed immunology miRNA panel was analysed utilising a miRNA Fireplex assay. MiRNA expression was further assessed in responders versus non responders according to the EULAR 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 healthy controls (all $p<0.05$), with high specificity and sensitivity determined by receiver operating characteristic (ROC) curve analysis. Analysis of responder vs non-responders 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 six-serum microRNA signature that could be attractive candidates as non-invasive markers for PsA, and may help to elucidate the disease pathogenesis.

Introduction

Small non-coding RNAs called microRNA (miRNA), that function as post-transcriptional regulators of target genes, have become increasingly recognised as key regulators in the development of autoimmune disease[1]. In addition to being capable of modulating numerous auto-immunity-related genes[2–4], several studies have also reported the potential utilisation of circulating miRNA (serum, plasma and PBMC) as predicative non-invasive biomarkers of therapeutic response[5,6].

As a complex, chronic, multifaceted and progressive autoinflammatory 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 clinics[9]. Diagnostic delays as short as six months might result in elevated bone erosion rates and significant deterioration in long-term physical function[10]. While the treatment of PsA has greatly improved in recent years[11], up to 47% of patients show evidence of bone erosions within the first two years of initial diagnosis, despite the use of conventional disease modifying agents[12].

miRNAs have been shown to be altered in the synovial tissue and PBMC of PsA patients[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 miRNAs, specifically focusing on a defined immunology miRNA panel, in patients with PsA, to identify miRNAs associated with the disease and response to therapy.

Methods

Patient Recruitment

31 patients with PsA were recruited from the outpatient clinics at the Centre for Arthritis and Rheumatic Diseases, St Vincent's University Hospital. All patients fulfilled the CASPAR criteria for the classification of PsA[15]. All patients were naïve to treatment and had active inflammation in one or both knees at the time of assessment. The control 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 non-responders to treatment according to the EULAR response criteria as previously described[16–19]. Assessments were made independent of treatment type (disease-modifying anti-rheumatic drugs) or time (3 months, 6 months and 9 months). Macroscopic synovitis and vascularity were quantified under direct visualisation at arthroscopy using a well-established visual analogue scale (VAS) 1–100 mm as previously described[20].

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 hr 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 a exploratory study examining 100s of miRNA, we performed a focussed immunology panel, designed based on a detailed analysis of published miRNA data by the Abcam Fireplex miRNA Biofluid Assay (FirePlex Bioworks Inc., Cambridge, MA, USA), as previously described[21]. Briefly a focussed panel 68 miRNA were analysed in 20 µL of serum by the Abcam Fireplex miRNA Biofluid Assay (FirePlex Bioworks Inc., Cambridge, MA, USA. Target miRNA were hybridised to miRNA target sequences on hydrogel FirePlex particles (35µL) at picomolar sensitivity and high specificity following incubation at 37 °C for 60 min with shaking. Bound miRNA were labelled with fluorescent adaptor molecules (75µL 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 re-hybridised and incubated with 75µL of Reporting Buffer at room temperature for 15 min with shaking. Following the addition of 175µL of Run Buffer, miRNA particles were quantitatively detected on an EMD Millipore Guava 8HT flow cytometer and analysed by the FirePlex Analysis Workbench (FirePlex Bioworks, USA). Red blood cell contamination was assessed by miRNA haemolysis makers: hsa-miR-451a and hsa-miR-486-5p[22]. Samples were also corrected for background signals determined by positive, negative and blank control wells. The GeNorm normalisation strategy was used for normalisation[23]. Heatmaps were generated in FirePlex Analysis Workbench and hierarchical clustering was performed with weighted and Ward's linkage. Six statistically significant miRNA (Bonferroni-corrected, $p \leq 0.02$) were chosen for further analysis.

Statistical Analysis

The statistical significance of intergroup differences were determined by Kruskal-Wallis one-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 Student T-Tests to determine statistical significance. Unsupervised two-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 microRNAs across all samples. The predicted probability of being a good, moderate or non-responder was used to construct receiver operating characteristic (ROC) curves. Area under the ROC curve (AUC) with 95% confidence interval (CI), sensitivity, specificity and likelihood ratios were calculated to determine the accuracy index for evaluating the predictive performance of the selected miRNA. Spearman's rank correlation coefficient was used to evaluate the association between miRNA levels and clinical parameters.

Results

Distinct miRNA expression profile in PsA patient serum compared to healthy controls

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 (**Fig. 1**). The baseline characteristics of the patients are provided in **Supplementary Table S1.** Using the high throughput Firefly Bioworks miRNA-profiling technology, we focussed on a 68 miRNA immunology panel based on their association with immune dysfunction (**Supplementary Table S2**). Unsupervised hierarchical clustering of the 20 most differentially expressed miRNA between PsA and HC serum showed independent clustering of PsA patient and HC groups (**Fig. 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 miRNAs were

decreased compared to HC **(Supplementary Table S3)**. Among the identified miRNAs, 6 miRNAs were significantly over-expressed in PsA compared to HC and further analysed: miR-221-3p, miR-130a-3p, miR-146a-5p, miR-151-5p, miR-26a-5p (all $p<0.001$) and miR-21-5p ($p<0.01$) (**Fig. 3**). Receiver operating characteristics (ROC) analysis were performed to determine the sensitivity and specificity of these miRNA to discriminate between PsA and HC (**Fig. 4**). Levels of miR-130a-3p and miR-26a-5p showed strong statistical separation between PsA and controls with an AUC of 0.866 and 0.894, 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 S3**). Furthermore, 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 due to disease-specific associations, miRNA serum expression was compared to the non-specific markers of inflammation, ESR and CRP (**Supplementary Figure S1 and S2)**. 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 [16–19], 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**. We found that demographic variables (age, sex), clinical scores (SJC, TJC, VAS) and markers of synovial inflammation (synovitis and vascularity) were not significantly different across good/moderate and non-responder 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 miR-26a-5p (all $p<0.05$) discriminated PsA responders and non-responders to treatment, with a good therapeutic response associated with a higher baseline miRNA expression (**Fig. 5**). This discrimination was found to be independent of disease duration as miRNA levels did not correlate with disease duration. Among the patients analysed, 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 two 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/non-responder treatment outcomes were miR-221-3p, miR-130-3p and miR-146a-3p, with AUC values of 0.747, 0.760 and 0.717 respectively (**Fig. 6**). Multivariate analysis did not improve the prognostic value of this 6-miRNA signature in distinguishing patients according to treatment response (**Supplementary Figure S4**).

Discussion

The identification of non-invasive, 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 miRNAs as potential biomarkers that can support the early diagnosis of PsA patients and retrospectively stratify patients as good/moderate and non-responders 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 healthy control 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 recently assessed circulating miRNA as biomarkers of disease diagnosis and treatment response in a number of diseases, including cancer and type 2 diabetes[24–26]. Recent efforts to identify serum-based biomarkers have increased as advances in technology have occurred. In addition to their stability across repeated freeze-thaw cycles[27], the ability of serum miRNA to correlate with disease activity scores are also feasible. Therefore, we hypothesised that serum miRNA could be utilised as potential biomarkers of both PsA diagnosis and treatment response. In this study, we identified for the first time several differentially expressed serum-derived miRNA in the PsA patient compared to healthy control subjects. Hierarchical clustering revealed a distinguished two-cluster miRNA heatmap profile, with most of the healthy control 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 miRNAs 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 that the potential value of these miRNA as biomarkers of early disease diagnosis. Many of these miRNAs are also elevated in the circulation of patients with other inflammatory driven autoimmune diseases, such as ulcerative colitis, RA and multiple sclerosis[28–30], but have not been previously reported as a serum miRNA biomarker for PsA diagnosis. Consistent with a recent miRNA expression profile in the plasma and peripheral blood of early PsA patients[13], serum based miR-21-5p was significantly increased in early PsA patients compared to healthy controls. 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[31–33]. A recent study demonstrated that inhibition of miR-21 ameliorated dermal thickness in patient-derived psoriatic skin xenografts and psoriasis models[34], 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 PsO patients and HC[35], and that a single nucleotide polymorphism in the miR-146a gene was associated with the progression of PsA[36], an association that appears to be functional in PsA but not RA patients[37]. Furthermore, in terms of therapeutic response, elevated serum levels of miR-130a-3p in patients with ovarian cancer have been associated with therapeutic resistance to cisplatin[38], which is consistent with other reports in glioblastoma patients and their response to a temazolamide treatment regimen[6]. Similarly, serum expression of miR-26a was significantly decreased in patients with PsO following effective treatment with the anti-TNFα agent, etanercept[39]. While our findings are similar to these external validation studies, larger multicentre studies will be required to validate our findings. As the majority of PsA patients develop PsO first, future studies to evaluate the association between this 6-miRNA signature and PsO may also further understanding of their pathogenic role in disease progression.

Another novel aspect of this work is the utilisation of the more clinically amenable, FirePlex Biofluids miRNA Assay. Previous protocols employed to detect circulating miRNA in inflammatory arthritis patients relied on laborious isolation techniques, high sample volume to obtain sufficient miRNA or pooled patient samples[13,40–42]. These approaches exhibit high yield variability amongst commercial isolation kits, require rigorous post-hoc confirmation analysis

and fail to provide individual patient information[43]. As such, none of these approaches have led to clinical use to date. In contrast to this, the FirePlex platform allows for miRNA detection in just 20 µL of crude non-isolated 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 parameters. 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) which distinguished good/moderate and non-responders in early PsA, independent of treatment type or duration. Interestingly, serum miRNAs better stratified patients according to treatment response compared to routine parameters 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 non-small cell lung cancer through MET-meditated regulation of miR-221/222[46]. 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 analyse the usefulness of serum miRNA as pharmacodynamic biomarkers to monitor response to treatment. Furthermore, identification of the cellular source of these circulating miRNAs 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 healthy controls and successfully stratified treatment response groups from baseline levels, supporting serum miRNA as potential diagnostic and prognosistic markers in PsA.

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Author Contributions

U.F., T.M. and S.M.W. conceived study and designed experiments. D.J.V., S.C.W. and S.M.W. Patient recruitment and sample collection. S.M.W., U.F. and D.J.V. Data analysis and interpretation. S.M.W., U.F. and D.J.V. wrote the manuscript. All authors read and approved the final manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

Figure Legends

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 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. DAS28, Disease activity score; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SJC, swollen joint count; TJC, tender joint count. Image has been modified with permission from the FirePlex miRNA Assay technical note. Copyright © 2017 Abcam.

Figure 2 Hierarchical clustering analysis of miRNA expression for the top 20 differentially expressed miRNAs in early PsA. Heatmap of individual serum miRNA expression in 31 patients with PsA and 20 healthy controls. Each column represents single miRNA and each row represents individual samples. The colour key indicates the degree of differential expression: green, lower expression; black, no change and red, higher expression.

Two major clusters were identified by unsupervised two-way hierarchical miRNA (weighted linkage) and sample (ward's linkage) clustering analysis.

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 student t-test when comparing the differences between two groups. *p<0.05, significantly different to comparator.

Figure 4 Receiver operating characteristic (ROC) curve analysis for PsA diagnosis. ROC curves of PsA patients (n=31) based on miRNA expression in serum compared to HC subjects (n=20). Area under the curve (AUC) was calculated with 95% confidence limits.

Figure 5 Relative expression of baseline serum miRNAs in PsA patients with good, moderate and non-response to treatment. Relative expression of a group of 6 baseline serum miRNA plotted as a function of treatment response. Non-responding patients (n=19) tended to have lower baseline serum miRNA levels than in those with a good - moderate response (n=16). P values were determined using student t-test when comparing the differences between two groups. *p<0.05, significantly different to comparator.

Figure 6 ROC curve analysis for baseline serum miRNA as a predictor of treatment response. Area under the curve (AUC) estimation for serum miRNA in responders (n=16) compared to non-responders (n=19). Area under the curve (AUC) was calculated with 95% confidence limits.

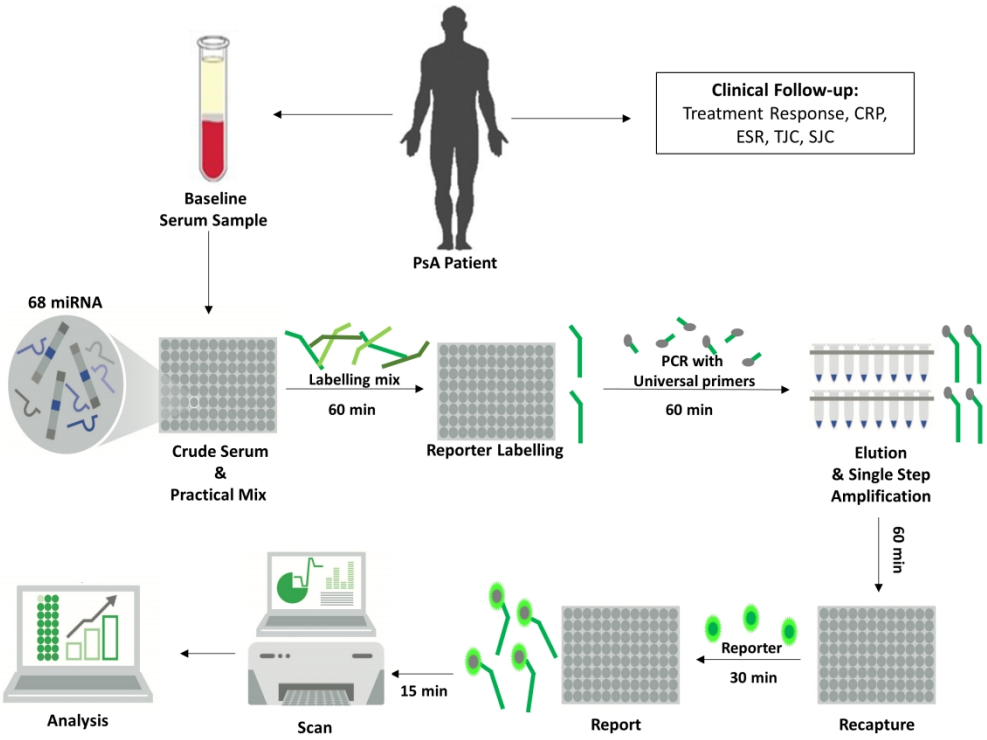

Figure 1 Process of PsA patient serum miRNA analysis. PsA patients naïve to treatment were recruited at baseline and their clinical progression followed. Fireplex Biofluid miRNA assays with 68 immune associated miRNA were used to identify candidate diagnostic biomarkers in a quick and easy step-wise protocol. Baseline miRNA levels were retrospectively stratified to identify potential serum miRNA biomarkers of treatment response in early disease. DAS28, Disease activity score; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SJC, swollen joint count; TJC, tender joint count. Image has been modified with permission from the FirePlex miRNA Assay technical note. Copyright © 2017 Abcam.

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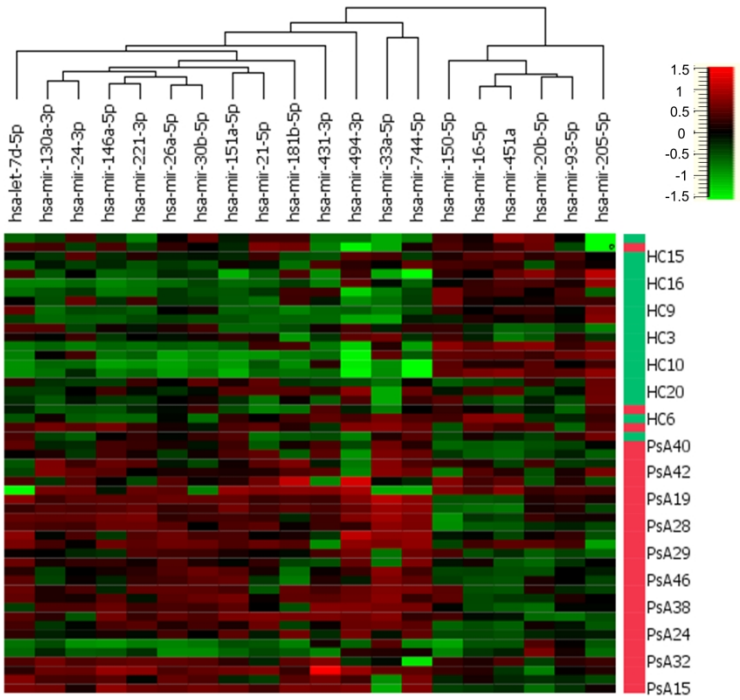

Figure 2 Hierarchical clustering analysis of miRNA expression for the top 20 differentially expressed miRNAs in early PsA. Heatmap of individual serum miRNA expression in 31 patients with PsA and 20 healthy controls. Each column represents single miRNA and each row represents individual samples. The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

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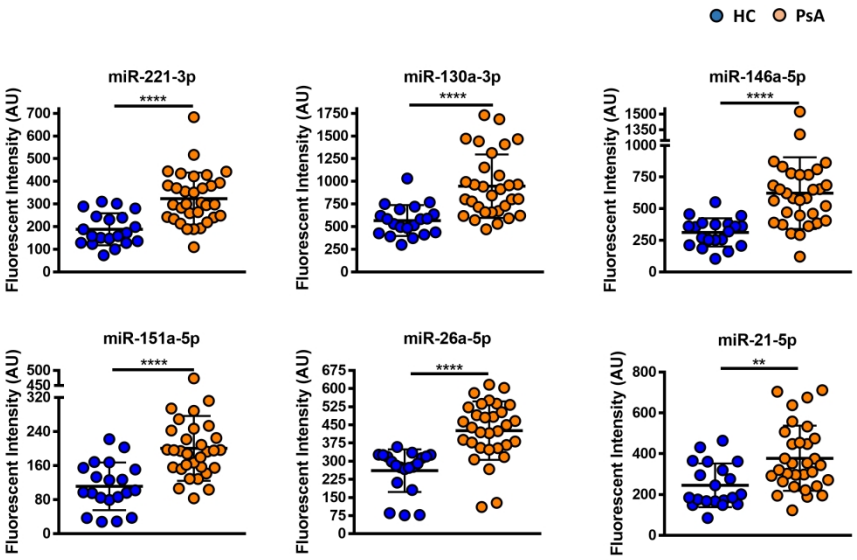

Figure 3 The expression of the 6 most differentially expressed miRNA in the serum from patients with early PsA compared to healthy controls. Relative expression of a group of 6 serum mRNA in PsA patients (n-=31) versus HC subjects (n=20). P values were determined using student t-test when comparing the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

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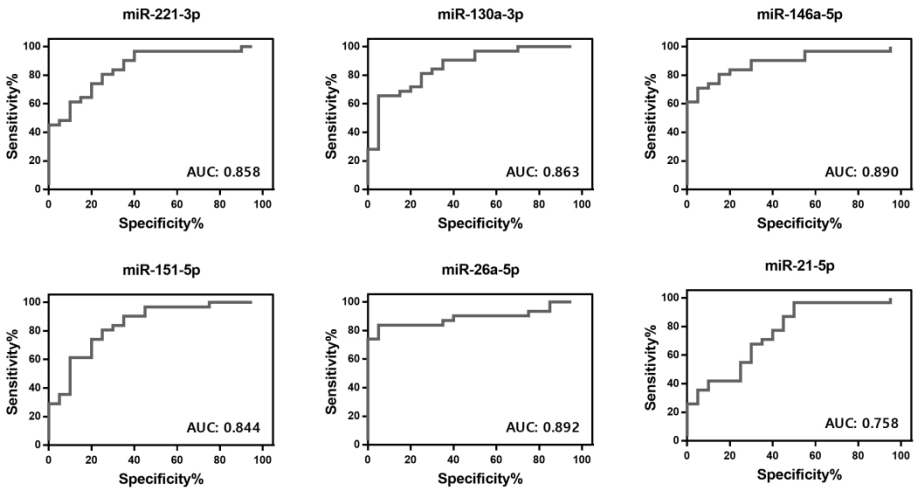

Figure 4 Receiver operating characteristic (ROC) curve analysis for PsA diagnosis. ROC curves of PsA patients (n=31) based on miRNA expression in serum compared to HC subjects (n=20). Area under the curve (AUC) was calculated with 95% confidence limits.

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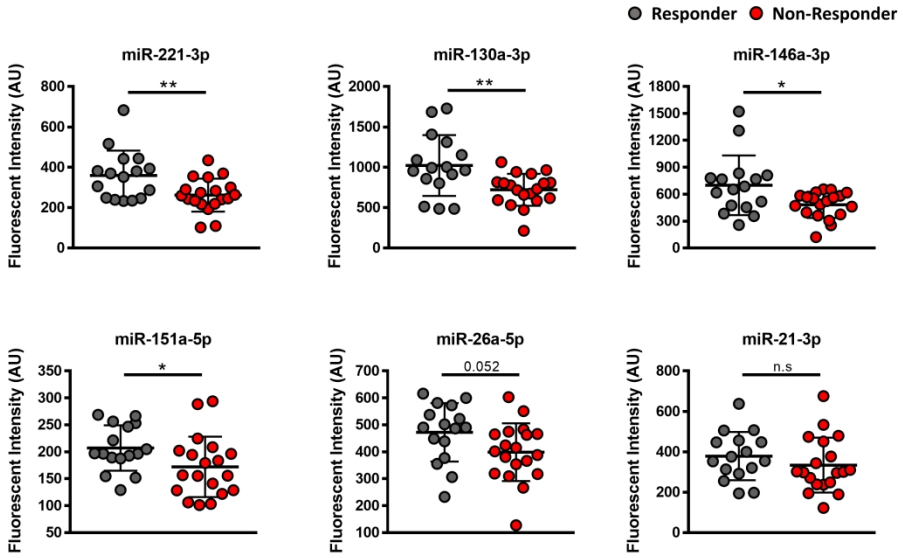

Figure 5 Relative expression of baseline serum miRNAs in PsA patients with good, moderate and non-response to treatment. Relative expression of a group of 6 baseline serum miRNA plotted as a function of treatment response. Non-responding patients (n=19) tended to have lower baseline serum miRNA levels than in those with a good - moderate response (n=16). P values were determined using student t-test when comparing the differences between two groups. *p<0.05, significantly different to comparator.

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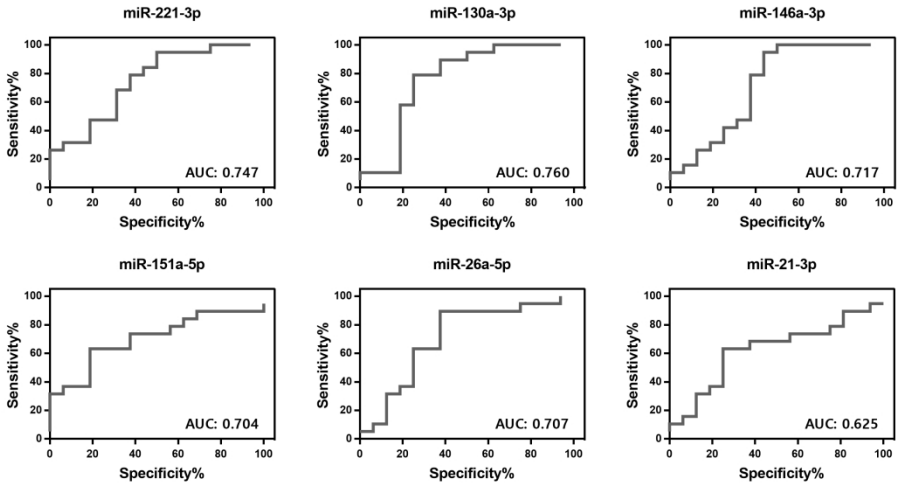

Figure 6 ROC curve analysis for baseline serum miRNA as a predictor of treatment response. Area under the curve (AUC) estimation for serum miRNA in responders (n=16) compared to non-responders (n=19). Area under the curve (AUC) was calculated with 95% confidence limits.

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