ORIGINAL ARTICLE

Circulating microRNAs as potential biomarkers of disease activity and structural damage in ankylosing spondylitis patients

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Abstract

Ankylosing spondylitis (AS) remains difficult to diagnose before irreversible damage to sacroiliac joint is noticeable. Circulating microRNAs have demonstrated to serve as diagnostic tools for several human diseases. Here, we analysed plasma microRNAs to identify potential AS biomarkers. Higher expression levels of microRNA (miR)-146a-5p, miR-125a-5p, miR-151a–3p and miR-22–3p, and lower expression of miR-150–5p, and miR-451a were found in AS versus healthy donors. Interestingly, higher miR-146a-5p, miR-125a-5p, miR-151a–3p, miR-22–3p and miR-451a expression was also observed in AS than psoriatic arthritis patients. The areas under the curve, generated to assess the accuracy of microRNAs as diagnostic bio-markers for AS, ranged from 0.614 to 0.781; the six-microRNA signature reached 0.957. Bioinformatics analysis revealed that microRNAs targeted inflammatory and bone remodeling genes, underlying their potential role in this pathology. Indeed, additional studies revealed an association between these six microRNAs and potential target proteins related to AS pathophysiology. Furthermore, miR-146a-5p, miR-125a-5p and miR-22–3p expression was increased in active versus non-active patients. Moreover, miR-125a-5p, miR-151a-3p, miR-150–5p and miR-451a expression was related to the presence of syndesmophytes in AS patients. Overall, this study identified a six-plasma microRNA signature that could be attractive candidates as non-invasive biomarkers for the AS diagnosis, and may help to elucidate the disease pathogenesis.

Introduction

Spondyloarthritis (SpA) is a chronic inflammatory rheumatic disorder that encompasses five major subtypes; ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis, arthritis related to inflammatory bowel disease and a subgroup of juvenile idiopathic arthritis (1). The most recognisable prototypical form of this disorder is the AS, which presents an estimated prevalence of 0.1–1.4% in general population (2). AS mainly affects the axial skeleton, frequently targeting the sacroiliac joint in the pelvis. The pathological process of this disease can be divided in two basic phases: an early inflammatory phase (3), followed by a pathological new bone formation phase and consequent ankylosis (4).

To date, AS remains difficult to diagnose before irreversible damage to sacroiliac joint is visible on X-ray and an overdiagnosis by wrongly including patients with mechanical back pain or fibromyalgia often occurs in these patients. Besides, the pathogenesis of disease causation and perpetuation has still not been fully identified. As a result, the management and treatment of this disease is not completely satisfactory. Although AS is closely associated with the human leukocyte antigen (HLA)-B27 hereditary (5), it can explain no more than 30% of the overall genetic risks of this disease (6). Thus, it is likely that other genetic disorders, not yet explored, can influence the AS pathogenesis.

MicroRNAs (miRNAs) are a group of single-stranded non-coding RNAs of 20–25 nucleotides in length that play a relevant role as regulators in the post-transcriptional control of gene expression (7). Over the past several years, it has been appreciated that miRNAs are not only important for regulating every physiological aspect of cellular function, including differentiation and development, metabolism, proliferation and apoptosis (8), but also in the pathologies of cancer, systemic rheumatic diseases and inflammation (9–11).

While the majority of miRNAs are found intracellularly, a significant number of them are detected in the circulation in a cell-free form (12). These extracellular miRNAs are protected from circulatory ribonuclease (RNase) activity (13) and show a remarkable stability even under severe conditions (e.g. extended storage and freeze-thaw cycles). Furthermore, it has been shown that diverse alterations in the expression of these plasma miRNAs are related to several disorders such as cancer (14) and autoimmune diseases (15,16). Thereupon, these features of circulating miRNAs have triggered widespread interest of these molecules as prognostic and diagnostic tools for various human diseases.

Recent studies have supported an important role for miRNAs in AS pathogenesis: altered expression of miRNAs has been detected in peripheral blood mononuclear cells (PBMCs), and T cells in AS patients, on which these miRNAs regulate specific pathways involved in pathogenesis of this disease (17–20). In contrast to cellular miRNAs, the expression profile of circulating miRNAs in AS has not been fully investigated (21), and their diagnostic potential has never been evaluated. Thus, additional studies are required to explore the role of plasma miRNAs for the AS diagnosis.

Therefore, the present study was designed to evaluate the expression profile of circulating miRNAs in AS patients, and to identify those miRNAs associated with the disease itself. Besides, we further evaluated the role of specific circulating miRNAs as potential biomarkers of disease activity and structural damage in these patients.

Results

Study population

The clinical features of the AS patients ($n = 53$) and the healthy age- and gender-matched donors ($n = 57$) are summarized in Table 1. Patients [43 males (81.1%) and 10 females (18.9%)] had an average age of 47.8 ± 11.3 years, with a disease duration of 13.4 ± 9.6 years and a mean score of total Bath Ankylosing Spondylitis Disease activity index (BASDAI) and modified Stoke Ankylosing Spondylitis Spine (mSASSS) of 3.8 ± 2.5 and 14.6 ± 20.8, respectively. Overall, patients exhibited greater mean levels of inflammatory markers [i.e. erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)] than healthy donors (HDs) ($P < 0.05$), and a total of 96.1% of patients presented HLA-B27 antigen. Regarding treatment, 100% were taking non-steroidal anti-inflammatory drugs (NSAIDs); 1.9% were on corticosteroids and 3.8% were using sulfasalazine.

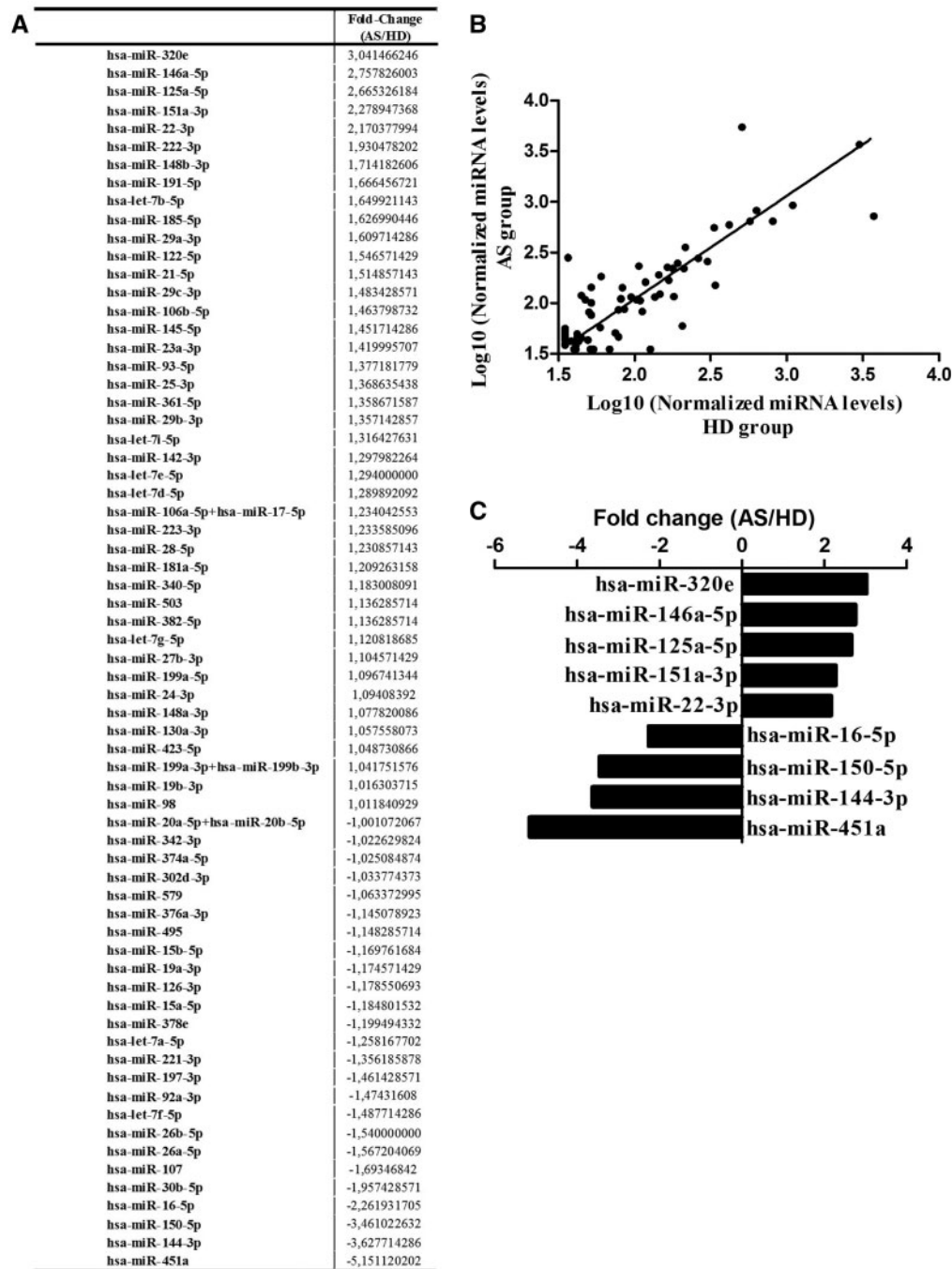### Identification and validation of miRNA expression profiling in AS patients and HDs

To evaluate the expression profiles of plasma miRNAs in AS patients and HDs, a total of 800 miRNAs were analysed in two sets of RNA samples (each of them consists of a pool of three RNA samples from AS patients or HDs), using a nCounter microRNA array (discovery cohort; Fig. 1). After normalization of the raw data, the expression levels of 42 miRNAs were found

Table 1. Clinical and laboratory parameters of the ankylosing spondylitis patients and the healthy donors enrolled for the validation phase

	AS Patients ($n = 53$)	Healthy donors ($n = 57$)	P
Clinical parameters			
Women/men, n/n	10/43	17/40	0.182
Age, years	47.8 ± 11.3	48.6 ± 11.3	0.740
BASDAI	3.8 ± 2.5	…	—
BASFI	4.0 ± 3.0	…	—
BASMI	2.9 ± 1.5	…	—
mSASSS	14.6 ± 20.8	…	—
ASQol	5.1 ± 4.8	…	—
Disease duration, y	13.4 ± 9.6	…	—
Laboratory parameters			
HLA-B27 (%)	49/51 (96.08%)	…	
CRP,[a] nmol/l	100.95 ± 114.67	14.28 ± 17.14	**<0.001**
ERS,[a] mm/h	17.1 ± 19.3	6.2 ± 5.2	**0.001**
Treatments			
NSAIDs (%)	53/53 (100%)	…	—
Corticosteroids (%)	1/53 (1.89%)	…	—
Sulfasalazine (%)	2/53 (3.77%)	…	—

Values are presented as mean $\pm$ SD. The data were analysed using an Independent Samples *t* test or a Mann–Whitney *U* test to evaluate statistical significance between AS patients and healthy donors.

[a]Non-normally distributed data. Differences in gender distribution were analysed by χ^2 analysis. AS indicates ankylosing spondylitis; ASQoL, Ankylosing Spondylitis Quality of Life questionnaire; BASDAI, Bath Ankylosing Spondylitis Disease Activity; BASFI; Bath Ankylosing Spondylitis Functionality Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; HLA, human leukocyte antigen; mSASSS, modified Stoke Ankylosing Spondylitis Spine Score; NSAIDs, non-steroidal anti- inflammatory drugs.

A	Fold-Change (AS/HD)
hsa-miR-320e	3,041466246
hsa-miR-146a-5p	2,757826003
hsa-miR-125a-5p	2,665326184
hsa-miR-151a-3p	2,278947368
hsa-miR-22-3p	2,170377994
hsa-miR-222-3p	1,930478202
hsa-miR-148b-3p	1,714182606
hsa-miR-191-5p	1,666456721
hsa-let-7b-5p	1,649921143
hsa-miR-185-5p	1,626990446
hsa-miR-29a-3p	1,609714286
hsa-miR-122-5p	1,546571429
hsa-miR-21-5p	1,514857143
hsa-miR-29c-3p	1,483428571
hsa-miR-106b-5p	1,463798732
hsa-miR-145-5p	1,451714286
hsa-miR-23a-3p	1,419995707
hsa-miR-93-5p	1,377181779
hsa-miR-25-3p	1,368635438
hsa-miR-361-5p	1,358671587
hsa-miR-29b-3p	1,357142857
hsa-let-7i-5p	1,316427631
hsa-miR-142-3p	1,297982264
hsa-let-7e-5p	1,294000000
hsa-let-7d-5p	1,289892092
hsa-miR-106a-5p+hsa-miR-17-5p	1,234042553
hsa-miR-223-3p	1,233585096
hsa-miR-28-5p	1,230857143
hsa-miR-181a-5p	1,209263158
hsa-miR-340-5p	1,183008091
hsa-miR-503	1,136285714
hsa-miR-382-5p	1,136285714
hsa-let-7g-5p	1,120818685
hsa-miR-27b-3p	1,104571429
hsa-miR-199a-5p	1,096741344
hsa-miR-24-3p	1,09408392
hsa-miR-148a-3p	1,077820086
hsa-miR-130a-3p	1,057558073
hsa-miR-423-5p	1,048730866
hsa-miR-199a-3p+hsa-miR-199b-3p	1,041751576
hsa-miR-19b-3p	1,016303715
hsa-miR-98	1,011840929
hsa-miR-20a-5p+hsa-miR-20b-5p	-1,001072067
hsa-miR-342-3p	-1,022629824
hsa-miR-374a-5p	-1,025084874
hsa-miR-302d-3p	-1,033774373
hsa-miR-579	-1,063372995
hsa-miR-376a-3p	-1,145078923
hsa-miR-495	-1,148285714
hsa-miR-15b-5p	-1,169761684
hsa-miR-19a-3p	-1,174571429
hsa-miR-126-3p	-1,178550693
hsa-miR-15a-5p	-1,184801532
hsa-miR-378e	-1,199494332
hsa-let-7a-5p	-1,258167702
hsa-miR-221-3p	-1,356185878
hsa-miR-197-3p	-1,461428571
hsa-miR-92a-3p	-1,47431608
hsa-let-7f-5p	-1,487714286
hsa-miR-26b-5p	-1,540000000
hsa-miR-26a-5p	-1,567204069
hsa-miR-107	-1,69346842
hsa-miR-30b-5p	-1,957428571
hsa-miR-16-5p	-2,261931705
hsa-miR-150-5p	-3,461022632
hsa-miR-144-3p	-3,627714286
hsa-miR-451a	-5,151120202

Figure 1. Plasma miRNA profiling using nCounter microRNA array. (**A**) Fold change of 67 miRNAs expressed in plasma after normalization of the raw data. (**B**) Scatter plot of miRNA expressed in AS patients (Y) and HDs (X). The expression profile of 67 miRNAs was plotted as $\log_{10}$ (2^-Delta Ct). Specific miRNA expression positioned on the mid-line indicates no differential expression. (**C**) Differentially expressed miRNAs (fold change ≥ 2) between AS patients and HDs. AS, Ankylosing spondylitis; HDs, Healthy Donors; miRNA, microRNA.

elevated, whereas 25 miRNAs were found lower in AS compared with HDs (Fig. 1A and B). Among them, there were nine differentially expressed miRNAs (fold change ≥ 2) when comparing both groups, with five circulating miRNAs increased [miRNA (miR)-320e, miR-146a-5p, miR-125a-5p, miR-151a-3p and miR22–3p] and four decreased (miR-16–5p, miR-150–5p, miR-144–3p and miR-451a) in AS patients compared with HDs (Fig. 1C). Next, real time PCR was performed on nine candidate miRNAs for further validation. In this second phase, a set of individual plasma samples including 53 AS patients and 57 HDs were compared (validation cohort). As shown in Figure 2A and Supplementary Material, Table S1, six of the nine miRNAs clearly distinguished AS plasma samples with high confidence level ($P < 0.05$). Specifically, the circulating levels of miR-146a-5p, miR-125a-5p, miR-151a–3p and miR-22–3p were significantly elevated, whereas those of miR-150–5p and miR-451a were significantly reduced in AS patients versus HDs ($P < 0.05$; Fig. 2A).
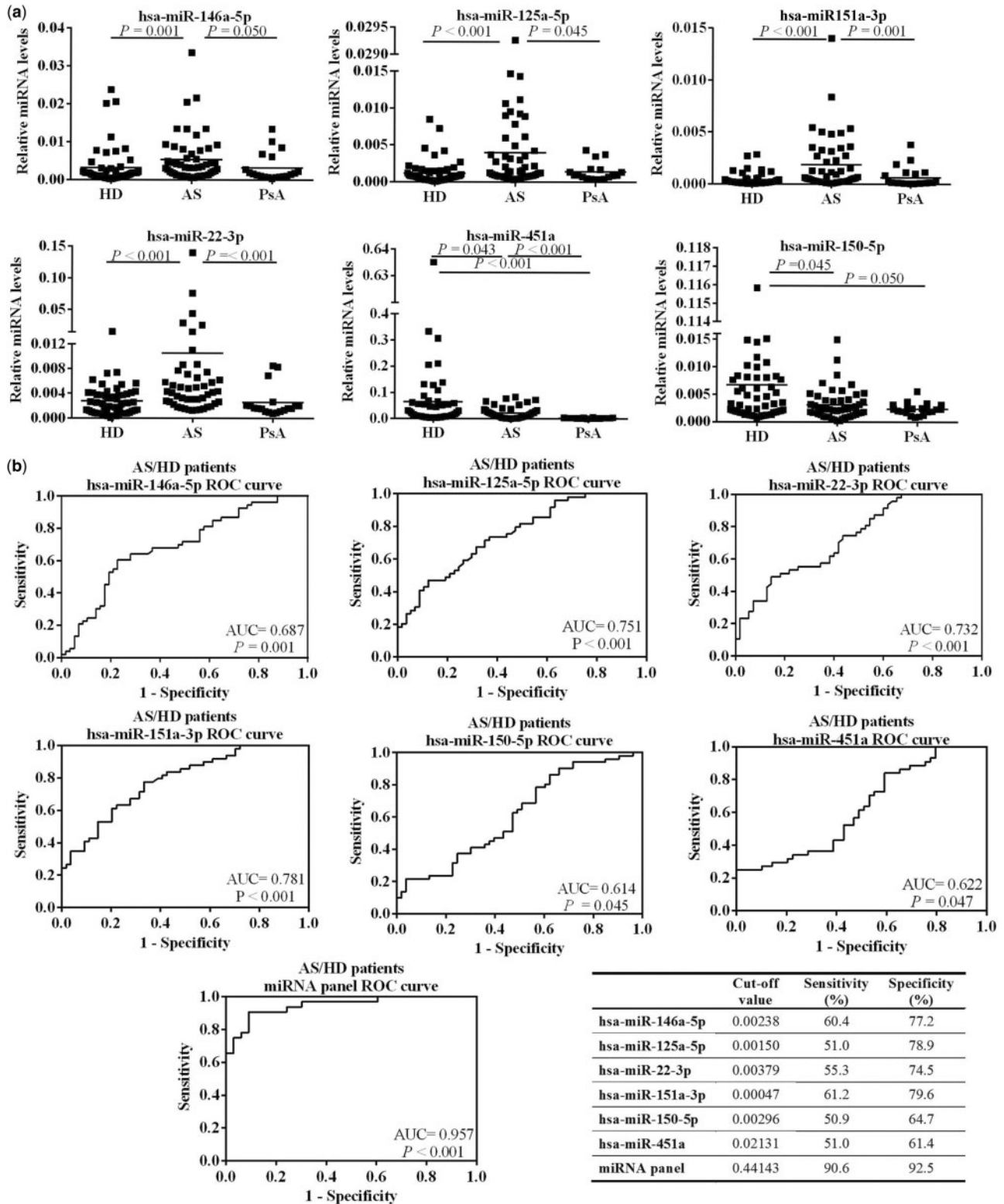
Figure 2. miRNA expression levels in the validation cohort and analyses of the diagnostic potential of differentially expressed miRNAs for discerning AS. (**A**) Relative expression levels of circulating miRNA are shown (HDs, $n = 57$; AS patients, $n = 53$; PsA patients, $n = 23$). The data were analysed using a Mann-Whitney U test to evaluate statistical significance between groups. A P-value < 0.05 was considered on the borderline of statistical significance. (**B**) ROC curve analyses of the six validated miRNAs and a combination of these as a panel were performed to assess their potential as diagnostic biomarkers for AS. AS, Ankylosing spondylitis; AUC, Area Under the Curve; HDs, Healthy Donors; miRNA, microRNA; PsA, Psoriatic Arthritis.

Subsequently, multiple linear regression analysis was used to determine the association of miRNA expression levels with age, gender, treatment [non-disease-modifying antirheumatic drugs (DMARDs)/non-corticoids versus DMARDs/corticoids] and diagnosis (AS patients versus HDs). The clinical characteristics of each patient and control are shown in Supplementary Material, Table S2. No gender, age and treatment rather than diagnosis was statistically proven to act as a confounding variable in miR-125a-5p, miR-150–5p, miR-151a–3p and miR-22–3p expression levels (Supplementary Material, Table S3). In the case of miR-146a-5p and miR-451a, age and diagnosis were found as significant predictors of their expression levels, indicative of association between both miRNAs and diagnosis for individuals with the same age (Supplementary Material, Table S3). In addition, we grouped AS patients in three blocks of disease duration (≤10 years, 11–20 years, >20 years) to evaluate the effect of this factor in the expression levels of circulating miRNAs. Comparison between groups demonstrated non-significant differences in the expression of plasma miRNAs (data not shown).

To further explore the specificity of the miRNA expression profile observed in AS, we next evaluated the expression of six validated miRNAs in a cohort of PsA patients (disease control; $n = 23$) (Fig. 2A). The clinical features of the PsA patients are summarized in Table 2 and Supplementary Material, Table S4. Patients [13 males (56.5%) and 10 females (43.5%)] had an average age of 50.7 ± 13.4 years, with a disease duration of 7.0 ± 5.8 years and a mean score of Disease Activity Score-28 (DAS28) of 4.3 ± 1.3. The expression levels of five miRNAs significantly discriminated the AS group from the PsA group. Thus, the expression levels of miR-146a-5p, miR-125a-5p, miR-151a–3p, miR-22–3p and miR-451a were higher in AS versus PsA patients. However, miR-150–5p expression levels did not show any significant difference from disease control. On the other hand, the expression levels of miR-150–5p and miR-451a, as in the AS patients, were also found substantially lower in PsA patients compared with HDs (Fig. 2A).

Table 2. Clinical and laboratory parameters of the psoriatic arthritis patients

	PsA patients ($n = 23$)
Clinical parameters	
Women/men, n/n	10/13
Age, years	50.7 ± 13.4
Disease duration, years	7.0 ± 5.8
RF positive (%)	0/23 (0%)
DAS28	4.3 ± 1.3
Axial involvement (%)	8/23 (34.8%)
Laboratory parameters	
HLA-B27 (%)	3/17 (17.65%)
CRP, nmol/l	71.43 ± 70.48
ERS, mm/h	15.3 ± 9.8
Treatments	
NSAIDs (%)	23/23 (100%)
Metotrexate (%)	13/23 (56.52%)
Leflunomide (%)	5/23 (21.74%)

Values are presented as mean ± SD. PsA indicates psoriatic arthritis; RF, rheumatoid factor; DAS28, disease activity score 28; HLA, human leukocyte antigen; CRP, C reactive protein; ERS, erythrocyte sedimentation rate; NSAIDs, non-steroidal anti-inflammatory drugs.

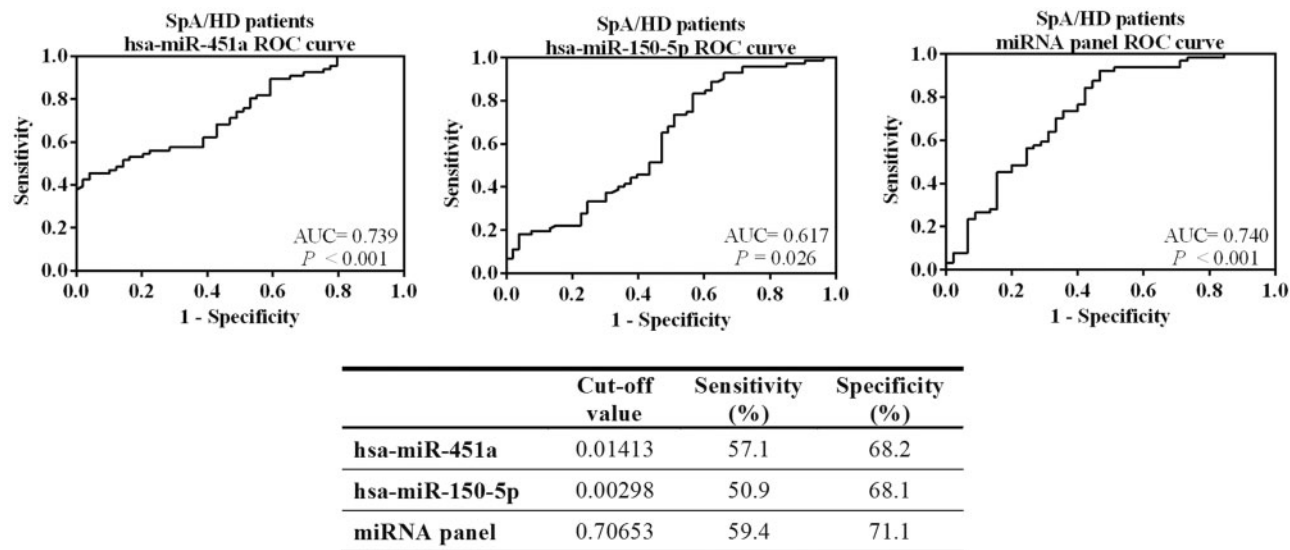## Analyses of the diagnostic potential of validated miRNAs

To investigate the relevance of the aforementioned circulating miRNAs as potential diagnostic biomarkers for discerning AS patients from HDs, receiver–operator characteristics (ROC) curve analysis was performed. As shown in Figure 2B, the ROC curves of miR-146a-5p, miR-125a-5p, miR-151a–3p, miR-22–3p, miR-150–5p and miR-451a exhibited a moderate distinguishing efficiency, with the areas under the curve (AUCs) for these miRNAs ranged from 0.614 to 0.781 ($P < 0.05$). To further explore the diagnostic accuracy of these miRNAs, a combination of the 6-miRNAs as a panel was carried out by using logistic regression on the data set and used to predict AS patients. After multivariate analysis, no gender, age and treatment rather than diagnosis was statistically proven to act as a confounding variable in the expression level of the six-miRNA signature (Supplementary Material, Table S5). Furthermore, comparative analysis between duration disease-stratified groups also showed non-significant differences in the miRNA panel expression (data not shown). The ROC curve for the 6-miRNA signature revealed a marked diagnostic accuracy, evidenced by the AUC of 0.957 ($P < 0.001$), which was much better than any individual miRNA in detecting AS (Fig. 2B). At the optimal cut-off value of 0.441, where the value of sensitivity + specificity was considered to be maximal, the diagnostic sensitivity and specificity of the 6-miRNA panel for AS detection was of 90.6% and 92.5%, respectively (Fig. 2B). These results suggest that the combination of the six miRNAs in a panel shows great potential as a biomarker for discriminating AS patients from HDs.

Since AS and PsA are the two main subtypes included within of SpA, and that levels of miR-150–5p and miR-451a expression were decreased in both AS and PsA patients versus HDs, we next grouped both disorders and studied the diagnostic potential of these miRNAs for SpA. The ROC curve analyses revealed that plasma miR-150–5p and miR-451a expression could accurately distinguish SpA patients from HDs, with a power AUC of 0.617 and 0.739, respectively (Fig. 3). In addition, the combination of the 2-miRNAs as a panel showed an evident diagnostic accuracy, evidenced by an AUC of 0.740 ($P < 0.001$) at a sensitivity of 59.4% and a specificity of 71.1% from a cut-off value of 0.706 (Fig. 3). Hence, plasma miR-150–5p and miR-451a may be suitable biomarkers for the prediction of SpA patients.

Multiple linear regression analysis was further performed to determine the association of the expression levels of miR-451a, miR-150–5p, and the combination of the 2-miRNAs as a panel with age, gender, treatment (non-DMARDs/non-corticoids versus DMARDs/corticoids) and diagnosis (SpA patients versus HDs). No gender, age and treatment rather than diagnosis was statistically proven to act as a confounding variable in the miR-150–5p and 2-miRNA signature (Supplementary Material, Table S6). miR-451a expression levels had a significant positive association with age and negative with diagnosis (Supplementary Material, Table S6). In addition, comparison between duration disease-stratified groups (≤10 years, 11–20 years, >20 years) demonstrated non-significant differences in the plasma content of miR-451a, miR-150–5p or the 2-miRNA signature (data not shown).

Determination of the AS-related processes and pathways potentially regulated by the differentially expressed plasma miRNAs

It is widely accepted that miRNAs regulate gene expression by causing either translational repression or mRNA degradation (22).

	Cut-off value	Sensitivity (%)	Specificity (%)
hsa-miR-451a	0.01413	57.1	68.2
hsa-miR-150-5p	0.00298	50.9	68.1
miRNA panel	0.70653	59.4	71.1

Figure 3. Analyses of the diagnostic potential of differentially expressed miRNAs for discerning SpA. ROC curve analysis of miR-451a, miR-150–5p and the 2-miRNA signature were performed to assess their potential as diagnostic biomarkers for SpA. A cut-off value with higher specificity and sensitivity was selected. The combination of the miRNAs as a panel was carried out by using logistic regression on the data set. AUC, Area Under the Curve; HDs, Healthy Donors; miRNA, microRNA; SpA, Spondyloarthritis.

Therefore, aberrant expression of miRNAs may alter multiple downstream pathways and promote various evident effects. By using the Ingenuity Pathway Analysis (IPA) software, we identified the biological functions and related diseases, as well as the potential gene targets and molecular networks related to the six validated miRNAs (Fig. 4).

A detailed analysis of these six miRNAs revealed their participation in cell-mediated immune response, immune cell trafficking, inflammatory response and in specific diseases and disorders such as immunological, inflammatory and gastrointestinal diseases or skeletal and muscular disorders. Therefore, all our differentially expressed miRNAs seem to be related to the functions previously demonstrated to be involved in the pathophysiology of AS (23) (Fig. 4A).

To better understand the significance of the results, the potential impact of the validated miRNAs on the inflammatory and bone remodeling pathways was further investigated (Fig. 4B). Notably, we observed that these six circulating miRNAs could target inflammatory and bone remodeling genes, underlying their potential role in this pathology. It is also interesting to remark that in this study, we found that several genes, grouped into inflammatory or bone-remodeling areas, were the targets of more than one of the validated miRNAs (Supplementary Material, Table S7). For example, the inflammatory genes that codify for CAMP responsive element binding protein 1 (CRBE1), colony-stimulating factor 1 receptor (CSF1R), Mannan-binding lectin serine peptidase 1 (MASP1) and Sp1 transcription factor (SP1) may be potentially regulated by three out of the six altered miRNAs. A number of genes directly related to both inflammation and bone remodeling in AS, such as AKT serine/threonine kinase 3 (AKT3), fibroblast growth factor receptor 1 (FGFR1), integrin subunit beta 3 (ITGB3), NRAS proto-oncogene, GTPase (NRAS), SMAD family member 4 (SMAD4) and tumor protein P53 (TP53) were also simultaneous targets for three of those miRNAs.

Associations between the six validated plasma miRNAs and the protein levels of various specific targets and clinical inflammatory parameters

Inasmuch as these miRNAs could target different pathways associated with AS pathogenesis (i.e. inflammation, bone remodeling), plasma levels of diverse cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-5], as susceptible indirect targets of validated miRNAs and involved in pathogenesis of AS (24–27), were next quantitatively measured to assess their relationship to the six validated plasma miRNAs. The key role of TNF-α and IL-1β in AS pathogenesis has been previously established by success of biological therapies against these cytokines (24–26,28), whereas IL-5 is a relevant anti-inflammatory cytokine produced by T helper (Th)2 lymphocytes. Compared with healthy controls, patients with AS have a higher prevalence of Th2 and Th17 cells to regulate their adaptive immunity (27), indicative of the general alteration in immune cell phenotypes in AS. In our study, we found that along with higher concentrations of ESR and CRP, patients exhibited significantly greater mean levels of TNF-α (12.05±11.25 vs. 1.45±2.21, $P = 0.001$), IL-1β (2.55±1.39 vs. 0.74±0.55, $P < 0.001$) and IL-5 (1.96±1.24 vs. 0.50±0.37, $P < 0.001$) than HDs.

Correlation studies showed that miR-146a–5p, miR-125a-5p, miR-151a-3p, miR-22–3p and miR-451a expression were associated with plasma levels of CRP and/or TNF-α. In addition, the expression levels of miR-22–3p, and miR-451a were correlated to plasma IL-1β and IL-5 levels, whereas miR-146a–5p expression was also positively associated with plasma ESR. No significant correlations of the miR-150–5p expression with the plasma ESR and cytokine levels were noted in our population (Table 3). Overall, these results support the relevance of these miRNAs in the pathophysiology of this disease.

Based on our correlation studies, we next selected miR-125a-5p, miR-451a and miR-22–3p, and investigated the effects of manipulating expression of these miRNAs on TNF-α, IL-1β
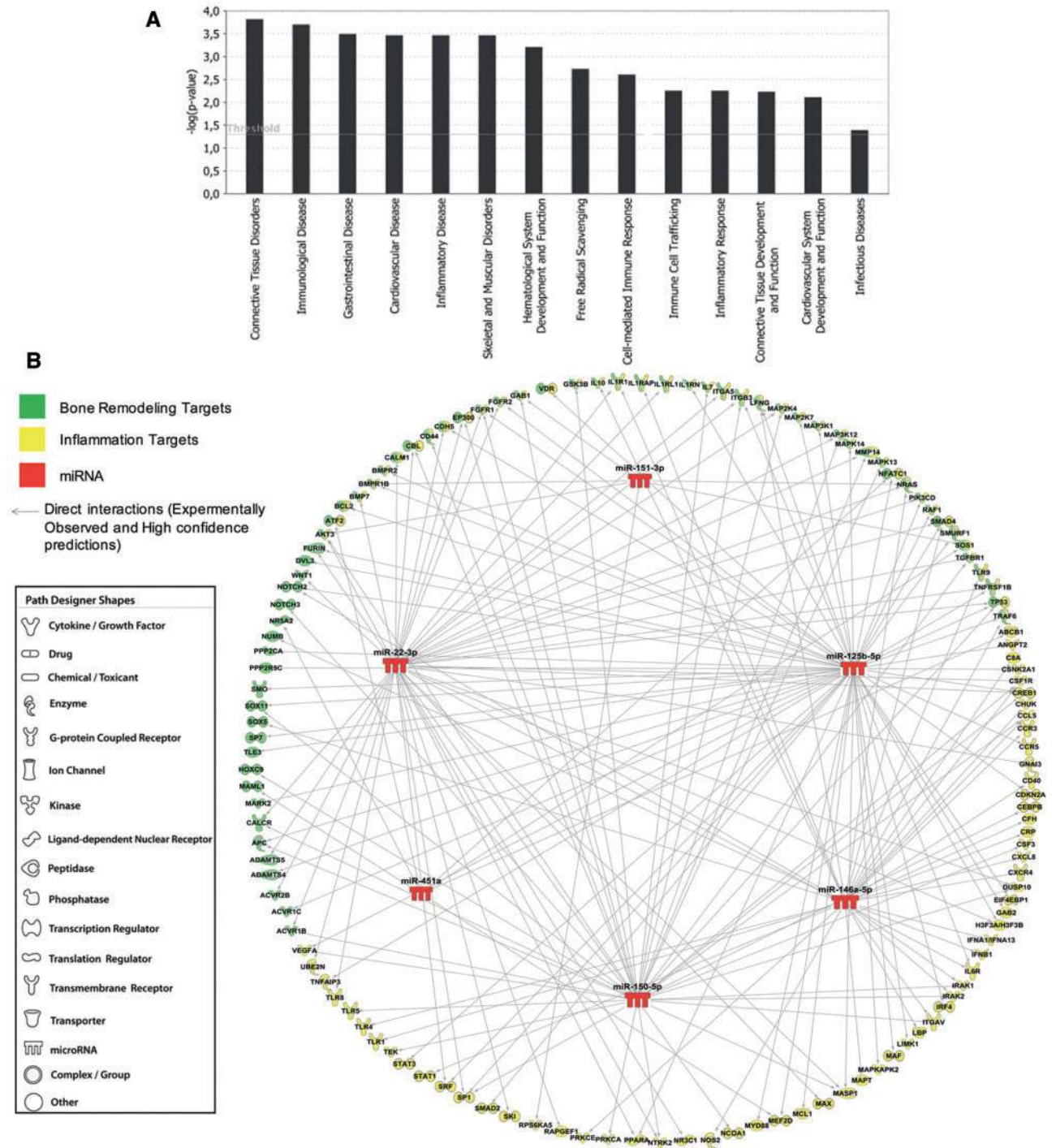
Figure 4. Participation of the six validated miRNAs in AS-related processes and pathways. (**A**) Ingenuity Pathway Analysis (IPA) uncovered the main enriched biological functions and pathways on which those miRNAs are involved. The analysis included only the functions and pathways with average IPA score >2 [indicated as -log (*P* value)]. Threshold bar indicates cut-off point of significance (*P* < 0.05), using Fisher's exact test. miRNAs, microRNAs. (**B**) By using the IPA software, a network including the selected miRNAs and their mRNA targets, filtered by inflammation and bone remodeling pathways, was generated. Only targets experimentally observed and predicted with high confidence are shown and related by direct interactions with their specific miRNA regulators.

and IL-5 levels in lymphocytes, key players in AS pathogenesis (23) and the main leukocyte subset involved in cytokine secretion. To address this point, we performed transient overexpression "gain-of function" of miRNA mimics, which is the most useful system in cells displaying high expression of miRNA targets (both, direct and indirect). Lymphocytes from AS patients (*n* = 3) transfected with miR-125a-5p, miR-451a and miR-22–3p mimics displayed a reduction in the expression of these inflammatory molecules, suggesting that the selected miRNAs might modulate the studied cytokines (*P* < 0.05) (Fig. 5).

Table 3. Spearman's rank correlations between differentially expressed plasma miRNAs and inflammatory parameters

	miR-146a–5p	miR-125a-5p	miR-151a-3p	miR-22–3p	miR-150–5p	miR-451a
CRP (mg/l)	$r = +0.353$	$r = +0.382$	$r = +0.258$	$r = +0.450$	$r = -0.225$	$r = -0.200$
	$P = 0.030$	$P = 0.020$	$P = 0.148$	$P = 0.009$	$P = 0.181$	$P = 0.266$
ESR (mm/h)	$r = +0.326$	$r = +0.254$	$r = +0.307$	$r = +0.318$	$r = -0.206$	$r = -0.282$
	$P = 0.040$	$P = 0.119$	$P = 0.068$	$P = 0.063$	$P = 0.209$	$P = 0.096$
TNF-α (pg/ml)	$r = +0.398$	$r = +0.405$	$r = +0.450$	$r = +0.464$	$r = -0.130$	$r = -0.434$
	$P = 0.049$	$P = 0.045$	$P = 0.027$	$P = 0.034$	$P = 0.535$	$P = 0.049$
IL-1β (pg/ml)	$r = +0.363$	$r = +0.328$	$r = +0.302$	$r = +0.653$	$r = -0.257$	$r = -0.641$
	$P = 0.081$	$P = 0.117$	$P = 0.151$	$P = 0.002$	$P = 0.225$	$P = 0.002$
IL-5 (pg/ml)	$r = +0.329$	$r = +0.342$	$r = +0.306$	$r = +0.482$	$r = -0.148$	$r = -0.434$
	$P = 0.100$	$P = 0.083$	$P = 0.136$	$P = 0.023$	$P = 0.470$	$P = 0.043$

r values of Spearman's rank correlation and P values of their null hypothesis are shown. The significance is the P value indicated in the bold. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IL, interleukin; miRNAs, microRNAs; TNF-α, tumor necrosis factor alpha.
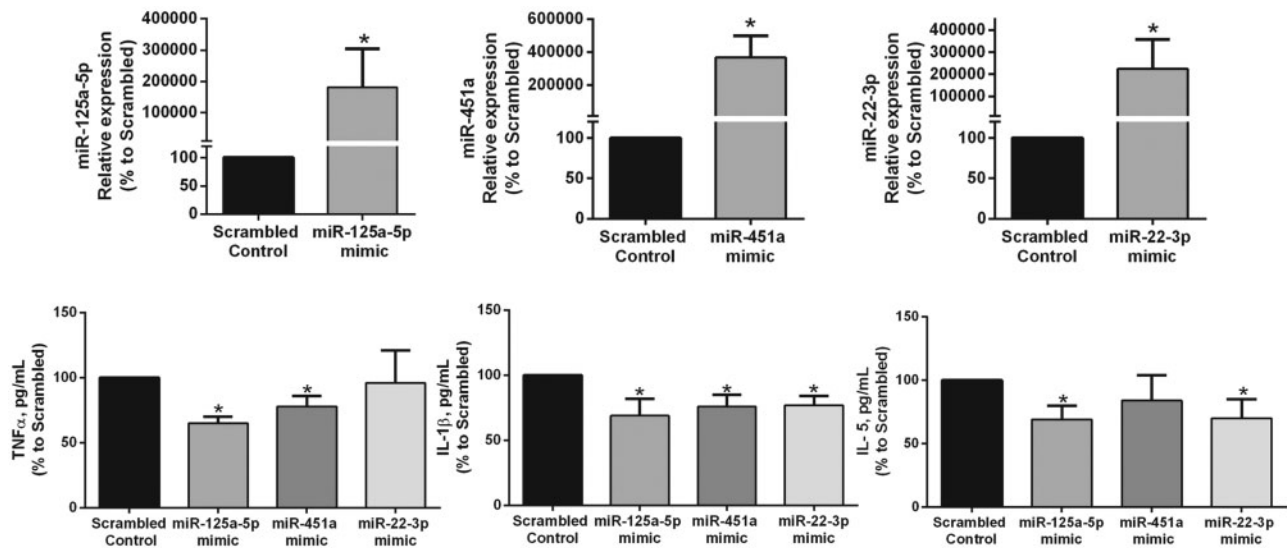

Figure 5. Effect of transfection of lymphocytes with miR-125a-5p, miR-451a and miR-22–3p on TNF-α, IL-1β and IL-5 expression levels. Lymphocytes isolated from AS patients were transfected with 100 nmol/l miR-125a-5p, miR-451a and miR-22–3p mimics and non-specific control (scrambled) by using siPORTTM NeoFXTM transfection agent following manufacturer's protocols. Forty-eight hours after transfection, miR-125a-5p, miR-451a and miR-22–3p content (**A**) and supernatant levels of TNF-α, IL-1β and IL-5 (**B**) were analysed. Results, obtained from three independent transfection experiments, were expressed as changes relative to the value of the cells transfected with scrambled control, and set as 100%. The data were analysed using an Independent Sample t test to evaluate statistical significance between groups. *$P < 0.05$. IL, Interleukin; miR, microRNA; TNF, tumor necrosis factor.

Evaluation of the validated six miRNAs for the discrimination of disease activity and structural damage in AS

To further explore the potential usefulness of the six screened miRNAs as disease biomarkers, we analysed the association of these plasma miRNAs with the clinical status of the AS patients. Firstly, the AS patients were divided into two subgroups based on CRP and ESR levels, and BASDAI score. Patients with a CRP level >5 mg/l and/or patients with a BASDAI score ≥ 4 and ESR >20 mm/h were considered as active patients ($n = 31$), while the others were classified as non-active patients ($n = 22$) (29). As shown in Figure 6A, the expression levels of plasma miR-146a-5p, miR-125a-5p and miR-22–3p were significantly increased in active AS patients as compared with non-active AS patients ($P < 0.05$). To evaluate whether these plasma miRNAs can be used as potential biomarkers for activity in AS patients, ROC curve analyses were performed. These analyses revealed that miR-146a-5p, miR-125a-5p and miR-22–3p showed a moderate distinguishing efficiency, with the AUCs for these miRNAs

ranged from 0.678 to 0.707 ($P < 0.05$). In addition, the combination of the 3-miRNAs as a panel showed an evident diagnostic accuracy, with a power AUC of 0.752 ($P = 0.005$) at a sensitivity of 59.3% and a specificity of 82.4% from a cut-off value of 0.639 (Fig. 6B). The remaining validated plasma miRNAs were not differentially expressed between active and non-active AS patients (Fig. 6A). Therefore, our results suggest that miR-146a-5p, miR-125a-5p and miR-22–3p may be suitable biomarkers for the discrimination of active versus non-active AS patients.

Next, the AS patients were separated into two subgroups depending on structural damage, measured by presence ($n = 31$) or absence ($n = 22$) of syndesmophytes (30). As shown in Figure 7A, there was no difference in disease duration between both groups (9.8 ± 8.6 in syndesmophyte-free patients versus 15.4 ± 10.3 in patients with syndesmophytes, $P = 0.06$). AS patients with more than or equal to one syndesmophyte exhibited significantly lower miR-125a-5p, miR-151a-3p, miR-150–5p and miR-451a expression than those syndesmophyte-free patients ($P < 0.05$) (Fig. 7B). The ROC curve analyses to evaluate the predictive power of miR-125a-5p, miR151a-3p, miR-150–5p
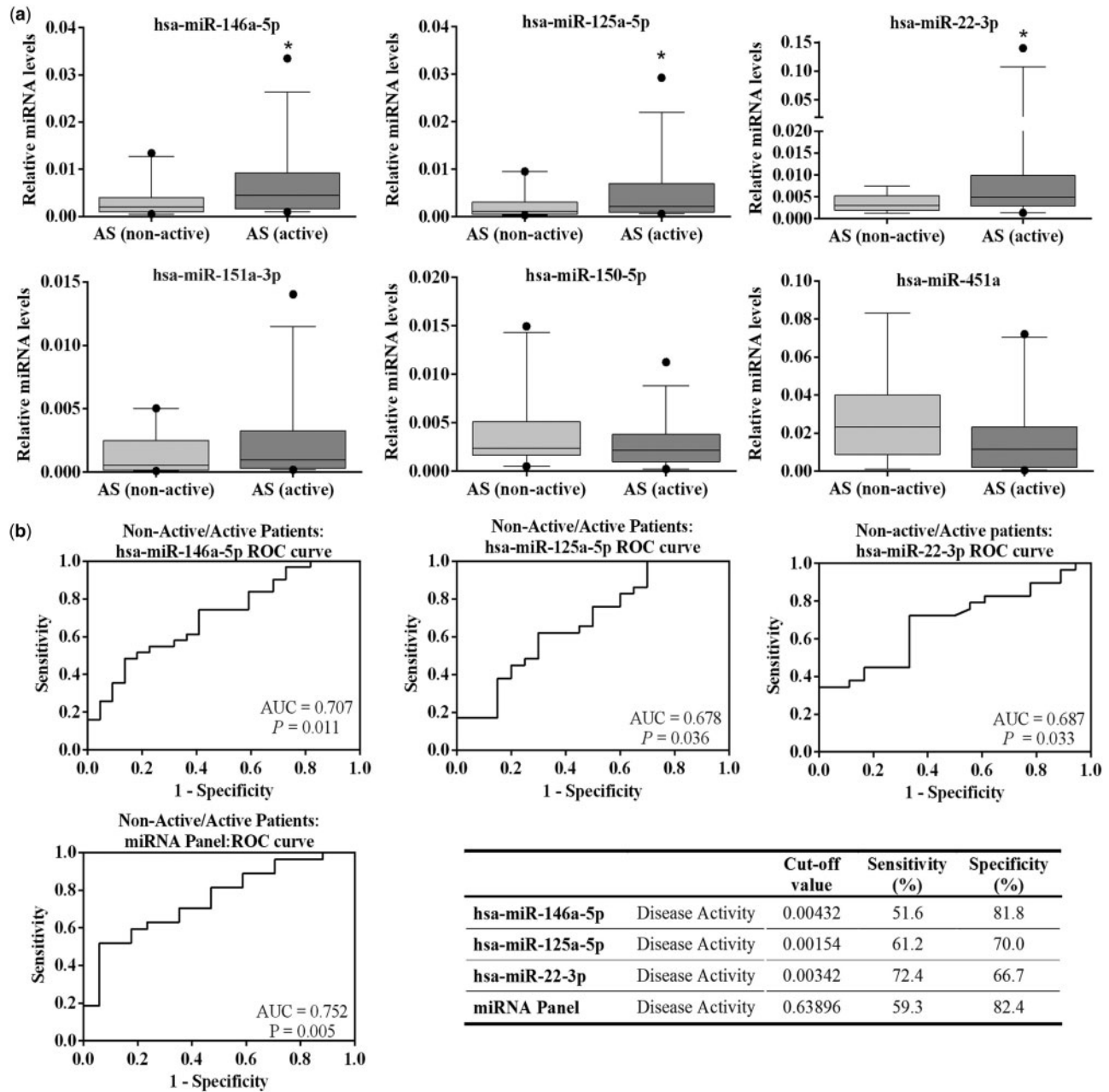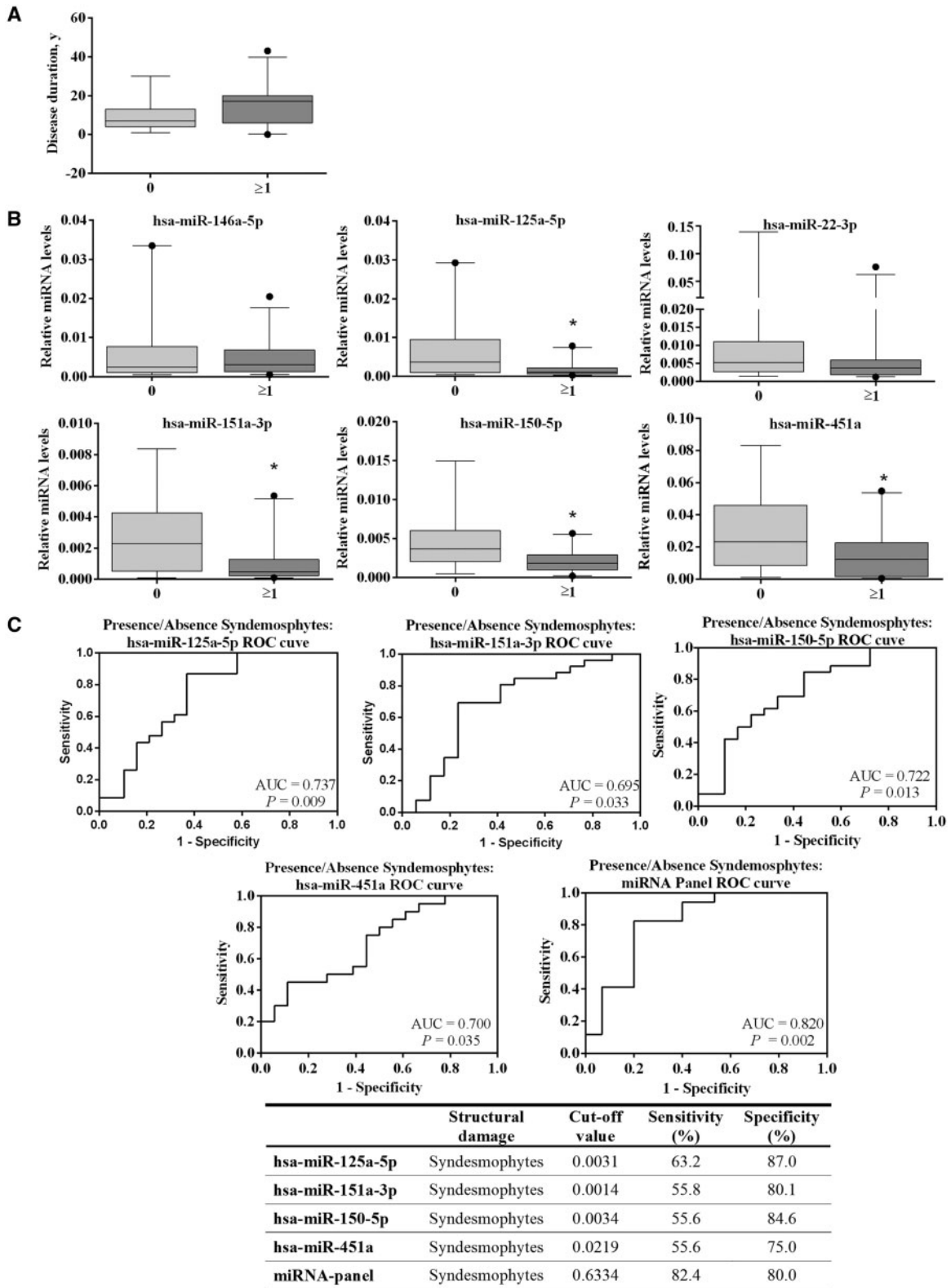
Figure 6. Evaluation of candidate miRNAs as biomarkers of disease activity in AS. Patients were divided into active and non-active based on CRP and ESR levels, and BASDAI score. (**A**) Relative expression levels of each miRNA in active ($n = 31$) and non-active AS patients ($n = 22$). Data shown as box and whisker plots (bar = median, box = interquartile range, whiskers = 5–95 percentile). The data were analysed using an Independent Samples *t* test or a Mann–Whitney *U* test to evaluate statistical significance between groups. $^{*}P < 0.05$. (**B**) ROC curve analyses of the three candidate miRNAs and a combination of these as a panel were performed to assess their potential as biomarkers for disease activity in AS patients. A cut-off value with higher specificity and sensitivity was tested. The combination of the 3-miRNAs as a panel was carried out by using logistic regression on the data set. AS, Ankylosing spondylitis; miRNA, microRNA; AUC, Area Under the Curve.

and miR-451a for structural damage in AS patients was carried out and illustrated in Figure 7C. Relative expression of these plasma miRNAs could accurately distinguish AS patients with radiographic severity, with a power AUC ranged from 0.695 to 0.737 ($P < 0.05$), respectively. To further explore the potential of these biomarkers to predict severe structural damage, a combination of the 4-miRNAs as a panel was carried out by using logistic regression on the data set. The ROC curve for the 4-miRNA signature revealed a marked diagnostic accuracy, evidenced by an AUC of 0.820 ($P < 0.002$), at a sensitivity of 82.4%

and a specificity of 80.0% from a cut-off value of 0.633 (Fig. 7C). Hence, plasma miR-125a-5p, miR-151a-3p, miR-150–5p and miR-451a may be suitable biomarkers for the prediction of severe structural damage.

Discussion

In the present study, we found a signature of six plasma miRNAs that could serve as biomarkers for the AS diagnosis. In addition, our study revealed that several of these circulating

	Structural damage	Cut-off value	Sensitivity (%)	Specificity (%)
hsa-miR-125a-5p	Syndesmophytes	0.0031	63.2	87.0
hsa-miR-151a-3p	Syndesmophytes	0.0014	55.8	80.1
hsa-miR-150-5p	Syndesmophytes	0.0034	55.6	84.6
hsa-miR-451a	Syndesmophytes	0.0219	55.6	75.0
miRNA-panel	Syndesmophytes	0.6334	82.4	80.0

Figure 7. Evaluation of candidate miRNAs as biomarkers of structural damage in AS. Patients were separated into two subgroups depending on structural damage measured by presence (≥ 1) ($n = 31$) or absence (0) ($n = 22$) of syndesmophytes. (**A**) Disease duration between AS patients with presence or absence of syndesmophytes. (**B**) Relative expression levels of each miRNA in AS patients with presence or absence of syndesmophytes. Data shown as box and whisker plots (bar = median, box = interquartile range, whiskers = 5–95 percentile). The data were analysed using an Independent Samples *t* test or a Mann-Whitney U test to evaluate statistical significance between groups. *$P < 0.05$. (**C**) ROC curve analyses of the four candidate miRNAs and a combination of these as a panel were performed to assess their potential as biomarkers for structural damage in AS patients. A cut-off value with higher specificity and sensitivity was tested. The combination of the 4-miRNAs as a panel was carried out by using logistic regression on the data set. AS, Ankylosing spondylitis; miRNA, microRNA; AUC, Area Under the Curve.

miRNAs might accurately discriminate among AS patients with different disease status.

AS is a form of chronic inflammatory arthritis characterized by pathologic new bone formation. To date, the aetiology and pathogenesis of AS remain unclear, and the diagnosis is still challenging. Traditionally, HLA-B27 antigen has been used as an important diagnostic biomarker due to its overwhelming prevalence in AS. However, this antigen does not account for more than 30% of the overall genetic risks of this pathology (6). In addition, X-ray fails to detect early radiological changes, and magnetic resonance imaging cannot be used as a screening test due to unfavourable manipulation and cost. Therefore, novel biomarkers are urgently needed for the disease screening and diagnosis of AS.

The present study, using a nCounter microRNA array containing 800 miRNAs that was followed by real time RT-PCR validation, demonstrated an altered expression of six plasma miRNAs (miR-146a-5p, miR-125a-5p, miR-151a–3p, miR-22–3p, miR-150–5p and miR-451a) in AS patients versus HDs. A previous study by Magrey *et al*. (21) screened 175 miRNAs using a PCR array-based miRNA analysis of plasma samples from AS and healthy controls, and identified two circulating miRNAs up-regulated (miR-32 and miR-34a) and five down-regulated (miR-16, miR-150, miR-10b, miR-30a and miR-154) in AS patients; however, they did not demonstrate the potential of these miRNAs as diagnostic biomarkers for AS. There are a number of discrepancies in miRNA profile between both studies, and several reasons might explain them: first, the AS and HDs populations included in both studies were different in terms of ethnicity (European origin in our study, and a mix of different ethnicities in the study of Magrey *et al*.); thus, discrepancies between both studies may be due to genetic background. Second, Magrey *et al*. determined 175 miRNAs using a PCR array, whereas we screened 800 miRNAs in our plasma samples. In addition, there were differences in population size; 53 AS patients and 57 HDs were included in our study, while only a total of 15 patients and five HDs were recruited by Magrey *et al*. (21).

Subsequently, we evaluated the potential of using these six miRNAs as diagnostic biomarkers for AS. ROC curve analyses were performed to estimate the sensitivity and specificity of each miRNA. Individual ROC curves of these six miRNAs showed moderate predictive values for the diagnosis of AS (AUC ranges from 0.614 to 0.781), but a combination of the six miRNAs reached 0.957 (sensitivity and specificity of 90.6% and 92.5%, respectively), which seems to harbor higher accuracy than single miRNA-based assays for AS diagnosis. The majority of recent studies tend to test the diagnostic value of miRNA signatures more than individual miRNAs as reliable screening tests (31,32), since they mimic more realistically the physiological *in vivo* state, with the simultaneous presence of various miRNAs in plasma, their interaction and effects, rather than individually.

In our present study, we also compared the profile of the validated miRNAs found in AS with a cohort of patients suffering from PsA- a chronic inflammatory condition with some similarities with AS-, to evaluate the specificity of the alterations found in their expression. The levels of miR-146a-5p, miR-125a-5p, miR-151a–3p, miR-22–3p and miR-451a were found to be significantly different among AS, HDs and PsA, suggesting that the altered levels of the validated miRNAs could accurately identify AS patients. In addition, we identified the expression levels of two miRNAs (miR-150–5p and miR-451a) significantly altered in AS and PsA patients as compared with HDs. A thorough analysis

of the ROC curve to study the diagnostic potential of these two miRNAs, as well as a combination of both of them in SpA patients (AS plus PsA individuals) showed an evident diagnostic accuracy (AUC ranged from 0.617 to 0.740), indicating that both miRNAs may also be suitable biomarkers for SpA.

The bioinformatic analysis showed that several predicted biological functions and related diseases of validated miRNAs were associated with the pathology of AS (i.e. immunological disease, inflammatory disease, skeletal and muscular disorders and cell-mediated immune response, among others). In addition, a more comprehensive analysis of potential gene targets and molecular networks controlled by these altered miRNAs further suggested that they could be involved in affecting various aspects of AS, such as signaling pathways related to inflammatory response and bone remodeling. Indeed, previous reports have already demonstrated that all these miRNAs are important regulators of both processes in the organism (33–42).

To additionally explore these bioinformatic results, we performed correlation and association studies, and confirmed that these miRNAs are associated with pathophysiological status of these patients. Specifically, we found that levels of miR-146a–5p, miR-125a-5p, miR-151a-3p, miR-22–3p and miR-451a expression were significantly correlated with the plasma cytokine levels (TNF-α, IL-1β and IL-5), CRP and ESR. In addition, transfection studies in lymphocytes with three of those miRNAs, proven to be involved in inflammatory signaling (35,39,43), further supporting the *in vivo* results. Subsequently, to analyse the potential association of our six screened plasma miRNAs with pathology of AS, we first divided our patients into two subgroups: active and non-active patients. We found that plasma miR-146a-5p, miR-125a-5p and miR-22–3p expression levels were significantly up-regulated in active AS patients as compared with non-active AS patients. Furthermore, the ROC curve analysis demonstrated that these miRNAs and the combination of them as a panel presented an evident diagnostic accuracy for disease activity. These three miRNAs have been previously reported to be important in inflammatory cascade. miR146a has been shown to target interleukin-1 receptor-associated kinase (IRAK)-1, IRAK-2, tumor necrosis factor-receptor-associated factor 6 (TRAF-6) and other transcripts associated with inflammatory signaling (44,45). miR-125a targets the tumor necrosis factor alpha-induced protein 3 (TNFAIP3) (35), a key player in the termination of nuclear factor (NF)-κB signaling. miR-22 controls the activation of antigen-presenting cells and Th17 responses through the activation of activator protein (AP)-1 transcription factor complexes and the histone deacetylase HDAC4 (43). Besides, miR-22 regulates peroxisome proliferator-activated receptor alpha (PPARA) and bone morphogenetic protein 7 (BMP7) expressions as its direct target genes, subsequently inhibiting the inflammatory and catabolic changes in osteoarthritis chondrocytes (36). An aberrant expression of these 3-miRNAs has been found in different diseases such as systemic lupus erythematosus (SLE) (46–48) and rheumatoid arthritis (RA) (49–51), supporting the possibility that these three miRNAs might be implicated in inflammatory processes common to both AS and autoimmune diseases.

The characteristics of the pathogenic progression of AS are the new bone formation, appearance of syndesmophytes at the vertebral body margin and, ultimately, ankylosis of the sacroiliac joints and vertebral column. The presence of inflammation at the areas of subsequent pathological new bone formation is considered to be a required trigger; however, the rate of ossification and progressive ankylosis observed in AS appears to be multifactorial, being the precise mechanisms unknown. Various studies

have identified some miRNAs related to bone remodeling with relevant roles in the regulation of osteogenesis and osteoclasto-genesis, via interaction with signaling molecules that control these processes (34,38). Among them, we found four miRNAs, named miR-125a-5p, miR-151a-3p, miR-150–5p and miR-451a, down-regulated in AS patients with more than or equal to one syndesmophyte as compared with those syndesmophyte-free patients. Moreover, our data of ROC curves revealed that these miRNAs, as well as the combination of them as a panel could accurately distinguish AS patients with radiographic severity. In support for those results, previous studies have shown that these four miRNAs are associated to bone remodeling. miR125a-5p has been reported not only to play a negative function in osteoclatogenesis through a novel TRAF6/nuclear factor of acti-vated T-cells 1 (NFATc1)/miR-125a-5p regulatory feedback loop (52) but also to present a critical role in the inhibition of osteo-blast differentiation (53). miR151a-3p, strongly associated with lower-body mineral density, has been demonstrated to be upre-gulated in the blood of post-menopausal Chinese women with osteoporosis versus those with osteopenia (38). miR-150–5p has been suggested to increase osteoblast function and promote bone mineralization by targeting matrix metalloproteinases (MMP)-14 (42), and miR-451a has been shown to suppress phos-phorylation of p38 mitogen activated protein kinase (MAPK), a crucial regulator of bone formation (39). Additional analyses are required to elucidate the possible mechanisms underlying the down-regulation and function of these four miRNAs in AS patients.

In conclusion, we have identified six differentially expressed circulating miRNAs in patients with AS that may serve as candi-date biomarkers for diagnosis of this disease. In addition, several identified plasma miRNAs were found to be associated with dis-ease activity and structural damage present in these patients, suggesting their potential involvement in the pathogenesis of AS. The identification of differentially expressed circulating miRNAs would signify a major insight in the understanding of AS by providing an important step towards finding useful, sensi-tive and specific non-invasive diagnostic biomarkers for this disease.

Materials and Methods

Study design and participants

Seventy-six European origin SpA patients [53 AS patients (43 men and 10 women) and 23 PsA patients (13 men and 10 women)] and 57 healthy European origin donors matched for age and sex (40 men and 17 women) were included in this cross-sectional study after obtaining approval from the ethics committee of the Reina Sofía Hospital from Cordoba (Spain). Eligible patients fulfilled the modified New York criteria for the classification of AS (54) and the Classification of Psoriatic Arthritis (CASPAR) criteria for PsA (55). Exclusion criteria were: pregnancy, malignancies, chronic infections, other rheumatol-ogy and chronic diseases, patients unable to understand the procedures related to the protocol, and those receiving biologi-cal therapy. The study was conducted according to the princi-ples of the Declaration of Helsinki. All participants enrolled provided written informed consent.

All subjects underwent a comprehensive medical history, physical examination and clinical chemistry analysis before enrolment. A case report form was used to collect following data: age, gender, disease duration, presence or absence of HLA-B27 antigen, administered therapy and rheumatoid factor in

PsA patients. Disease activity was determined by the CRP (mg/l), ESR (mm/h), BASDAI and DAS28 in PsA patients. Spinal mobility of AS patients was measured by the Bath Ankylosing Spondylitis Metrology Index (BASMI). Structural damage was calculated by the mSASSS. Functionality was measured by the Bath Ankylosing Spondylitis Functionality Index (BASFI). Quality of life was quantified by the Ankylosing Spondylitis Quality of Life questionnaire (ASQoL).

Blood sample collection and assessment of biological parameters

Peripheral venous blood samples were collected in sterile tubes containing 0.129 M sodium citrate (Becton Dickinson, Meylan, France), 1.8 mg/ml K_3EDTA (Becton Dickinson) and Z Serum Sep Clot Activator (Vacuette, Madrid, Spain). To avoid blood compo-sition changes promoted by diet and circadian rhythms, sam-ples were always collected in the early hours in the morning and after a fasting period of 8 h. All the blood was processed within 4 h of collection. Plasma samples were transferred to a fresh RNase-free tube and stored at $-80°C$ until their analysis. Laboratory markers of inflammation (ESR, CRP), autoimmunity (rheumatoid factor) and genetic factors (HLA-B27) were deter-mined as part of routine patient management.

MicroRNA study phases

Two phases were designed to identify circulating miRNA profiles to act as potential biomarkers for AS. The flow chart of miRNA study is illustrated in Figure 8. Briefly, six plasma samples from three AS patients and three age- and gender-matched HDs were selected for the initial discovery phase. miRNAs from these sam-ples were extracted, pooled and used to quantify a total of 800 miRNAs by a nCounter microRNA array (NanoString Technology, Seattle, WA, USA). The nCounter miRNA panel contained probes for >95% of all human miRBase reads denoted as "high con-fidence". In addition, NanoString Technology employed propriet-ary metrics to screen potential content, and carried out literature reviews to ensure that clinically relevant miRNAs were included in the panel.

In the validation phase, the expression of candidate miRNAs from discovery phase was further validated by real time PCR in our complete cohort of AS patients ($n = 53$) and HDs ($n = 57$). A disease control cohort included 23 PsA patients.
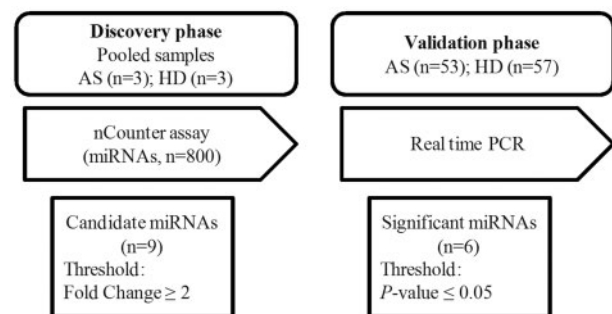
Figure 8. Flow chart of miRNA study. AS, Ankylosing spondylitis; HDs, Healthy Donors; miRNA, microRNA.

Isolation of microRNAs from plasma

Total RNA, including the miRNA fraction, was extracted from plasma by using the QIAzol miRNeasy kit (Qiagen, Valencia, CA, USA) with some modifications. A total of 200 μl of plasma were thawed on ice and lysed in 1 ml QIAzol Lysis Reagent (Qiagen). Samples were then incubated at RT for 5 min to inactivate RNases. To adjust for variations in RNA extraction and/or copurification of inhibitors, 5fmol of spike-in non-human synthetic miRNA (*C. elegans* miR-39 miRNA mimic: 5′-UCACCGGGUG UAAAUCAGCUUG-3) were added to the samples after the initial denaturation. The remaining extraction protocol was performed according to the manufacturer′s instruction. Total RNA was eluted in 14 μl of RNase-free water and stored at −80°C.

MicroRNA expression profiling

To identify the changes that occurred in the expression levels of miRNAs in plasma from AS patients as compared with HDs, a nCounter microRNA array (NanoString Technology) was performed. This array profiles the expression of 800 human microRNAs in each sample. Totally, 3 μl of RNA of two samples (each of them consist of a pool of three RNA samples from AS patients or HDs), were prepared by ligating a specific oligonucleotide tag (miR-tag) onto the 3′ end of each mature microRNA followed by an overnight hybridization (65°C) to nCounter Reporter and Capture probes. Excess Reporter and Capture probes were removed using a two-step magnetic bead-based purification on the automated nCounter prep station and probe/ target complexes were aligned and immobilized in the nCounter Cartridge. Cartridges were then placed in the nCounter digital analyzer for data collection. Abundance of specific target molecules was quantified using the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing target molecules. nSolver Analysis software (NanoString) (V2.5) was used for data analysis. A global mean normalization by using the count of the total expressed miRNAs was performed.

cDNA synthesis and real time RT-PCR

The expression levels of plasma miRNAs were measured by RT-PCR using a LightCycler® Thermal Cycler System (Roche Diagnostics, Indianapolis, Indiana, USA). Specifically, 3 μl of RNA eluate were reverse transcribed in 10 μl reactions using the miRCURY LNA™ Universal RT mi-RNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon, Vedbaek, Denmark). Real time PCR was carried out with 4 μl cDNA diluted 40x, 6 μl of reaction mixture [5 μl of SYBR Green master mix (GoTaq® qPCR Master Mix; Promega, Madison, WI, USA) and 1 μl of the corresponding PCR primer mix (microRNAs LNA™ PCR primer set, Exiqon, Vedbaek, Denmark)]. The primers are listed in Supplementary Material, Table S8. After an initial hold of 10 min at 94°C, samples were cycled 40 times at 95° for 10 s and at 60°C for 1 min. The expression levels of miRNAs were normalized to the mean of spiked-in miRNA Cel-miR-39. BestKeeper software was used to evaluate whether this miRNA was a good reference miRNA (56). After uploading each Ct value in the excel spreadsheet, the BestKeeper standard deviation value was lower than 1, thus considering this miRNA as a good stable housekeeping gene for our experimental conditions. The expression levels of miRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. All measurements were performed in duplicate. Controls consisting of reaction mixture without cDNA were negative in all runs.

For *in vitro* experiments, 200 ng of total RNA were used to quantify the expression levels of miR-125a-5p, miR-451a and miR-22–3p by RT-PCR as previously described in this section. The relative abundance of miRNAs was normalized to the mean of U6 snRNA expression.

Target gene prediction and integrated analysis by IPA

The selected miRNAs were further analysed to obtain information about biological functions, pathways and networks by using the web-based bioinformatics tool IPA (Ingenuity Systems, http://www.INGENUITY.com). For this purpose, all differentially regulated miRNAs and fold changes were imported into IPA. The right-tailed Fisher's exact test was used to calculate a P-value determining the statistical probability that the association between a set of molecules and a pathway or function might be due to chance alone. Additionally, specific targets (experimentally observed and predicted with high confidence) regulated by the differentially expressed miRNAs were also identified by using the different databases integrated in IPA software (TargetScan, TarBase, miRecords and Ingenuity® knowledge Base).

Cytokine assay

The serum concentrations of TNF-α, IL-1β and IL-5, as susceptible indirect targets of the validated miRNAs, and directly related to the pathophysiology of AS, were quantified using a Bio-Plex Pro Assay (BioRad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions, in a Bio-Plex 200 System (BioRad Laboratories). Data were obtained and analysed using the Bio-Plex Manager software version 6.0 (BioRad Laboratories).

For *in vitro* studies, the supernatant concentrations of TNF-α, IL-1β and IL-5 were determined in duplicate with commercially available ELISA kits (BioNova, Madrid, Spain) following the manufacturer's instructions.

Cell transfection

AS PBMCs from whole blood were separated using Ficoll gradient centrifugation (StemCell Technology, Oslo, Norway). Thereafter, the separation of lymphocytes from the mononuclear layer was performed by the immunomagnetic depletion of monocytes, using commercially available kits (CD14 Microbeads, human, Miltentyi Biotec Bergisch Gladbach, Germany) in an autoMACS™ Separator system (Miltentyi Biotec). The purity of the lymphocyte fraction was evaluated with specific antibodies [Fluorescein IsoTioCyanate (FITC)-conjugated anti-CD3 antibody, and phycoerythrin (PE)-conjugated anti-CD19 antibody (Miltentyi Biotec)] by flow cytometry (single-laser FACScalibur cytometer, BD Biosciences, San Jose, CA). By this method, > 90% viable lymphocytes were obtained.

Next, lymphocytes purified from AS patients were plated 1 h before transfection in 12-well plates with complete medium without antibiotics (Opti-MEM, Life Technologies, Madrid, Spain). Cell were transfected with 100 nmol/l miRNA mimic (Life Technologies, Madrid, Spain) for miR-125a-5p, miR-22–3p, miR-451a and a non-specific control (scrambled) by using siPORT™ NeoFX™ transfection agent (Life Technologies). After 48 h, the selected miRNAs and potential indirect targets (i.e. TNF-α, IL-1β, IL-5) were analysed.

Data analysis and statistics

Statistical analyses used SPSS statistical software, version 19.0 for WINDOWS (SPSS Inc., Chicago, IL). For our cross-sectional study, a statistical power calculation indicated that accepting an alpha risk of 0.05 in a two-sided test with 53 subjects in the AS group and 57 in the HD group, the statistical power in the miR-125a-5p was 98% to recognize as statistically significant the difference of means (0.004 in AS group and 0.0011 in HD group). Although we used the miR-125a-5p to set statistical power, because this miRNA was a potential biomarker for the prediction of activity and structural damage in AS patients, we were equally interested in changes in the other miRNAs of our study. The statistical power in the remaining miRNAs was at least 80%. All data in text and tables are expressed as mean ± SD. The normal distribution of variables (Gaussian data) to characterize differences in the analysed parameters was assessed using the Kolmogorov-Smirnow test. For continuous data, comparisons among variables were performed using Student's t tests or One-Way ANOVA tests for Gaussian data and using Mann–Whitney rank sum tests or Kruskal-Wallis tests for non-normally distributed data. Categorical data were analysed using the χ^2 test. Multiple linear regression analysis was performed to exclude the influences of potential confounding variables in the expression levels of miRNAs. Correlations were assessed by Spearman's rank correlation. Differences were considered significant at $P < 0.05$.

ROC curves, plotting the true positive rate (sensitivity) versus the false positive rate (1-specificity) at various threshold settings, and the AUC analysis were used to determine the sensitivity, specificity and corresponding cut-off values for each plasma miRNA using SPSS. Logistic regression was used to develop composite panels of biomarkers to identify signatures that could distinguish AS from control, SpA from control, active patients from non-active patients or presence/absence of syndesmophytes with the greatest sensitivity and specificity. ROC analysis for miRNA-combined was calculated. $P < 0.05$ was considered statistically significant.

Supplementary Material

Supplementary Material is available at *HMG* online.

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