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ORIGINAL PRE-CLINICAL SCIENCE

Diagnostic value of serum miR-144-3p for the detection of acute cellular rejection in heart transplant patients

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BACKGROUND: The development of noninvasive approaches for the early diagnosis of acute cellular rejection (ACR), an important complication of cardiac transplantation, is of great importance in clinical practice. We conducted a nontargeted transcriptomic study focused on identifying serum miRNAs to evaluate their diagnostic accuracy for detecting rejection episodes.

METHODS: We included consecutive serum samples from transplant recipients undergoing routine endomyocardial biopsies. In the discovery phase (n = 40), an RNA sequencing analysis (Illumina HiSeq 2500 sequencer) was performed. We focused on the validation of miR-144-3p in a larger patient cohort (n = 212), selected based on the criteria of higher accuracy for ACR detection. ACR was assessed according to the International Society for Heart and Lung Transplantation.

RESULTS: In the discovery phase, 26 altered miRNAs were identified as potential markers for detecting ACR. miR-144-3p showed the best results, it was the only molecule with an AUC greater than 0.95 to detect Grade ≥2R ACR and it showed significant differences in its levels when we compared Grade 1R ACR with the nonrejection group. In the validation phase, we confirmed this finding, and it had an excellent diagnostic capacity for clinically relevant rejection (Grade ≥2R AUC = 0.801, $p < 0.0001$), detecting mild rejection (Grade 1R AUC = 0.631, $p < 0.01$) and was an independent predictor for the presence of ACR (odds ratio of 14.538, $p < 0.01$).

CONCLUSIONS: ACR is associated with the differential expression of specific serum miRNAs that correlate with the severity of the episode. Circulating miR-144-3p is a candidate noninvasive ACR biomarker that could contribute to improving the surveillance of cardiac transplanted patients.

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Heart transplantation is the treatment of choice for many patients with end-stage heart failure.[1] However, despite considerable advances in transplantation, patients are at risk for developing numerous complications, such as acute rejection, which remains a major issue leading to allograft loss and mortality.[1] Currently, histological analysis of allograft biopsies is the standard rejection screening procedure but presents inherent risks and discomfort to patients, has technical limitations and has a significant economic cost.[2] Therefore, it is essential to identify noninvasive methods to improve the rejection diagnosis and surveillance of transplanted patients. Numerous studies have been carried out based on this topic.[3-13]

Developing technologies in the fields of multiomics, such as transcriptomics, are favoring the discovery of new biomarkers to improve the understanding of disease and to develop instruments for predicting and monitoring pathological conditions or treatment responses.[14] Currently, microRNAs (miRNAs) are emerging as promising biomarkers in a wide range of pathologies.[15,16] These endogenous small, noncoding RNAs that act on a posttranscriptional level in gene regulation can be easily detected in the circulation and remain in a highly stable state resistant to temperature changes.[16,17] Their remarkable stability makes these molecules potential biomarker candidates.

There is evidence indicating that miRNAs play a critical role in the cellular and immunological mechanisms regulating the process of organ rejection.[18] In recent years, these circulating molecules have attracted increasing attention as potential noninvasive biomarkers of cardiac rejection.[8-12] In fact, several studies have been performed to elucidate the possible role of serum miRNAs as cardiac rejection biomarkers, with their discovery phase based on a transcriptomic approach conducted using microarrays and PCR panels or making use of bibliographic criteria.[9,12] However, neither of these is based on a next-generation sequencing (NGS) approach. Although all of the established transcriptomic methods can be considered competing technologies, each tool possesses distinct complementary features and has been reported to have several advantages of sequencing-based genomics compared to microarray or PCR panel analysis. Specifically, RNA sequencing is an NGS method that offers increased sensitivity to detect rare sequences, and accurate single-nucleotide resolution permits the discrimination between highly related sequences. In addition, the sequence-based digital data format simplifies comparisons between datasets and permits unlimited quantitative ranges within a sample.[19] This nonpredetermined and nontargeted method makes it possible to discover markers in an unbiased manner to obtain nonselective information.

For this reason, we designed this study with a discovery phase based on RNA-sequencing methodology to evaluate whether the assessment of serum miRNAs posttransplant could serve as a noninvasive diagnostic method of acute cellular rejection (ACR). New specific biomarkers are required to detect early stages of organ injury and improve routine clinical practice. Thus, we evaluated serum miR-144-3p as a potential noninvasive marker of cardiac ACR.

Methods

Sample collection

This study included serum samples from heart transplant patients (> 18 years) who were referred for EMB as a scheduled routine screening from a single center. The samples and associated clinical data were collected from follow-up visits of cardiac transplantation recipients from The University and Polytechnic Hospital La Fe (February 2015 - June 2018). At the time of EMB, blood samples were collected for laboratory analysis. The serum was separated by centrifugation at 1500 x g for 15 min at 4˚C, aliquoted, and immediately stored at −80˚C until analysis.

First, a preliminary RNA-sequencing-based study included 40 consecutive samples from heart transplant patients. Of the patients studied, 28 had a diagnosis of biopsy-proven allograft rejection (Grade 1R, n = 16; Grade ≥2R, n = 12 (Grade 2R, n = 11 and Grade 3R, n = 1)). These samples were compared with samples from 12 patients who did not experience allograft rejection (Grade 0R). Next, we used an additional validation cohort of 212 consecutive samples (Grade 0R, n = 93; Grade 1R, n = 81; Grade ≥2R, n = 38 (Grade 2R, n = 36 and Grade 3R, n = 2)).

Patients were maintained on a standard immunosuppression regimen, and rejection episodes were assessed according to the International Society for Heart and Lung Transplantation (ISHLT) consensus report.[20] Histology of EMBs was assessed by an expert pathologist blinded to the clinical information. For each sample, we recorded age, gender, body mass index, primary heart disease, interval between transplantation and study enrolment, biochemical markers, echocardiographic parameters, and other clinical characteristics at the time of each biopsy (Table 1). Experimenters were blind to group assignment and outcome assessment, for all the experiments.

The study was approved by the Ethics Committee (Biomedical Investigation Ethics Committee of University and Polytechnic Hospital La Fe of Valencia, Spain) and was conducted in accordance to the principles outlined in the Declaration of Helsinki.[21] Prior to sample collection, an informed consent was obtained from each patient.

RNA sequencing discovery phase

This information has been extensively described in the Supplementary Appendix Material. Briefly, cDNA libraries were

Table 1 Patient Characteristics at the Time of Biopsy and Blood Sample Extraction. Serum Samples Matched in Time With Biopsies

	Discovery Phase (RNA-sequencing)		Validation Phase (RT-qPCR)	
	Non-ACR (n = 12)	ACR (n = 28)	Non-ACR (n = 93)	ACR (n = 119)
Age, years	48±15	47±13	48±13	50±12
Male sex, n (%)	9 (75)	24 (86)	76 (82)	108 (91)
Indication for cardiac transplantation				
Ischemic cardiomyopathy, n (%)	3 (25)	11 (39)	35 (38)	55 (46)
Idiopathic dilated cardiomyopathy, n (%)	5 (42)	12 (43)	42 (45)	48 (40)
Other, n (%)	4 (33)	5 (18)	16 (17)	17 (14)
Time between Tx and study enrolment, months	8.1±3.8	5.3±3.8[a]	6.5±4.1	5.8±4.1
Body mass index (kg/m^2)	25±5	25±3	24±4	25±4
Hypertension, n (%)	7 (58)	10 (36)	43 (46)	50 (42)
Diabetes mellitus, n (%)	7 (58)	16 (57)	33 (35)	52 (44)
Dyslipemia, n (%)	5 (42)	12 (43)	42 (45)	40 (34)
Ventricular assist device before Tx, n (%)	10 (82)	14 (50)	41 (44)	45 (38)
Primary graft failure, n (%)	3 (27)	5 (18)	22 (24)	37 (31)
Infection, n (%)	2 (18)	2 (7)	19 (20)	36 (30)
Echo-Doppler study				
Ejection fraction (%)	60±8	61±10	62±5	60±6
LV end systolic diameter (mm)	25±3	29 ±4[a]	28±5	28±4
LV end diastolic diameter (mm)	41±3	44±5	44±5	44±4
Hemodynamic parameters				
Mean right atrial pressure (mm Hg)	3.6±1.8	6.3±3.0	5.5±3.5	7.3±4.1[a]
Systolic right ventricular pressure (mm Hg)	33±5	37±7	36±7	37±8
Diastolic right ventricular pressure (mm Hg)	4.2±1.6	5.9±3.3	5.9±4.2	7.1±4.2
Immunosuppressive therapy				
Tacrolimus, n (%)	12 (100)	28 (100)	93 (100)	119 (100)
Mycophenolic acid, n (%)	12 (100)	28 (100)	93 (100)	115 (97)
Steroids, n (%)	12 (100)	28 (100)	93 (100)	117 (98)
Induction therapy				
Basiliximab, n (%)	12 (100)	28 (100)	93 (100)	119 (100)
Neutrophils (thousands/mm^3)	4.8 ± 4.1	5.5 ± 4.7	5.0 ± 4.1	4.8 ± 3.5
Leukocytes (thousands/mm^3)	7.1±3.8	8.2±4.8	7.5±5.0	7.2±3.7
Lymphocytes (thousands/mm^3)	1.5±0.5	1.9 ±0.7	1.7±0.9	1.9±2.5
Hemoglobin (mg/dl)	11.6±2.4	12.3±1.6	12.0±1.8	12.0±1.9
Hematocrit (%)	37±8	39±4	37±5.5	37±5.8
NT-proBNP (pg/ml)	152 (113-467)	572 (184-1513)	313 (144-687)	432 (261-1354)[a]
Troponin T (ng/l)	19 (11-66)	15 (13-33)	18 (11-50)	24 (12-52)

ACR, acute cellular rejection; LV, left ventricular; NT-proBNP, N-terminal fragment of B-type natriuretic peptide, Tx, transplantation.
[a]Non-ACR versus ACR ($p < 0.05$). Continuous variables are presented as mean ± standard deviation. NT-proBNP and Troponin T are presented as median and interquartile range and categorical variables as number of patients (n) and percentage (%).

obtained following Illumina's recommendations. The quality and quantity of the cDNA libraries were analyzed using the High-Sensitivity D1000 ScreenTape Assay and the 4200 TapeStation System (Agilent Technologies, CA, USA). The cDNA libraries were then pooled and sequenced in two lanes of 100 bp paired-end sequencing using an Illumina HiSeq 2500 sequencer.

RT-qPCR validation phase

RNA isolation, RT reaction, qPCR, and data analysis have been extensively described in the Supplementary Appendix Material. In this phase, qPCR was performed using TaqMan Advanced miRNA Assays (Applied Biosystems, Thermo Fisher Scientific, CA, USA). The geometric mean as the average Ct of the previously selected reference genes, miR-486-3p and miR-25-3p, was used to normalize the results.

Statistical analysis

Results for each variable were tested for normality using the Kolmogorov-Smirnov method. Continuous variables are presented as mean ± standard deviation (variables with normal distribution) or median and interquartile range (non-normal distributions), and categorical variables as percentage. Continuous variables not following a normal distribution were compared using the Mann-Whitney test and variables with a normal distribution were compared using the Student's *t*-test. Fisher's exact test was used to compare discrete variables. Temporal changes in miR-144-3p levels were analyzed using the paired Student's t-test.

The relativity, sensitivity, specificity, and predictive value of the miR-144-3p serum levels for the presence of transplant rejection were assessed by the construction of receiver operating characteristic (ROC) curves. To determine whether miR-144-3p was an independent predictor for grade ≥2R ACR, binary logistic regression analyses were

Table 2 Circulating Altered miRNAs for Detecting Heart Transplant Rejection

	Ensembl ID	miRNA name	*p* value[a]	
			Grade 1R ACR	Grade ≥2R ACR
1	ENSG00000199165	let-7a-5p	0.479	0.007
2	ENSG00000199133	let-7d-3p	0.086	0.044
3	ENSG00000283785	miR-15a-5p	0.858	0.046
4	ENSG00000284043	miR-20b-5p	0.181	0.045
5	ENSG00000284459	miR-24-3p	0.910	0.036
6	ENSG00000207547	miR-25-5p	0.075	0.040
7	ENSG00000199075	miR-26a-5p	0.554	0.034
8	ENSG00000199153	miR-30d-5p	0.075	0.022
9	ENSG00000284157	miR-106a-5p	0.022	0.045
10	ENSG00000207654	miR-128-3p	0.387	0.019
11	ENSG00000208017	miR-140-5p	0.113	0.007
12	ENSG00000283819	miR-144-3p	0.024	< 0.0001
13	ENSG00000283819	miR-144-5p	0.316	0.001
14	ENSG00000199122	miR-148b-3p	0.993	0.003
15	ENSG00000254324	miR-151a-3p	0.720	0.029
16	ENSG00000207759	miR-181a-5p	0.954	0.001
17	ENSG00000207581	miR-199b-5p	0.084	0.039
18	ENSG00000199038	miR-210-3p	0.276	0.012
19	ENSG00000207870	miR-221-3p	0.907	0.001
20	ENSG00000198995	miR-340-3p	0.279	0.009
21	ENSG00000199168	miR-374a-5p	0.289	0.033
22	ENSG00000207628	miR-651-5p	0.472	0.024
23	ENSG00000208013	miR-652-3p	0.023	0.001
24	ENSG00000284191	miR-671-3p	0.333	0.021
25	ENSG00000284458	miR-1304-3p	0.403	0.035
26	ENSG00000276102	miR-6747-3p	0.038	0.018

ACR, acute cellular rejection.
[a]Non-ACR versus ACR (Grade 1R and ≥Grade 2R).

performed with all relevant variables included in the models. An incremental approach was used for the continuous variables (the calculated odds ratios are related to the increase in the indicated continuous variable by a given amount), and dichotomous risk factors were coded with an indicator variable of 1 for having the condition and 0 for its absence. Discrimination was assessed through the C statistic. In addition, a multivariate linear regression analysis was performed using miR-144-3p serum levels as the dependent variable and included the same variables in the model as in the binary logistic regression analysis. All statistical analyses were performed using SPSS software (version 20.0; IBM SPSS Inc.). A $p < 0.05$ was considered statistically significant.

For the NGS process, the reads were mapped against the last version of the human genome (hg38) using bowtie algorithms.[22] No mismatches were allowed in this step. Then, the low-quality mapped reads were eliminated using a mapping quality threshold of 20. For the quantification process, the mature coordinates of the human miRNAs were obtained from the miRBase database,[23] and the htseq-count method was used.[24] For differential expression, the DESEQ algorithm was applied.[25] Finally, for differential miRNA expression selection, a *p*-value adjusted by FDR of 0.05 and fold change of +/- 1.5 was used.

Results

Clinical characteristics

Both populations of the study, the transcriptomic analysis and the validation cohort, had similar clinical characteristics (Table 1). The ACR and non-ACR groups were similar with regard to variables such as age, sex, body mass index, hypertension, diabetes, dyslipidemia, immunosuppressive and induction therapy, lymphocyte number, hemoglobin concentration and hematocrit percentage, among others. In the validation cohort, patients with ACR displayed significant differences between specific clinical characteristics when we compared them with the nonrejection group and they showed worse hemodynamic function and an increase in N-terminal pro−B-type natriuretic peptide (NT-proBNP) levels.

Circulating miRNAs predict acute cellular rejection

Transcriptomic differences between the rejection and nonrejection groups were examined by large-scale screening of serum samples using RNA-sequencing technology. The number of useful reads obtained from all samples ranged between 11 and 29 million. We found a total of 26 differentially expressed miRNAs in the serum of patients with clinically relevant rejection (Grade ≥2R) (Table 2), and 4 of them maintained these differences in mild grade (Grade 1R): miR-144-3p (Figure 1A), miR-106a-5p (Figure 1B), miR-6747-3p (Figure 1C) and miR-652-3p (Figure 1D). The main differences were the expression levels of miR-

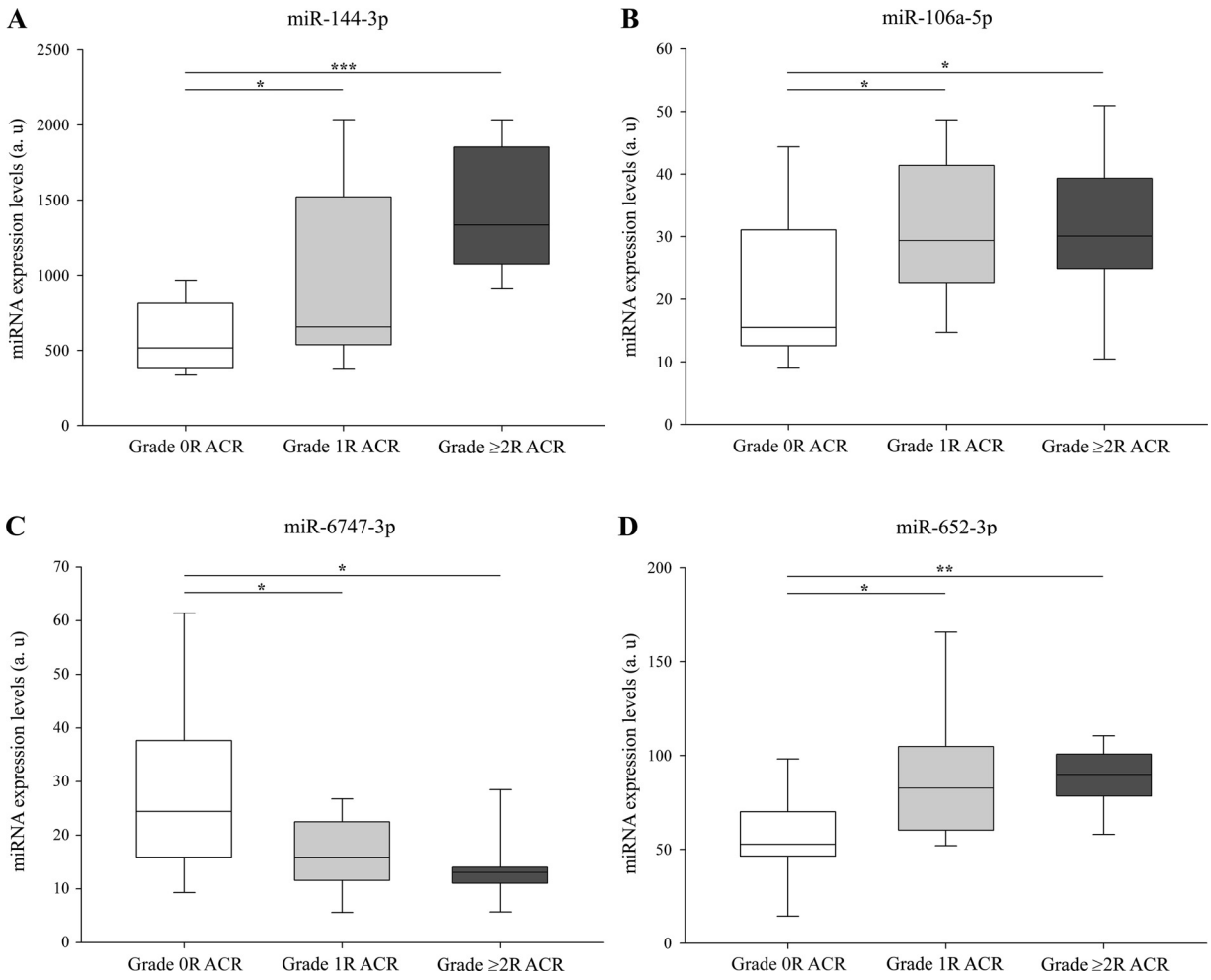

Figure 1 Serum levels of miRNAs between normal and rejected heart allografts from the discovery cohort. Comparison of the levels of miR-144-3p (A), miR-106a-5p (B), miR-6747-3p (C), and miR-652-3p (D) among the groups of patients (nonrejection Grade 0R, acute cellular rejection (ACR) of Grade 1R and Grade ≥2R). The middle line in the boxplots represents the median, the lower box bounds the first quartile, the upper box bounds the third quartile, and the whiskers represent the 95% confidence interval of the mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

144-3p, which showed a significant increase for the grade ≥2R group ($p < 0.0001$) and a significant fold change (median 1.83 [1.47 - 2.56], $p < 0.0001$) with respect to the nonrejection group (Figure 2A).

ROC curves were generated to analyze the capability of the altered miRNAs to detect heart transplant rejection. These data are summarized in Table 3. The best ROC curve to detect heart transplant rejection (Grade ≥2R) corresponded to miR-144-3p, obtaining a significant area under the curve of 0.983 with a standard error of 0.022 [95% CI, 0.941 - 1.000; $p < 0.0001$]; (Figure 2B) with high sensitivity, specificity, positive predictive value, and negative predictive value (92%, 90%, 92%, and 100%, respectively). Our results show that miR-144-3p is the only molecule that has an AUC greater than 0.95 to detect patients with a moderate or severe degree of rejection (Grade ≥2R), and it presents significant differences in its levels when we compare the group of patients without rejection with the group of patients with mild rejection (Grade 1R, $p < 0.05$ Figure 1A, Table 2).

Validation of circulating miR-144-3p

Taking into account the excellent diagnostic value observed with miR-144-3p for grade ≥2R ACR (Table 3 and Figure 2A), its high expression serum levels and the significant differences found between the grades of rejection (Figure 1A), we decided to validate these results in a larger patient cohort (n = 212 consecutive serum samples). As shown in Figure 3A, miR-144-3p serum levels were higher in patients with heart transplant rejection (all grades) than in the nonrejection group (median 6.36 [2.06 - 13.77] arbitrary units versus 2.70 [1.05 - 6.05] arbitrary units, $p < 0.0001$). When we compared patients with allograft rejection of Grade 1R and ≥2R independently with the nonrejection group (Figure 3B), we found significant differences for all comparisons (Grade 1R median: 5.12 [1.47 - 10.44] arbitrary units $p < 0.05$; Grade ≥2R median: 8.63 [4.13 - 19.33] arbitrary units, $p < 0.0001$). In addition, we found significant differences between Grade 1R and ≥2R ($p < 0.05$). We obtained a significant fold change for Grade ≥2R

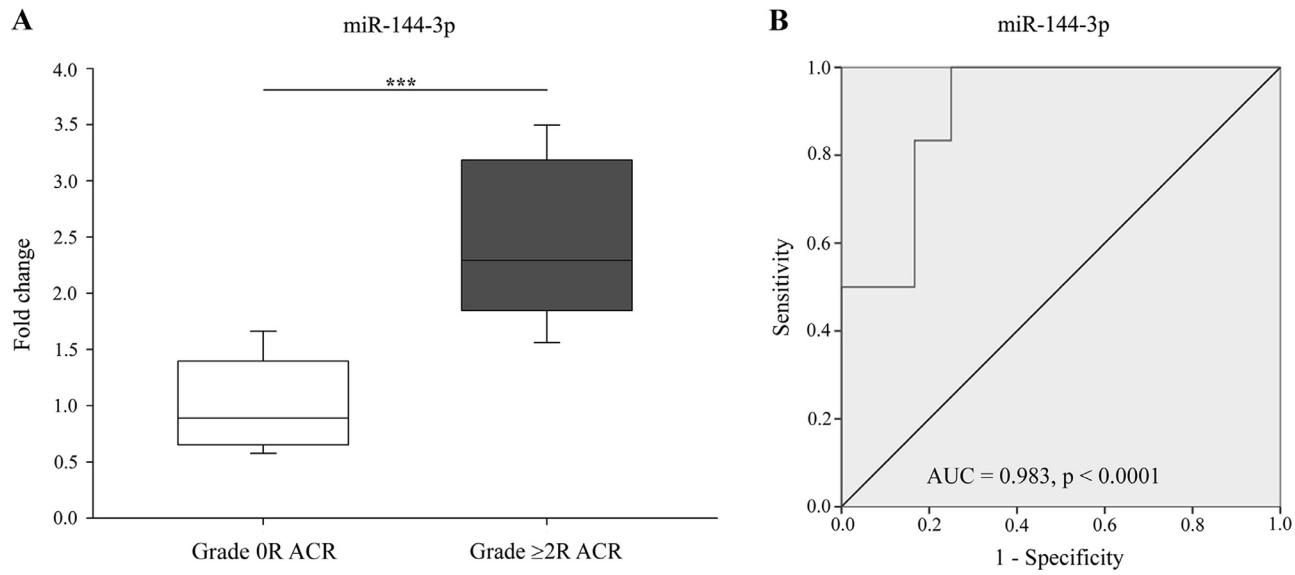

Figure 2 miR-144-3p expression in the discovery phase. Comparison of the miR-144-3p fold change value between Grade 0R non-acute cellular rejection (ACR) and grade ≥2R ACR (A). The middle line in the boxplots represents the median, the lower box bound the first quartile, the upper box bound the third quartile, and the whiskers represent the 95% confidence interval of the mean. ***$p < 0.0001$. Receiver operating characteristic (ROC) curve based on the primary analysis of serum levels of miR-144-3p for the detection of ACR of Grade ≥2R (B).

(median 2.29 [1.09 - 5.14], $p < 0.0001$) with respect to the nonrejection group (Figure 4A).

Furthermore, to investigate whether circulating miR-144-3p is an independent predictor of grade ≥2R ACR, binary logistic regression was performed, adjusting the model by age, sex, and immunosuppressor treatment and including other variables of interest, specifically hypertension, diabetes mellitus, time between transplant and EMB, ventricular assist device before transplantation, infection, mean right atrial pressure, and primary graft dysfunction. Serum miR-144-3p (value of fold change ≥1.5, optimum cut-off point obtained from the ROC curve) was the most significant predictor for the presence of grade ≥2R ACR, with an odds ratio of 14.538 (95% CI 2.348 - 90.02, $p < 0.01$) and C statistic of 0.920 (95% CI 0.844 - 0.995, $p < 0.0001$). The time between transplantation and study enrolment also was independent predictor of rejection ($p < 0.01$), but with an odds ratio < 1. All other variables included in the model failed to reach statistical significance, but the mean right atrial pressure showed a statistical trend ($p = 0.060$) (*Supplementary Table 1*). Then, a multivariate linear regression analysis was performed using log-

Table 3 Receiver-Operating Characteristic (ROC) Curve of Circulating Altered miRNAs for Detecting Heart Transplant Rejection (ACR grade ≥2R). Sensitivities, Specificities and Predictive Values (%) for the Diagnosis of Cardiac Rejection (Cut-off Point FC > 1.5)

miRNA	AUC	*p* value	95% CI	SS	SP	VPP	VPN
miR-144-3p	0.983	<0.0001	0.941-1.000	92	90	92	100
miR-652-3p	0.896	0.001	0.754-1.000	58	92	89	73
miR-144-5p	0.889	0.001	0.749-1.000	58	92	88	69
miR-221-3p	0.868	0.002	0.715-1.000	17	100	100	55
let-7a-5p	0.861	0.003	0.705-1.000	17	92	67	52
miR-181a-5p	0.861	0.003	0.705-1.000	0	100	0	50
miR-6747-3p	0.813	0.009	0.615-1.000	75	83	81	77
miR-140-5p	0.806	0.011	0.620-0.991	42	100	100	67
let-7d-3p	0.806	0.011	0.620-0.991	17	100	67	52
miR-340-3p	0.792	0.015	0.610-0.973	25	100	100	60
miR-210-3p	0.785	0.018	0.598-0.972	50	92	86	65
miR-651-5p	0.764	0.028	0.570-0.958	25	92	80	58
miR-1304-3p	0.764	0.028	0.573-0.954	42	92	86	65
miR-374a-5p	0.764	0.028	0.560-0.968	42	83	75	63
miR-671-3p	0.757	0.033	0.560-0.954	33	92	80	58
miR-20b-5p	0.750	0.038	0.539-0.961	58	83	70	64
miR-26a-5p	0.743	0.043	0.543-0.943	17	92	67	52
miR-25-5p	0.743	0.043	0.533-0.953	50	92	88	69

AUC, area under the curve; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; SS, Sensitivity; SP, specificity.

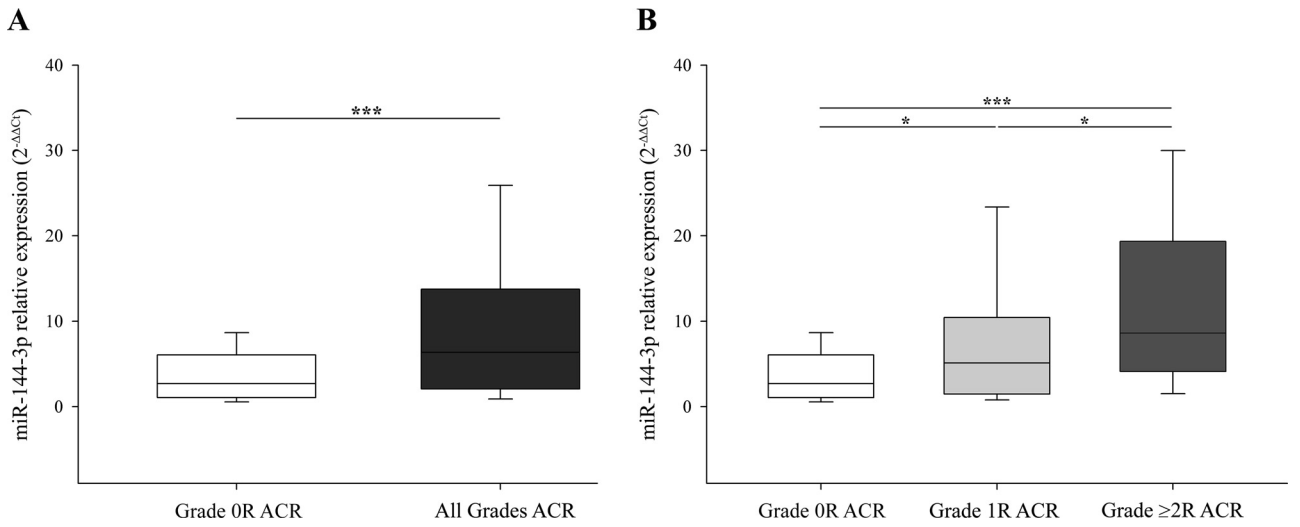

Figure 3 Validation of serum levels of miR-144-3p between nonrejection and rejection heart allografts. Comparison between nonrejection vs. all grades of rejection heart allografts (A). Comparison of the levels of miR-144-3p among the groups of patients (nonrejection Grade 0R, acute cellular rejection (ACR) of Grade 1R and Grade ≥2R). The middle line in the boxplots represents the median, the lower box bound the first quartile, the upper box bound the third quartile, and the whiskers represent the 95% confidence interval of the mean. *$p < 0.05$, ***$p < 0.0001$.

transformed miR-144-3p as the dependent variable to test the independent predictive power of the same covariates included in the binary logistic regression in addition to the presence of grade ≥2R ACR. This model included grade ≥2R ACR as the only independent factor of the levels of miR-144-3p ($p < 0.0001$) (*Supplementary Table 2*).

ROC curves confirmed the capability of miR-144-3p to detect heart transplant rejection (Grade ≥2R), obtaining a significant area under the curve of 0.801 with a standard error of 0.042 [95% CI, 0.719 - 0.884; $p < 0.0001$] (Figure 4B). The sensitivity, specificity, positive predictive value, and negative predictive value for the diagnosis of rejection were 68%, 73%, 50%, 85%, respectively. In addition, miR-144-3p had a significant discrimination capability for Grade 1R (area under the curve of 0.631 with a standard error of 0.043 [95% CI, 0.547 - 0.715; $p < 0.01$].

The levels of miR-144-3p were dynamics with allograft status, not only increased during rejection but that also decreased as the rejection resolved. We included the patients with a Grade ≥2R and a subsequent Grade 0R (n = 10), Grade 0R corresponds to the first 0R biopsy after the ≥2R rejection (in most of the patients, before the Grade 0R biopsy that followed the Grade ≥2R, there were several 2R or 1R episodes). miR-144-

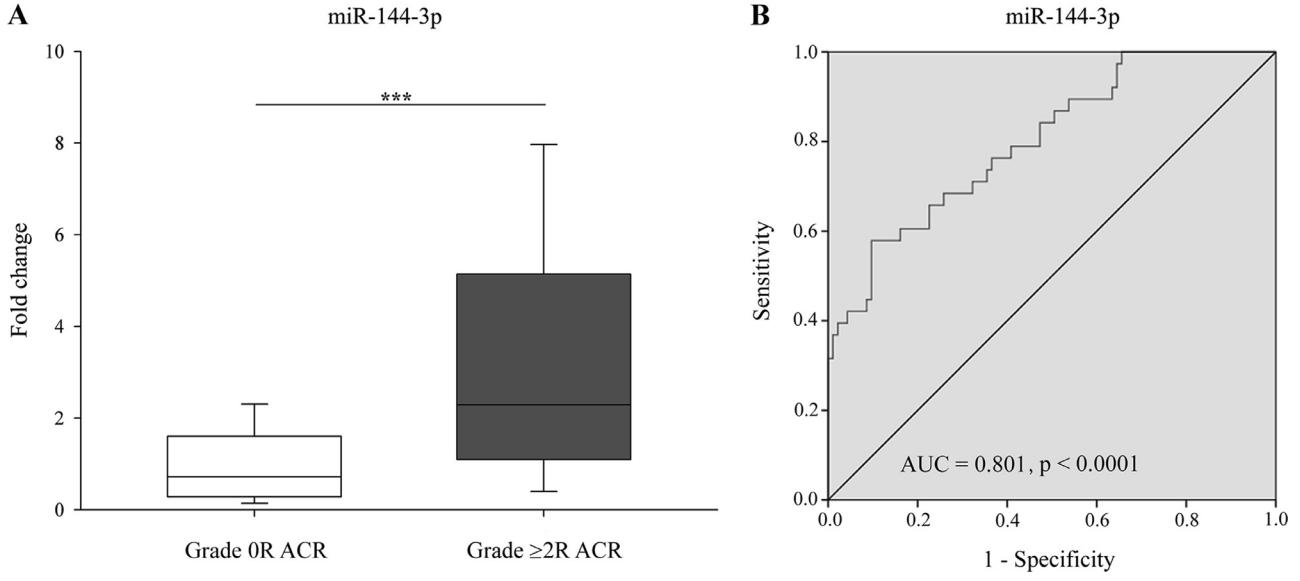

Figure 4 miR-144-3p expression in the validation phase. Comparison of the miR-144-3p fold change value between Grade 0R non-acute cellular rejection (ACR) and grade ≥2R ACR (A). The middle line in the boxplots represents the median, the lower box bound the first quartile, the upper box bound the third quartile, and the whiskers represent the 95% confidence interval of the mean. ***$p < 0.0001$. Receiver operating characteristic (ROC) curve based on the primary analysis of serum levels of miR-144-3p for the detection of ACR of Grade ≥2R (B).

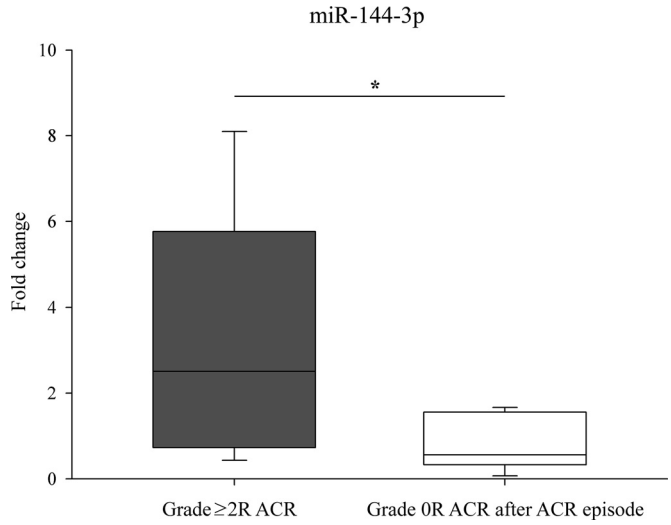

Figure 5 Significant differentially expressed miR-144-3p during ACR episode. Comparison of the miR-144-3p fold change value between grade ≥2R ACR and the subsequent Grade 0R. The middle line in the boxplots represents the median, the lower box bound the first quartile, the upper box bound the third quartile, and the whiskers represent the 95% confidence interval of the mean. *$p < 0.05$.

3p returns to basal levels when the rejection is resolved ($p < 0.05$) (Figure 5).

Discussion

Avoiding and treating acute rejection is an essential challenge in the heart transplantation area because immune injury is an important contributor to graft failure[26] and cardiac allograft vasculopathy,[27] which remain a leading cause of death. Therefore, deeper knowledge of the mechanisms preceding rejection and a diagnosis in early stages is required to further improve the survival of transplanted patients. Although the gold standard of the diagnosis of cardiac allograft rejection is EMB,[28,29] this procedure has negative consequences for patients[2] and is subject to sampling error and interobserver variability.[30] Due to these important risks and limitations, numerous studies have focused on identifying a method of noninvasive monitoring of acute and chronic rejection after cardiac transplantation.[3-13] It is crucial to develop complementary methods for the present tools, as well as provide a non-invasive alternative to EMB, to achieve an optimal diagnostic approach to diagnose cardiac allograft rejection, especially in its early phase.

To the best of our knowledge, this is the first RNA-sequencing analysis applied in a discovery phase to explore serum miRNAs as potential candidate biomarkers of cardiac allograft rejection. With NGS technology, we detected and quantified circulating miRNAs with good accuracy and showed significant differential levels of 26 of them. We focused the study on miR-144-3p, a molecule with a remarkable detection power for Grade ≥2R ACR and with significant overexpression in mild grade rejection patients compared with nonrejection patients. These findings were confirmed in the validation phase using a larger cohort of patients. We showed that miR-144-3p strongly discriminated patients with allograft rejection Grade 2R or greater, while maintaining a significant detection capability for rejection episodes of mild grade. In addition, when multivariate analyses were performed, only miR-144-3p was an independent predictor of ACR after adjusting the models for age, sex, immunosuppressive treatment and relevant clinical variables. Thus, serum miR-144-3p is not altered by these conditions, which adds validity to our results. In addition, it is important to note that the levels of miR-144-3p are sensitive to changes in the allograft status, its levels increased during rejection and decreased as the rejection resolved.

Their remarkable stability at room temperature and the fact that they are minimally affected by freeze-thawing cycles,[31] make serum miRNAs suitable as biomarkers in clinical practice where variations in the treatment of samples after collection occur. miRNAs have emerged as promising biomarker candidates in the field of heart transplantation, reflecting their involvement in the regulation of processes such as endothelial activation,[32] cardiac tissue remodeling[33] and inflammation.[34] In fact, increasing the levels of miR-144-3p by mimics has been shown to enhance the expression of inflammatory factors, including IL-1β, IL-6 and TNFα, in vivo and in vitro.[35]

Sukma Dewi et al,[10] in a small pilot study, selected seven miRNAs in an initial screening to assess the possibility of using serum miRNAs of biomarkers of ACR after heart transplantation. Six of them maintained significant discrimination between normal and diseased states in a subsequent validation phase by qPCR, and miR-144-3p was presented as a potential biomarker candidate for noninvasive allograft surveillance after heart transplantation, as further confirmed in this study. Next, they conducted a new study in an independent cohort showing that the levels of the same miRNAs, including miR-144-3p, were significantly higher in the ACR group than in the controls and that each miRNA could discriminate between patients with and without ACR.[11] It should be noted that among the hundreds of miRNAs detected in the serum of transplanted patients included in our study, our findings agreed with these two previous studies to propose miR-144-3p as a potential

candidate, but from a larger cohort with 212 consecutive serum samples. We found this concordance despite the large heterogeneity among the studies in terms of clinical setting, experimental design, and analytical methods used by the authors to identify candidate biomarkers of ACR. All of these variables, in addition to the miRNA biological variation, add extra complexity and hinder the reproducibility of the different analyses. In fact, other authors have proposed different miRNAs as ACR biomarkers.[9,12] In this context, with the purpose of contributing to the analytic standardization, we have carefully followed the different recommendations in every phase of the process.

On the other hand, with RNA-sequencing technology, we detected a higher number of serum miRNAs in contrast to previous studies based on qPCR panels or microarrays with a priori selected molecules. In addition, we chose next-generation sequencing for the considerable advantages of this methodology as previously described.[36] Thus, we opted for a nonpredetermined and nontargeted method to discover cardiac rejection markers in an unbiased manner to obtain nonselective information. In fact, some markers of rejection found in our study, such as miR-6747-3p, were not included in the qPCR panels used to detect ACR biomarkers previously.[12]

The discovery of biomarkers of cardiac rejection not only in clinically relevant grades but also in mild rejection grades could improve our knowledge of underlying physiopathological mechanisms by early detection of damage, when pathological changes are only taking place at the molecular level, and could be used to predict an upcoming rejection prior to any organ damage. Unlike previous studies, we also assessed the relationship of the serum miRNAs detected with the severity of the rejection episode, obtaining significant changes in miR-6747-3p, miR-106a-5p, and miR-652-3p in addition to miR-144-3p in patients with the rejection of Grade 1R compared with the nonrejection group.

Nowadays, Gene Expression Profiling (Allomap) is the only non-invasive analytical test included in ISHLT guidelines to identify the risk of ACR in heart transplant recipients. However, it lacks a good positive predictive value, it can only be used to rule out the presence of ACR grade 2R or greater, and it has not shown to distinguish between antibody-mediated rejection (AMR) and cellular rejection.[3] In addition, other genomic analyses have been emerging. Specifically, percent donor-derived cell-free DNA (%ddcfDNA) is a precise and reproducible marker of acute rejection. %ddcfDNA it has demonstrated excellent performance characteristics for both ACR and AMR, correlate with severity of acute rejection grades and allograft dysfunction, and unmasks pathology earlier than existing tools.[13]

In comparing miR-144-3p to other promising noninvasive expression analysis tests, such as AlloMap[3] and %ddcfDNA,[13] there are some similarities and differences. In the present study, we have worked on serum samples using a relatively inexpensive, established, and feasible technology based on standard qPCR isolating the small RNA directly from the serum of transplanted patients. The Allomap test is also based on qPCR, but first is necessary to isolate peripheral blood mononuclear cells, a more complex process. AlloMap test showed an AUC of 0.70, lower than the AUC here obtained for miR-144-3p or the obtained for %ddcfDNA. These three approaches to monitoring organ health present good negative predictive values for the respective cut-off points selected (Allomap 96%, %ddcfDNA 99%, miR-144-3p 85%), but only %ddcfDNA and miR-144-3p correlate with the severity of rejection. Our work has focused on cellular rejection and have not specifically evaluated AMR, however monitoring with ddcfDNA, demonstrated excellent performance characteristics for both ACR and AMR. Thus, although miR-144-3p could be a promising biomarker, we show a preliminary analysis that may be validated in larger patient cohorts to lead to the use of this relatively simple non-invasive determination as a complement of other analytical approaches and also as an alternative to EMB.

Our study has several limitations, and the results must be interpreted within this context. This investigation only involved a single center, and the findings obtained should be assessed in a larger multicenter study in which the effect of the different treatments will also be analyzed. In addition, our study is focused on cellular rejection and has not specifically evaluated AMR. However, we believe that our findings have provided substantial evidence and represent a necessary first step to support future research in which these limiting factors could be overcome.

In conclusion, we have demonstrated that episodes of ACR are associated with differential expression of specific serum miRNAs and that they correlate with the severity of the rejection episode. Circulating miR-144-3p is a candidate to be integrated into future panel studies as a noninvasive ACR biomarker. This finding contributes to the progress in diagnostic and preventive methods for the surveillance of cardiac transplanted patients.

Author contributions

E.R.L. conceived the original idea, supervised the project and wrote the main manuscript text. E.R.L. M.P. and E.T. planned the experiments and study design. I.S.L. and L.M.D. provided the samples. L.P.C. collected and processed the samples. E.T. and J.T. carried out the experiments. F.L., J.R.G.J., S.F.B., L.M.D., I.S.L., J.T., E.T., E.R.L. and M.P. contributed to data analysis and interpretation of results. All authors contributed to the final version of the manuscript.

Disclosure statement

The authors of this manuscript have no conflicts of interest to disclose.

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Supplementary materials

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References

1. Khush KK, Cherikh WS, Chambers DC, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-sixth adult heart transplantation report - 2019; focus theme: Donor and recipient size match. J Heart Lung Transplant 2019;38:1056-66. https://doi.org/10.1016/j.healun.2019.08.004.
2. Cooper LT, Baughman KL, Feldman AM, et al. The role of endomyocardial biopsy in the management of cardiovascular disease: a scientific statement from the American Heart Association, the American College of Cardiology, and the European Society of Cardiology. Endorsed by the Heart Failure Society of America and the Heart Failure Association of the European Society of Cardiology. J Am Coll Cardiol 2007;50:1914-31. https://doi.org/10.1016/j.jacc.2007.09.008.
3. Crespo-Leiro MG, Stypmann J, Schulz U, et al. Clinical usefulness of gene-expression profile to rule out acute rejection after heart transplantation: CARGO II. Eur Heart J 2016;37:2591-601. https://doi.org/10.1093/eurheartj/ehv682.
4. Tarazón E, Ortega A, Gil-Cayuela C, et al. SERCA2a: A potential non-invasive biomarker of cardiac allograft rejection. J Heart Lung Transplant 2017;36:1322-8. https://doi.org/10.1016/j.healun.2017.07.003.
5. - Kennel PJ, Saha A, Maldonado DA, et al. Serum exosomal protein profiling for the non-invasive detection of cardiac allograft rejection. J Heart Lung Transplant 2018;37:409-17. https://doi.org/10.1016/j.healun.2017.07.012.
6. Tarazón E, Corbacho-Alonso N, Barderas MG, et al. Plasma CD5L and non-invasive diagnosis of acute heart rejection. J Heart Lung Transplant 2020;39:257-66. https://doi.org/10.1016/j.healun.2019.11.004.
7. Tarazón E, Pérez-Carrillo L, García-Bolufer P, et al. Circulating mitochondrial genes detect acute cardiac allograft rejection: Role of the mitochondrial calcium uniporter complex. Am J Transplant 2021;21:2056-66. https://doi.org/10.1111/ajt.16387.
8. Di Francesco A, Fedrigo M, Santovito D, et al. MicroRNA signatures in cardiac biopsies and detection of allograft rejection. J Heart Lung Transplant 2018;37:1329-40. https://doi.org/10.1016/j.healun.2018.06.010.
9. Duong Van Huyen JP, Tible M, Gay A, et al. MicroRNAs as non-invasive biomarkers of heart transplant rejection. Eur Heart J 2014;35:3194-202. https://doi.org/10.1093/eurheartj/ehu346.
10. Sukma Dewi I, Torngren K, Gidlöf O, Kornhall B, Ohman J. Altered serum miRNA profiles during acute rejection after heart transplantation: potential for non-invasive allograft surveillance. J Heart Lung Transplant 2013;32:463-6. https://doi.org/10.1016/j.healun.2012.12.007.
11. Sukma Dewi I, Hollander Z, Lam KK, et al. Association of serum MiR-142-3p and MiR-101-3p levels with acute cellular rejection after heart transplantation. PLoS One 2017;12:e0170842. https://doi.org/10.1371/journal.pone.0170842.
12. Constanso-Conde I, Hermida-Prieto M, Barge-Caballero E, et al. Circulating miR-181a-5p as a new biomarker for acute cellular rejection in heart transplantation. J Heart Lung Transplant 2020;39:1100-8. https://doi.org/10.1016/j.healun.2020.05.018.
13. Agbor-Enoh S, Shah P, Tunc I, et al. Cell-free DNA to detect heart allograft acute rejection. Circulation 2021;143:1184-97. https://doi.org/10.1161/CIRCULATIONAHA.120.049098.
14. Shah P, Valantine HA, Agbor-Enoh S. Transcriptomics in transplantation: more than just biomarkers of allograft rejection. Am J Transplant 2021;21:2000-1. https://doi.org/10.1111/ajt.16429.
15. Reilly SN, Liu X, Carnicer R, et al. Up-regulation of miR-31 in human atrial fibrillation begets the arrhythmia by depleting dystrophin and neuronal nitric oxide synthase. Sci Transl Med 2016;8. https://doi.org/10.1126/scitranslmed.aac4296. 340ra74.
16. Wang J, Chen J, Sen S. MicroRNA as biomarkers and diagnostics. J Cell Physiol 2016;231:25-30. https://doi.org/10.1002/jcp.25056.
17. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-97. https://doi.org/10.1016/s0092-8674(04)00045-5.
18. Sarma NJ, Tiriveedhi V, Ramachandran S, Crippin J, Chapman W, Mohanakumar T. Modulation of immune responses following solid organ transplantation by microRNA. Exp Mol Pathol 2012;93:378-85. https://doi.org/10.1016/j.yexmp.2012.09.020.
19. Hurd PJ, Nelson CJ. Advantages of next-generation sequencing versus the microarray in epigenetic research. Brief Funct Genomic Proteomic 2009;8:174-83. https://doi.org/10.1093/bfgp/elp013.
20. Stewart S, Winters GL, Fishbein MC, et al. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. J Heart Lung Transplant 2005;24:1710-20. https://doi.org/10.1016/j.healun.2005.03.019.
21. Macrae DJ. The Council for International Organizations and Medical Sciences (CIOMS) guidelines on ethics of clinical trials. Proc Am Thorac Soc 2007;4:176-8. https://doi.org/10.1513/pats.200701-011GC.
22. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009;10:R25. https://doi.org/10.1186/gb-2009-10-3-r25.
23. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. Nucleic Acids Res 2019;47:D155-62. https://doi.org/10.1093/nar/gky1141.
24. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 2015;31:166-9. https://doi.org/10.1093/bioinformatics/btu638.
25. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550. https://doi.org/10.1186/s13059-014-0550-8.
26. Lund LH, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirtieth Official Adult Heart Transplant Report—2013; focus theme: age. J Heart Lung Transplant 2013;32:951-64. https://doi.org/10.1016/j.healun.2013.08.006.
27. Stoica SC, Cafferty F, Pauriah M, et al. The cumulative effect of acute rejection on development of cardiac allograft vasculopathy. J Heart Lung Transplant 2006;25:420-5. https://doi.org/10.1016/j.healun.2005.11.449.
28. Gradek WQ, D'Amico C, Smith AL, Vega D, Book WM. Routine surveillance endomyocardial biopsy continues to detect significant rejection late after heart transplantation. J Heart Lung Transplant 2001;20:497-502. https://doi.org/10.1016/s1053-2498(01)00236-4.
29. Costanzo MR, Dipchand A, Starling R, et al. The International Society of Heart and Lung Transplantation Guidelines for the care of heart transplant recipients. J Heart Lung Transplant 2010;29:914-56. https://doi.org/10.1016/j.healun.2010.05.034.
30. Nielsen H, Sørensen FB, Nielsen B, Bagger JP, Thayssen P, Baandrup U. Reproducibility of the acute rejection diagnosis in human cardiac allografts. The Stanford Classification and the International Grading System. J Heart Lung Transplant 1993;12:239-43.
31. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105:10513-8. https://doi.org/10.1073/pnas.0804549105.
32. Loyer X, Potteaux S, Vion AC, et al. Inhibition of microRNA-92a prevents endothelial dysfunction and atherosclerosis in mice. Circ Res 2014;114:434-43. https://doi.org/10.1161/CIRCRESAHA.114.302213.
33. Ji X, Takahashi R, Hiura Y, Hirokawa G, Fukushima Y, Iwai N. Plasma miR-208 as a biomarker of myocardial injury. Clin Chem 2009;55:1944-9. https://doi.org/10.1373/clinchem.2009.125310.

34. Fang Y, Shi C, Manduchi E, Civelek M, Davies PF. MicroRNA-10a regulation of proinflammatory phenotype inathero-susceptible endotheliumin vivo and in vitro. ProcNatl Acad Sci USA 2010;107:13450-5. https://doi.org/10.1073/pnas.1002120107.
35. Hu YW, Hu YR, Zhao JY, et al. An agomir of miR-144-3p accelerates plaque formation through impairing reverse cholesterol transport and promoting pro-inflammatory cytokine production. PLoS One 2014;9: e94997. https://doi.org/10.1371/journal.pone.0094997.
36. Rai MF, Tycksen ED, Sandell LJ, Brophy RH. Advantages of RNA-seq compared to RNA microarrays for transcriptome profiling of anterior cruciate ligament tears. J Orthop Res 2018;36:484-97. https://doi.org/10.1002/jor.23661.