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Validation of the clinical utility of microRNA as noninvasive biomarkers of cardiac allograft rejection: A prospective longitudinal multicenter study

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KEYWORDS:

heart transplantation;; allograft rejection;; noninvasive biomarker;; endomyocardial biopsy.

List of abbreviations:

ACR, acute cellular rejection; AMR, antibody-mediated rejection; EMB, endomyocardial biopsy; miR, micro-RNA; OR, odds ratio

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While studies have shown an association between microRNAs and cardiac rejection, the clinical relevance of a pre-identified miRNA signature as a noninvasive biomarker has never been assessed in prospective multicentric unselected cohorts. To address this unmet need, we designed a prospective study (NCT02672683) including recipients from 11 centers between August 2016 to March 2018. The objective was to validate the association between 3 previously identified circulating microRNA (10a, 92a, 155) and the histopathological diagnosis of rejection. Both relative and absolute (sensitivity analysis) quantifications of microRNAs were performed. Overall, 461 patients were included (831 biopsies, 79 rejections). A per-protocol interim analysis (258 biopsies, 49 rejections) did not find any association between microRNA and rejection (microRNA 10a: odds ratio (OR) = 1.05, 95% confidence intervals (CI) = 0.87-1.27, $p = 0.61$; 92a: OR = 0.98, 95%CI = 0.87-1.10, $p = 0.68$; 155: OR = 0.91, 95%CI = 0.76-1.10, $p = 0.33$). These results were confirmed in the sensitivity analysis. The analysis of the remaining sera was stopped for futility. This study shows no clinical utility of circulating microRNAs 10a, 92a, and 155 monitoring in heart allograft recipients

Circulating microRNAs (miR) have been identified as potential valuable noninvasive biomarkers of cardiac allograft rejection that may help clinicians minimize the number of protocol endomyocardial biopsies (EMB).¹ However, the majority of literature is based on retrospective cross-sectional studies at risk of bias.²⁻⁵ The prospective validation of an miR rejection signature in independent cohorts is therefore crucial. In a multicenter case-control study, we previously identified an miR signature of rejection with differential tissue and serum expression between rejecting and normal heart allografts.⁶ The assessment of circulating miR permitted discrimination with notably high accuracy between patients with and without allograft rejection. We designed a longitudinal prospective multicenter observational study aimed at validating this circulating miR signature of cardiac rejection (Non-Invasive Detection of Cardiac Allograft Rejection by Circulating microRNAs, NCT02672683).

Patients were recruited from 11 transplant centers across the country following written informed consent for a protocol approved by our local institutional review board (Ile de France IV, IRB number = 00003835, protocol 201554NICB). Our study complies with the ISHLT ethics statement. The primary objective was to prospectively validate the association between 3 previously identified circulating miR and the histopathological diagnosis of rejection on concomitant EMB. The rejection was defined as an acute cellular rejection (ACR) $\geq 2R$ and/or an antibody-mediated rejection (AMR) $\geq pAMR1$ according to international guidelines.^{7,8} Patients were included from August 2016 to March 2018 and followed during 1 year.

The inclusion criteria were adult heart transplant recipient with < 10 post-transplant years undergoing EMB. The exclusion criterion was multiorgan transplantation. The sample size was calculated based on an estimation of a 33% differential expression of circulating miR between rejection and no rejection, with a 0.05 significance level and 0.90 power and considering a rejection rate of 7% (n = 820). Sera samples were collected just before the EMB (i) for all for-cause biopsies; (ii) at 1-, 3-, 6-, and 12- months post- transplant for de-novo transplant recipients; and (iii) during annual visits for recipients transplanted after 1 year. miR 10a, miR 92a, and miR 155 were measured in triplicate in sera. Relative quantification of the miR of interest was performed by normalizing miR copy numbers using cel- miR-39 to obtain ΔCt values, as previously described.⁶ $\Delta\Delta\text{Ct}$ was then obtained by subtracting the average of ΔCt to each ΔCt values. Finally, we determined fold values for each sample ($2^{(-\Delta\Delta\text{Ct})}$). Sensitivity analysis was performed with an absolute quantification analysis of the miR copy numbers using miR mimics standard dilution curves (Supplementary Methods).⁹ We followed the STROBE statement checklist for the report of observational cohort studies.¹⁰ The selection of sera for interim analyses is described in the Supplementary Methods. The association between circulating miR expression and rejection was tested using a 3-level mixed-effect logistic regression with a random intercept (random effects: “subject-level” nested in the “center-level,” fixed effects: patient gender, integration method = adaptive Gauss–Hermite quadrature, number of integration points = 7) to account for the clustering of patients within a center and the clustering of biopsies from the same subject. The receiver-operating characteristics were drawn with calculations of the area under the curve and 95% confidence intervals (CI). We used STATA/MP (version 17.0) for all analyses and considered p-values below 0.05 to be significant. All the tests were two tailed.

A total of 461 patients were included representing 831 EMB, mostly protocol biopsies (n = 773, 93.0%). The baseline clinical characteristics at inclusion are described in Table 1. The median time between heart transplantation and EMB was 8 months (interquartile range: 3-16 months). A total of 79 rejection episodes were diagnosed, including 25 ACR \geq 2 R and 56 AMR \geq pAMR1 (Supplementary Table 1). In a per-protocol interim analysis based on a subset of 258 EMB from 204 patients and including 49 rejection episodes (ACR \geq 2R = 17, pAMR1(H+) = 14, pAMR1(I+) = 9, pAMR2 = 11), no association between the relative expression of any circulating miR and rejection was found (miR 10a: OR = 1.05, 95%CI = 0.87-1.27, p = 0.61; miR 92a: OR = 0.98, 95%CI = 0.87-1.10, p = 0.68; miR 155: OR = 0.91, 95%CI = 0.76-1.10, p = 0.33; Figures 1 and 2A)

The sensitivity analysis using an absolute quantification of circulating miR on a subset of 191 EMB from 163 patients and including 74 rejection episodes (ACR \geq 2R = 23, pAMR1(H+) = 25, pAMR1(I+) = 12, pAMR2 = 16) confirmed the lack of association between circulating miR and rejection (miR 10a: OR = 1.05, 95%CI = 0.98-1.11, p = 0.18; p = ; miR 92a: OR = 1.00, 95%CI = 0.999-1.001, p = 0.60, miR 155: OR = 1.00, 95%CI = 0.999-1.002, p = 0.07; Figures 1 and 2B). The a posteriori power calculation to detect a 33% differential expression of circulating miR between rejection and no rejection was 0.75 and 0.77 for the main and sensitivity analysis, respectively. Defining allograft rejection differently or considering

separately ACR and AMR did not have any impact on our results (Supplementary Figures S1-S4). The analysis of the remaining sera was then stopped for futility.

Table 1 Characteristics of Patients at Inclusion

Characteristics	<i>n</i>	Overall cohort
<i>Recipient characteristics</i>		
Age (years)	437	48.9 ± 12.6
Sex (female)—no. (%)	460	96 (20.9)
Body mass index (kg/m ²)	405	25.9 ± 14.4
Etiology of heart failure—no. (%)	405	
Dilated		168 (41.5)
Ischemic		142 (35.1)
Congenital		20 (4.9)
Redo heart transplantation		4 (1.0)
Others		71 (17.5)
Positive CMV serology—no. (%)	393	234 (59.5)
<i>Donor characteristics</i>		
Age (years)	380	43.4 ± 13.7
Sex (female)—no. (%)	380	132 (34.7)
Cause of death—no. (%)	380	
Traumatic		127 (33.4)
Cerebrovascular		167 (44.0)
Other		86 (22.6)
Positive CMV serology—no. (%)	385	164 (42.6)
<i>Transplant characteristics</i>		
Sex mismatch (female D, male R)—no. (%)	380	85 (22.4)
Ischemic time (minutes)	402	191 ± 56
HLA A/B/DR/DQ mismatch (<i>n</i>)	380	5.8 ± 1.5
Anti-HLA DSA at day 0 ≥ 500 MFI—no. (%)	317	83 (26.1)
Induction therapy—no. (%)	324	410 (98.8)
Antithymocyte globulines		288 (88.9)
Basiliximab		36 (11.1)
Routine immunosuppressive regimen—no. (%)		
Cyclosporine	348	324 (93.1)
Tacrolimus	345	28 (8.1)
Mycophenolate mofetil	364	363 (99.7)
Everolimus	364	12 (3.3)
Azathioprine	364	0 (0)
Corticosteroids	364	363 (99.7)

Abbreviations: CMV, cytomegalovirus, D, donor; DSA, donor-specific antibody; HLA, human leukocyte antigen; MFI, mean fluorescence intensity; R, recipient.

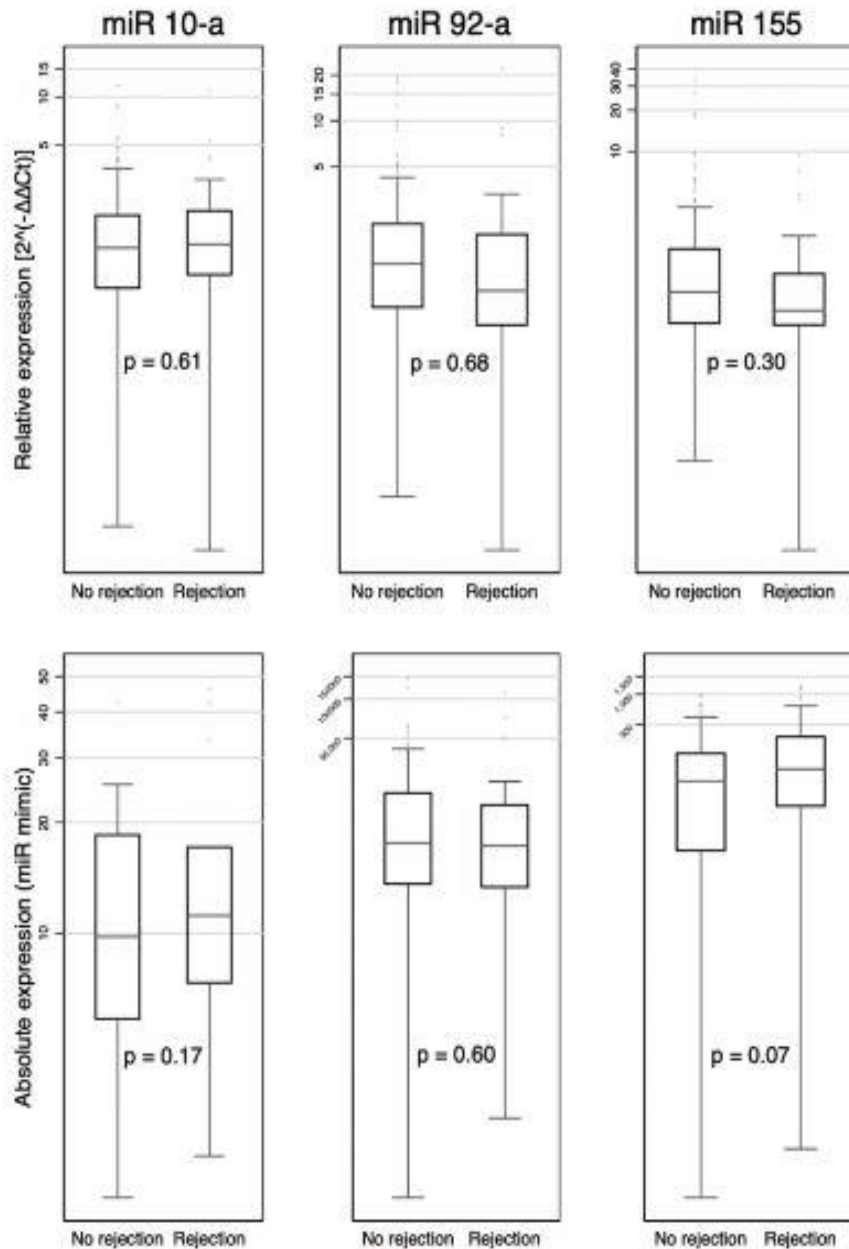


Figure 1 Relative and absolute expression of circulating microRNA 10a, 92a, and 155 according to the rejection status. Upper panel: relative quantification. Lower panel: absolute quantification. Rejection was defined as an acute cellular rejection $\geq 2R$ and/or an antibody-mediated rejection $\geq pAMR1$. The expression of circulating microRNA did not significantly differ between rejection and no-rejection groups. miR, microRNA.

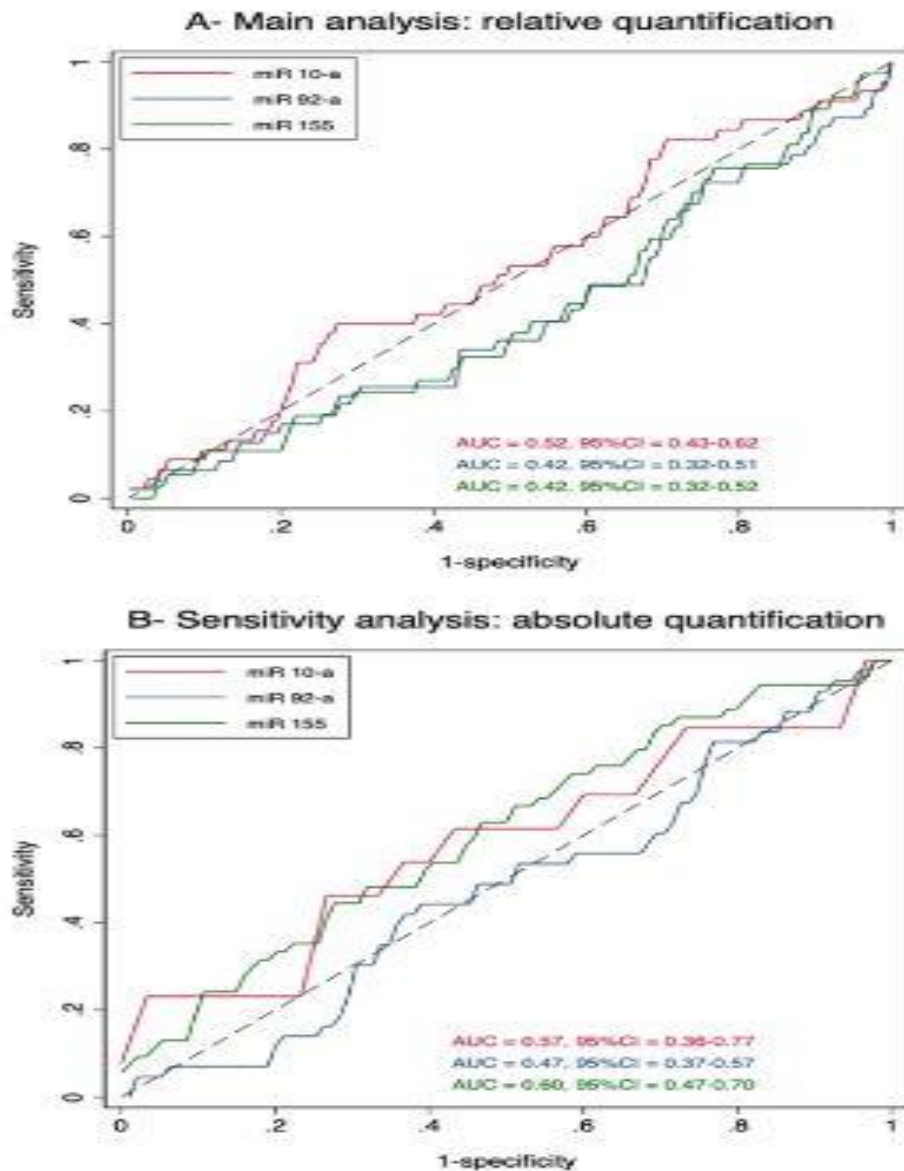


Figure 2 Receiver operating characteristics curves of circulating microRNA expression to detect concomitant biopsy-proven rejection. (A) Relative quantification and (B) absolute quantification. Rejection was defined as an acute cellular rejection $\geq 2R$ and/or an antibody-mediated rejection $\geq pAMR1$. The expression of circulating micro-RNA did not discriminate rejecting patients from nonrejecting patients. miR, microRNA.

In this large prospective cohort of unselected patients, the previously identified circulating miR signature of cardiac rejection was not validated. First, this absence of prospective validation may reflect important bias inherent to retrospective case-control studies (limited sample size, selection bias). Second, an important issue limiting the clinical application of miR is the high variability of the miR signature identified across studies (miR-144-3p,² miR-181a-5p,³ miR-142-3p, and miR-101-3p,⁵ miR-29c-3p, and miR-486-5p⁴) and a drop in discrimination when externally validating an miR rejection signature.¹ Among the 39 miR associated with rejection in the largest prospective study published, only 1 has been found to

be associated with rejection in previous studies.¹ Third, the normalization step using an endogenous miR expression may induce measurement bias since there is no consensus in the literature regarding the best normalization miR. However, even an absolute quantification of miR failed to find any relevant association. Finally, our analysis was restricted to 3 circulating miR. An analysis of a larger number of miR may have identified stronger and more stable associations required for the external validation process, as in a recent large prospective study performed without any a priori selection of miR of interest.¹ However, our aim was to prospectively validate a previously identified rejection signature.

This large prospective longitudinal multicenter study showed no clinical utility of circulating miR 10a, 92a, and 155 monitoring in heart allograft recipients.

Author contributions (ICJME)

GC and MR contributed to the acquisition of data and analysis, drafted the work, approved the final version, and agreed to be accountable for all aspects of the work. GB, RG, FR, KB, EE, AS, SP, AB, and AG contributed to the acquisition of data, revised the manuscript critically, approved the final version, and agreed to be accountable for all aspects of the work. FT contributed to the acquisition of data and all technical aspects of the study, approved the final version, and agreed to be accountable for all aspects of the work. PB contributed to the conception, the design and interpretation, approved the final version, and agreed to be accountable for all aspects of the work. XJ, AL, and JPVDH contributed to the conception, the design and interpretation, revised the manuscript critically for important intellectual content, approved the final version, and agreed to be accountable for all aspects of the work.

Disclosure statement

The authors of this manuscript have no conflicts of interest to disclose as described by the Journal of Heart and Lung

Transplantation.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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