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Circulating mitochondrial genes detect acute cardiac allograft rejection: Role of the mitochondrial calcium uniporter complex

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Abstract

Acute rejection after heart transplantation increases the risk of chronic dysfunction. Disturbances in mitochondrial function may play a contributory role, however, the relationship between histological signs of rejection in the human transplanted heart and expression levels of circulating mitochondrial genes, such as the mitochondrial Ca^{2+} uniporter (MCU) complex, remains unexplored. We conducted an RNA-sequencing analysis to identify altered mitochondrial genes in serum and to evaluate their diagnostic accuracy for rejection episodes. We included 40 consecutive samples from transplant recipients undergoing routine endomyocardial biopsies. In total, 112 mitochondrial genes were identified in the serum of posttransplant patients, of which 28 were differentially expressed in patients with acute rejection ($p < .05$). Considering the receiver operating characteristic analysis with an area under the curve (AUC) >0.900 to discriminate patients with moderate or severe degrees of rejection, we found that the MCU system showed a strong capability for detection: *MCU* (AUC = 0.944, $p < .0001$), *MCU/MCUR1* ratio (AUC = 0.972, $p < .0001$), *MCU/MCUB* ratio (AUC = 0.970, $p < .0001$), and *MCU/MICU1* ratio (AUC = 0.970, $p < .0001$). Mitochondrial alterations are reflected in peripheral blood and are capable of discriminating between patients with allograft rejection and those not experiencing rejection with excellent accuracy. The dysregulation of the MCU complex was found to be the most relevant finding.

KEYWORDS
biomarker, cell death: apoptosis, genomics, heart biology, heart transplantation/cardiology, molecular biology, rejection: acute, translational research/science

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1 | INTRODUCTION

The specific molecular mechanisms involved in the evolution of heart transplant rejection are not completely understood. Disturbances in mitochondrial function may play a contributory role in this process. In fact, some authors have observed increased expression of mitochondrial genes in peripheral blood derived from patients with acute renal rejection, compared with patients who experienced no rejection.[1,2] However, there is currently a lack of data to correlate mitochondrial dysfunction and rejection development in the human transplanted heart.

Calcium uptake into the mitochondrial matrix is crucial for cellular functions. This influx has an effect on energy production and can also initiate cell death.[3-7] The calcium transient in cardiac myocytes produces repetitive Ca^{2+} modulations that envelope the mitochondria with every heartbeat. This Ca^{2+} is rapidly extruded from the cytosol through the sarco/endoplasmic reticulum Ca^{2+} ATPase pump (SERCA) and the sarcolemma Na^+/Ca^{2+} exchanger (NCX). Mitochondria can also sequester Ca^{2+} from the cytosol through the mitochondrial Ca^{2+} uniporter (MCU) complex, and this pathway is currently a hot research topic.[3-7] It has been reported that differences in the MCU complex composition can affect the ability of mitochondria to uptake Ca^{2+}, and that the ratio between MCU and the different regulatory subunits are altered in pathological conditions.[8-10] Additionally, SERCA2a has received significant attention due to its crucial role in regulating calcium cycling; it also plays a key role in the progression of heart failure.[11] Our previous work demonstrated that SERCA2a dysregulation is involved in cardiac rejection by promoting alterations in myocardial calcium homeostasis.[12]

Acute rejection after heart transplantation increases the risk of chronic dysfunction and worsens allograft outcomes. Currently, the histological examination of allograft biopsies is the standard rejection screening procedure, despite its inherent risks to patients and technical limitations. Thus far, the Federal Drug Administration has approved only one panel of biomarkers for acute rejection for application in cardiac transplantation; however, this panel presents some limitations. Therefore, it is necessary to identify a novel set of biomarkers to complement the current assays and integrate novel potential molecules to the studies in progress.

To our knowledge, this study is the first to report a correlation between histological signs of rejection in the human transplanted heart and expression levels of circulating mitochondrial genes. Changes in the expression of genes involved in relevant mitochondrial functions were found, mainly in the mitochondrial calcium uniporter complex, intramitochondrial protein transport, the respiratory chain, and apoptosis. The panel of mitochondrial dysfunction markers identified by us could contribute to a new noninvasive approach to identify acute transplant rejection.

2 | METHODS

2.1 | Sample collection

A total of 40 consecutive endomyocardial biopsies (EMB) and blood samples were collected from heart transplant patients (>18-years) who were referred for EMB for routine surveillance prior to the availability of the gene expression profiling analysis at the University and Polytechnic Hospital La Fe (October 2016 to April 2017). The transcriptomic analysis was performed on a single occasion for each patient. The associated clinical data were also collected. We recorded the age, sex, body mass index, primary cause of heart disease, interval between transplantation and study enrollment, biochemical markers, echocardiographic parameters, and other clinical characteristics at the time of each biopsy (Table 1). Patients were maintained on a standard immunosuppressive regimen. Rejection episodes were assessed according to the International Society for Heart and Lung Transplantation (ISHLT) consensus report.[13] Histology of EMBs was assessed by an expert pathologist blinded to the clinical information. Only in doubtful cases was involved a second pathologist to reach a consensus. 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 the 12 patients who did not experience allograft rejection. Experimenters were blinded to group allocation and outcome assessment.

At the time of EMB, blood samples were collected for laboratory analysis, just before the EMB. Serum was separated from the blood samples by centrifugation at 1500 g for 15 min at 4°C, aliquoted and stored immediately at −80°C.

This study was approved by the Ethics Committee (Biomedical Investigation Ethics Committee of the University and Polytechnic Hospital La Fe of Valencia, Spain) and was conducted in accordance with the principles outlined in the Declaration of Helsinki.[14] Informed consent was obtained from each patient prior to sample collection.

2.2 | RNA sequencing

RNA extraction was carried out using NucleoSpin® miRNA Plasma of Macherey Nagel, following the protocol and instructions provided by the manufacturer. RNA quantification was performed using a NanoDrop 1000 spectrophotometer and the Qubit 3.0 fluorometer (Thermo Fisher Scientific). The purity and integrity of RNA samples were determined using the RNA 6000 Nano Kit and Small RNA Kit with 0.8% agarose gel and the Agilent 2100 Bioanalyzer (Agilent Technologies). Extracted RNA were only considered valid samples with a 260:280 absorbance ratio greater than 2.0, and an RNA integrity parameter (RIN software algorithm), equal to or greater than 7. A reduced time between sample receipt and storage yielded higher-quality samples, as evidenced by the RNA integrity numbers of ≥9.

TABLE 1 Patient characteristics at the time of biopsy and blood sample extraction

		Rejection (n = 28)		
	Nonrejection (n = 12)	Grade 1R (n = 16)	Grade ≥2R (n = 12)	p value[a]
Age, years	48 ± 15	51 ± 10	42 ± 15	.328
Male sex (%)	75	94	75	1.000
Indication for cardiac transplantation				
Ischemic cardiomyopathy (%)	25	50	25	1.000
Idiopathic dilated cardiomyopathy (%)	42	31	58	.684
Other (%)	33	19	17	.320
Time between transplantation and study enrollment, months	8.1 ± 3.8	6.4 ± 3.2	3.8 ± 4.2	.019
Body mass index (kg/m^2)	25 ± 5	25 ± 3	24 ± 3	.615
Hypertension (%)	58	31	42	.684
Diabetes mellitus (%)	58	63	50	.930
Dyslipemia (%)	42	56	25	.667
Echo-Doppler study				
Ejection fraction (%)	72 ± 8	64 ± 9	70 ± 10	.826
LV end systolic diameter (mm)	25 ± 3	28 ± 4	31 ± 3	.019
LV end diastolic diameter (mm)	41 ± 3	44 ± 5	45 ± 4	.133
Hemodynamic parameters				
Mean right atrial pressure (mm Hg)	3.6 ± 1.8	5.9 ± 3.2	7.7 ± 1.5	.018
Systolic right ventricular pressure (mm Hg)	33 ± 5	36 ± 7	42 ± 4	.037
Diastolic right ventricular pressure (mm Hg)	4.2 ± 1.6	5.9 ± 3.3	8.7 ± 3.8	.054
Immunosuppressive therapy				
Tacrolimus (%)	100	100	100	1.000
Mycophenolic acid (%)	100	95	100	1.000
Steroids (%)	100	95	100	1.000
Induction therapy				
Basiliximab (%)	100	100	100	1.000
Neutrophils (thousands/mm^3)	4.8 ± 4.1	3.7 ± 1.7	8.2 ± 6.3	.158
Leukocytes (thousands/mm^3)	7.1 ± 3.8	6.2 ± 2.1	11.0 ± 6.3	.087
Lymphocytes (thousands/mm^3)	1.5 ± 0.53	1.8 ± 0.6	2.0 ± 0.8	.075
Hemoglobin (mg/dl)	11.6 ± 2.4	12.6 ± 1.4	11.9 ± 1.7	.752
Hematocrit (%)	37 ± 8	40 ± 4	37 ± 4	.923
NT-proBNP (pg/ml)	152 (113–467)	280 (122–572)	1209 (736–2382)	.016
Troponin T (ng/L)	19 (11–66)	15 (10–21)	25 (13–40)	.840

Abbreviations: LV, left ventricular; NT-proBNP, N-terminal fragment of B-type natriuretic peptide.

[a]Nonrejection vs rejection grade ≥2R group.

cDNA libraries were obtained following Illumina's recommendations. Briefly, 3′ and 5′ adapters were sequentially ligated to RNA prior to reverse transcription and cDNA synthesis. Size selection was performed using a 6% polyacrylamide gel. The quality and quantity of cDNA libraries were analyzed using the High-Sensitivity D1000 ScreenTape Assay and the 4200 TapeStation System (Agilent Technologies). cDNA libraries were then pooled and sequenced by two lanes of 100 bp paired-end sequencing using an Illumina HiSeq 2500 sequencer.

Quality control of the raw sequence data was performed using FastQC software. Bias was prevented through adapter identification and elimination using Trim Galore (http://www.bioinformatics.babra ham.ac.uk/projects/trim_galore/). Following this, the raw paired-end reads were mapped against the human hg38 genome using the bowtie algorithm.[15] Insufficient quality reads, with a phred score ≤20, were eliminated using the SAMtools method.[16] RNA quantification was then estimated using HTSeq software (version 0.6.0).[17] Lastly, differential expression analysis between conditions were assessed using the DESeq2 method (version 3.4).[18] The FDR method adjusts the original p value using the number of tests. An interpretation of this method is implemented in DESeq2 in which the genes are rank in function of the p value and then are multiply by a correction

rank value, similar to original FDR method. Differentially expressed RNAs with fold change (FC) values ±1.5, and with false discovery rate (FDR) adjusted $p \leq .05$ were included to avoid identification of false positives across the differential expression data.[18]

2.3 | Statistical analysis

Clinical characteristics were expressed as mean ± SD for continuous variables and percentages for discrete variables. Results for each variable were assessed for normality using the Kolmogorov–Smirnov test. Continuous and discrete variables that did not follow a normal distribution were compared using the Mann–Whitney test, and the chi-square test, respectively. Variables with a normal distribution were compared using the Student's t test when continuous, and Fisher's exact test when discrete.

The diagnostic capability of mitochondrial serum markers for the presence of transplant rejection was assessed by the construction of receiver operating characteristic (ROC) curves. $p < .05$ was considered statistically significant. All statistical analyses were performed using SPSS software (version 20.0; SPSS Inc.).

3 | RESULTS

3.1 | Clinical characteristics

As shown in Table 1, all groups of posttransplant patients included in this transcriptomic study were similar with regards to variables such as age, sex, body mass index, hypertension, diabetes mellitus, dyslipidemia, and immunosuppressive therapy. We found alterations in hemodynamic parameters such as the mean right atrial pressure ($p = .018$) and systolic right ventricular pressure ($p = .037$) in the group of patients with rejection grade ≥2R compared to nonrejection group. With regard to the echo-Doppler study we found an increase in the values of left ventricular end systolic diameter in grade ≥2R compared with nonrejection patients ($p = .019$). We found an increase in N-terminal pro–B-type natriuretic peptide (NT-proBNP) levels in the group with rejection grade ≥2R when we compare with the nonrejection group ($p = .016$).

3.2 | Gene expression analysis

Transcriptomic differences between 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 (Table S1).

Focusing on mitochondrial genes, we found a total of 112 circulating genes in the serum of posttransplant patients (Table S2), of which 28 were differentially expressed in patients experiencing rejection (all $p < .05$). We displayed gene expression levels according to the rejection grade and found changes in genes involved in mitochondrial calcium uptake, specifically in the MCU complex (Figure 1). We also found alterations in several genes involved in intramitochondrial transport, metabolism, and energy production, as well as the stress response, and apoptosis (Figure 2). Relative expression levels (Figure 3A) and a hierarchical clustering heat map (Figure 3B), created using MeV software (version 4.9.0), are displayed to compare all altered mitochondrial genes in samples from patients with clinically relevant rejection (grade ≥2R) with the corresponding genes in nonrejection samples. Notably, this analysis identified two divergent protein expression profiles, indicating a clear demarcation between the rejection grade ≥2R and the nonrejection group.

3.3 | Diagnostic capability

As summarized in Table 2 and Figure 4, ROC curves were obtained to analyze the capability of the mitochondrial genes altered to detect heart transplant rejection. Specifically, the best ROC curves to detect heart transplant rejection corresponded to genes involved in mitochondrial calcium uptake, intramitochondrial transport, the respiratory chain, and cell death. Taking into account the molecules that generated an AUC > 0.900 to detect patients with moderate or severe degree of rejection (grade ≥2R), we found *MCU* (AUC = 0.944 ± 0.047, $p < .0001$), *TIMM50* (AUC = 0.917 ± 0.055, $p < .01$), *NDUFA10* (AUC = 0.951 ± 0.042, $p < .0001$), *TBRG4* (AUC = 0.972 ± 0.028, $p < .0001$), and *BNIP3* (AUC = 0.965 ± 0.037, $p < .0001$) as the most relevant rejection markers. Considering the functional interaction of the individual regulatory mechanisms of three known molecular components altered within the mitochondrial calcium uniporter, we obtained a better capability of detection than *MCU* alone: *MCU/MCUR1* (AUC = 0.972 ± 0.028, $p < .0001$), *MCU/MCUB* ratio (AUC = 0.970 ± 0.033, $p < .0001$), and *MCU/MICU1* (AUC = 0.970 ± 0.033, $p < .0001$). We also list in Table 2 the sensitivities, specificities, and predictive values for the diagnosis of cardiac rejection.

Some of these markers maintained good capability when detecting mild rejection (grade 1R): *MCU/MCUR1* ratio (AUC = 0.724 ± 0.099, $p < .05$), *TIMM50* (AUC = 0.833 ± 0.078, $p < .01$), *NDUFA10* (AUC = 0.729 ± 0.103, $p < .05$), and *TBRG4* (AUC = 0.849 ± 0.075, $p < .01$). Furthermore, as seen in Table 2, there were molecules that presented an AUC lower than 0.900 to detect patients with moderate or severe degrees of rejection (grade ≥2R), but at the same time presented significant differences in AUC scores when we compared the group of patients without rejection to the group of patients with mild rejection (grade 1R), maintaining a remarkable and significant detection power.

4 | DISCUSSION

In this study, we have investigated the expression of circulating mitochondrial genes in acute rejection for the first time. Our findings suggest that patients with transplant-induced cellular rejection have a molecular signature consistent with impaired mitochondrial
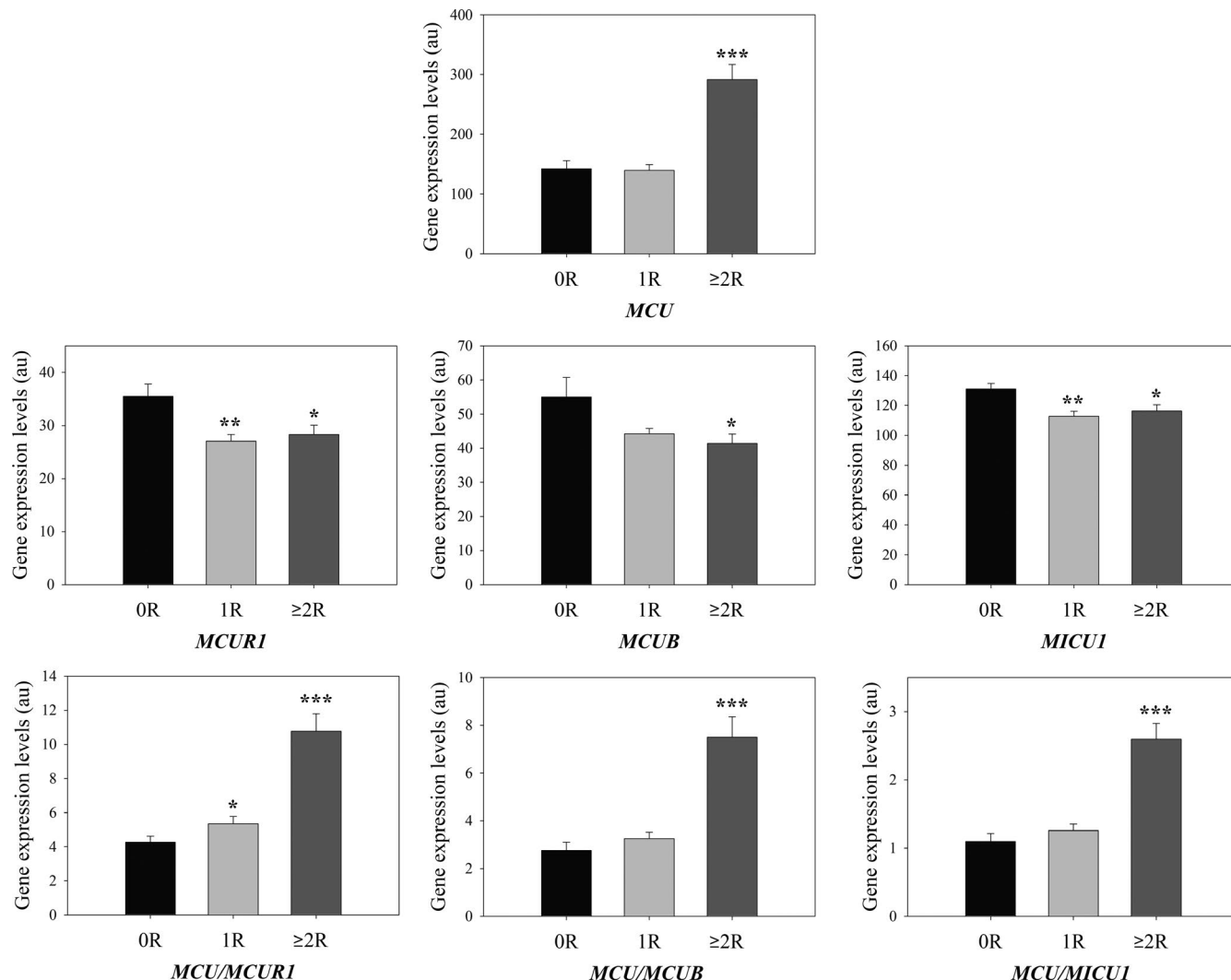
FIGURE 1 Circulating MCU complex genes are differentially expressed between normal and rejected heart allografts. Values were obtained by Illumina HiSeq 2500 sequencing. Differential expression levels for molecules involved in mitochondrial calcium uptake (*MCU*, *MCUR1*, *MCUB*, and *MICU1*) and their respective ratios (*MCU/MCUR1*, *MCU/MCUB*, and *MCU/MICU1*) in each group of patients (nonrejection, rejection grade 1R and grade ≥2R) are displayed. Bars indicate protein expression levels ±SEM (standard error of the mean). Au, arbitrary units; *$p < .05$ and **$p < .01$ against the nonrejection group

function, with differential expression in 25% of mitochondrial genes detected in serum.

Although the gold standard for the diagnosis of cardiac allograft rejection is currently EMB, this technique has negative consequences for the patient and is prone to sampling error and inter-observer variability. Because of these important risks and limitations, it could be greatly beneficial to further the existing knowledge in this field and to complement the current tools available to produce an optimal diagnostic method. In addition, an improved understanding of the mechanisms leading to rejection is needed to enhance the survival rate of cardiac transplant recipients and to aid the development of more specific immunosuppressive drugs.

The importance of mitochondria in cardiovascular pathophysiology and the potential of targeting mitochondrial dysfunction for clinical intervention to improve cardiac function has been increasingly

recognized over the past decade.[19-23] The calcium-mediated contractile activity of cardiomyocytes makes them acutely dependent on an uninterrupted energy supply and precise calcium buffering, both provided by mitochondria. The mitochondrial Ca^{2+} uniporter complex is a central pathway of mitochondrial Ca^{2+} influx that allows for the membrane potential-dependent transport of Ca^{2+} ions across the mitochondrial inner membrane into the mitochondrial matrix. An average of 4 subunits of the mitochondrial calcium uniporter protein (MCU) compose the core of the calcium channel,[24,25] in addition to the regulatory molecules MICU1,[26] MICU2,[27] EMRE,[28] MCUB,[29] and MCUR1.[30] In vivo models of MCU deletion and inactivation have clearly defined the contribution of the MCU complex to the regulation of cardiac energetics. Many researchers have studied the MCU complex by focusing on MCU loss-of-function mouse models.[7,31,32] Currently, there is a dynamic debate over whether the MCU complex is required for basal heart function, or if its abilities are limited
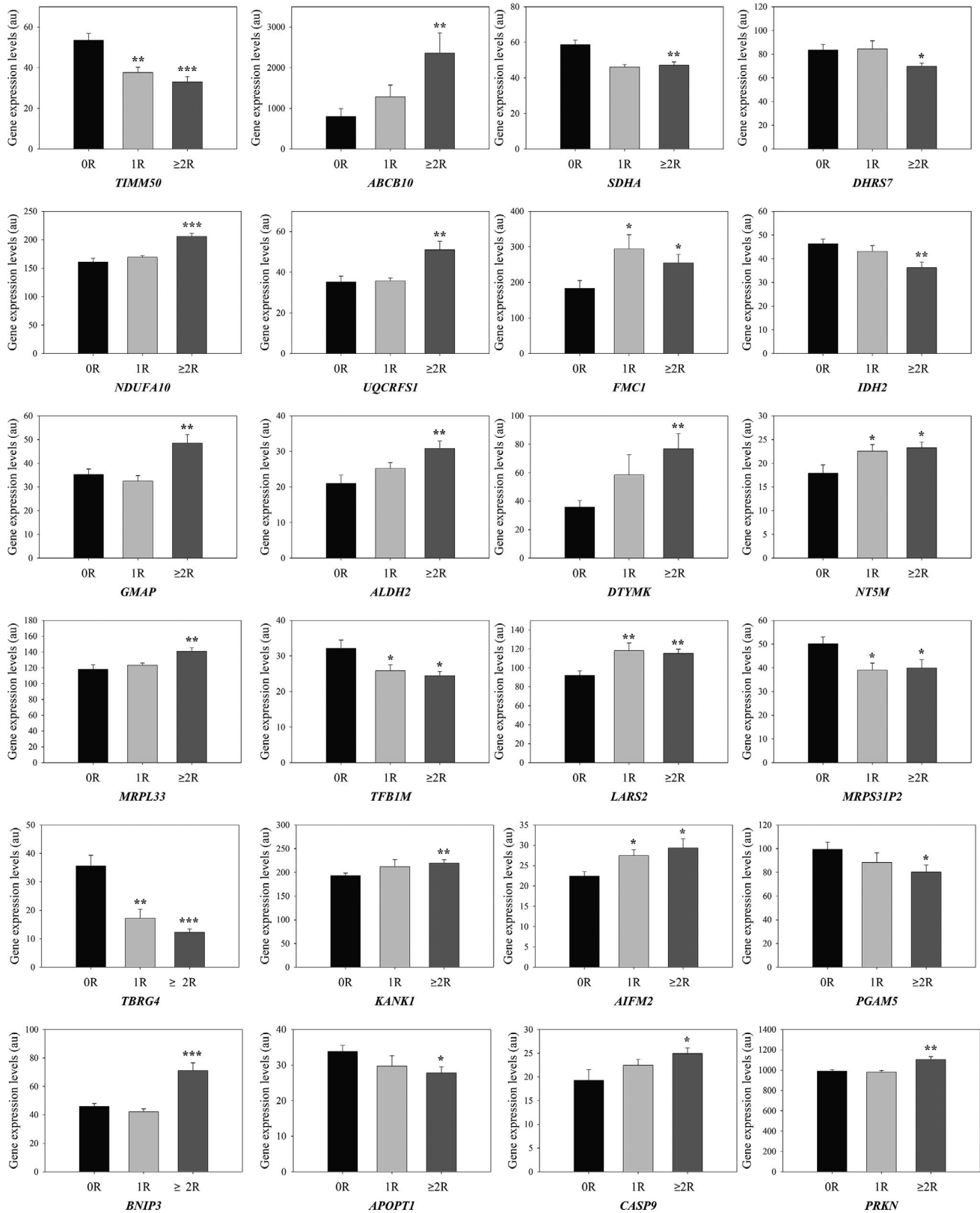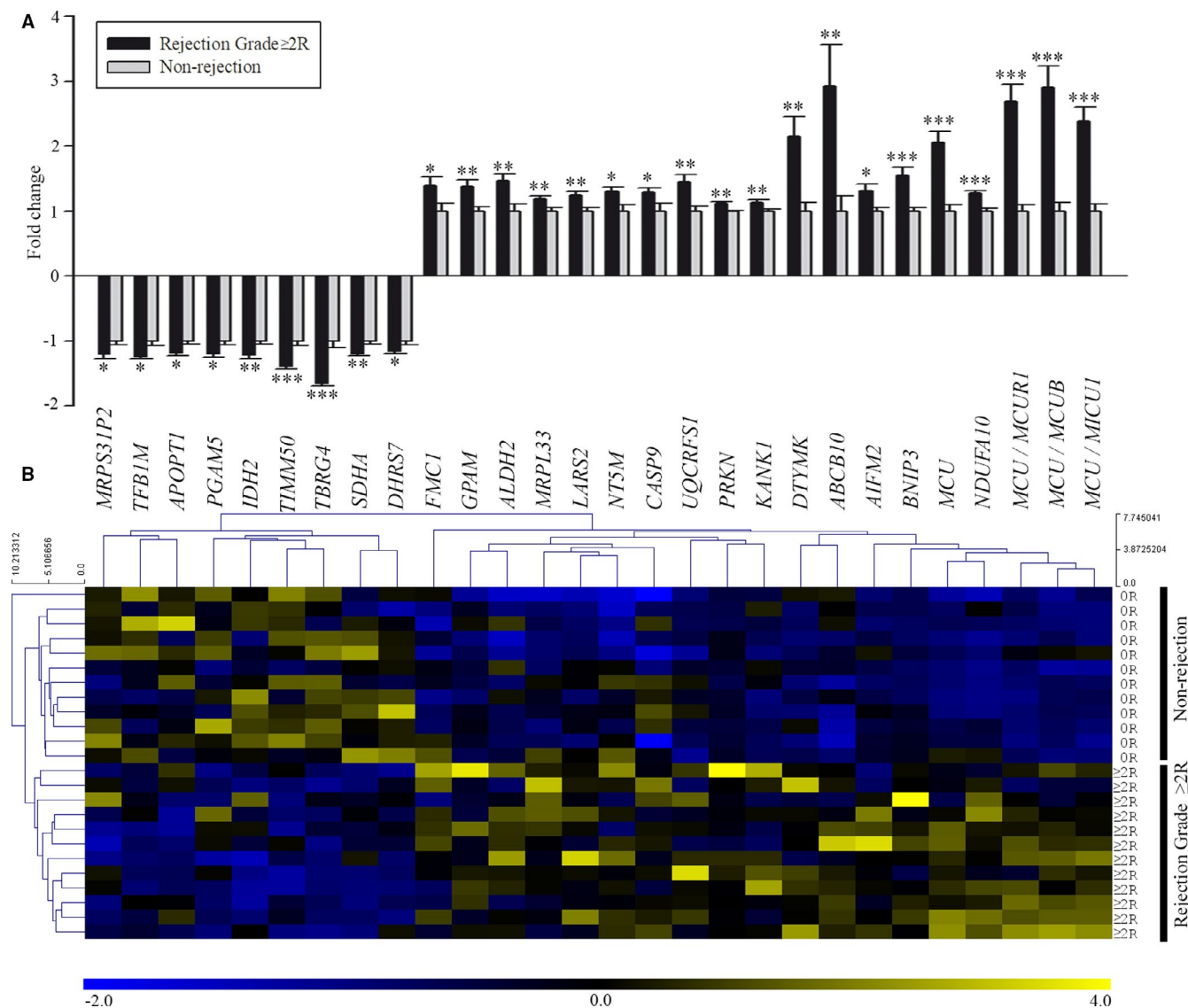
FIGURE 2 Circulating mitochondrial genes are differentially expressed between normal and rejected heart allografts. Values obtained by Illumina HiSeq 2500 sequencing. Differential expression levels for molecules involved in intramitochondrial protein transport (*TIMM50*, *ABCB10*), metabolism and energy production (*SDHA*, *DHRS7*, *NUDFA10*, *UQCRFS1*, *FMC1*, *IDH2*, *GMAP*, *ALDH2*, *DTYMK*, *NT5M*, *MRPL33*, *TFB1M*, *LARS2*), pseudogene (*MRPS31P2*), transcription, stress response, and apoptosis (*TBRG4*, *KANK1*, *AIFM2*, *PGAM5*, *BNIP3*, *APOPT1*, *CASP9*, *PRKN*) in each group of patients (nonrejection, rejection grade 1R and grade ≥2R) are displayed. Bars indicate protein expression levels ±SEM (standard error of the mean). Au, arbitrary units; *p < .05 and **p < .01 against the nonrejection group

FIGURE 3 Relative expression levels of altered circulating mitochondrial genes in rejection grade ≥2R. Controls values were set to 1. FC units represent the fold change over the control RNA relative expression levels (nonrejection group). Bars indicate FC±SEM (standard error of the mean) (A). Hierarchical clustering heat map based on the relative expression level of genes between normal and rejected heart allografts. The hierarchical clustering heat map analyses show the separation of the ≥2R rejection group and nonrejection group for circulating mitochondrial genes. Genes are listed in the columns and samples in the rows. Colors depict the relative expression level of each molecule with blue being the lowest and yellow the highest (B). *p < .05 and **p < .01 against the nonrejection group [Color figure can be viewed at wileyonlinelibrary.com]

to managing the heart's functional response to physiological or pathological stress.[7] In addition, it has been shown that other MCU-independent calcium-uptake mechanisms contribute to maintaining normal mitochondrial function.[33]

In our study, we observed notable changes in this system in cardiac transplant patients experiencing cellular rejection. Specifically, we found an increase in *MCU* expression and a decrease in the expression of the MCU regulators *MCUR1*, *MCUB*, and *MICU1*. The serum levels of *MCU* discriminated with great accuracy between patients with clinically relevant rejection and those without. Our interest in calculating the ratio between the regulators of MCU and MCU itself is owing to the fact that the activity of the MCU

channel is affected by changes in the relationship between the different subunits that make up the channel (MCU, MCUB, MCUR1, MICU1). Specifically, it has been observed that the contents of MCU, MCUB, and MICU1, as well as their stoichiometry, could regulate mitochondrial transport of Ca^{2+} according to physiological conditions. The effect of the relationship of these subunits on calcium transport parameters in the mitochondria has been described previously.[8-10] In this study, we found that the capability of detecting rejection was improved upon calculation of these ratios.

On the other hand, it is important to highlight that a low percentage of cytosolic Ca^{2+} is estimated to be removed by the mitochondria during cardiac beating, whereas the vast majority

TABLE 2 Receiver-operating characteristic (ROC) curve of circulating altered mitochondrial genes for detecting heart transplant rejection (grade ≥2R). Sensitivities, specificities, and predictive values (%) for the diagnosis of cardiac rejection (cut-off point FC ≥1.3)

Gene name	AUC	95% CI	SS	SP	VPP	VPN
Mitochondrial calcium uptake						
MCU	0.944***	0.853–1.000	92	91	92	92
MCU/MCUR1	0.972***	0.918–1.000	92	92	85	91
MCU/MCUB	0.970***	0.904–1.000	100	92	92	100
MCU/MICU1	0.970***	0.904–1.000	100	92	92	100
Transport and respiratory chain						
TIMM50	0.917**	0.809–1.000	83	80	82	77
SDHA	0.861**	0.716–1.000	100	50	100	67
DHRS7	0.833**	0.655–1.000	92	20	75	55
NDUFA10	0.951***	0.870–1.000	58	100	100	71
Apoptosis and stress response						
TBRG4	0.972***	0.918–1.000	75	100	75	100
BNIP3	0.965***	0.893–1.000	92	100	100	92
APOPT1	0.770*	0.575–0.967	100	30	100	60
AIFM2	0.803*	0.603–1.000	50	91	86	63

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

*p < .05.

**p < .01.

***p < .0001.

is mobilized by SERCA and NCX.[3] In a previous study, we found that SERCA2a serum levels were lower in transplant rejection patients. Together, these data suggest that calcium regulation has an important role in the pathophysiology of transplant rejection. Additionally, the upregulation of MCU expression observed in patients experiencing rejection could be a cellular mechanism to compensate for the downregulation of SERCA2a or the low levels of MCU regulators. However, further investigation is required to define the role that these alterations have in the pathogenesis of heart transplant rejection.

It is well understood that calcium uptake into the mitochondrial matrix is critically important to cellular function, and that this flux influences energy production and can initiate cell death. In our study, we observed a significant upregulation in three known mitochondrial molecules involved in calcium-induced apoptosis,[34,35] CASP9, AIFM2, and BNIP3. In addition, we found a significant downregulation of the transforming growth factor beta regulator 4 (TBRG4) under rejection conditions, which is known to promote apoptosis in knockdown experiments.[36] Our results are in concordance with those of previously published studies, supporting the idea that transplantation is associated with programmed cell death including apoptosis, resulting in delayed graft function and organ rejection.[37-39]

Lastly, we observed significant changes in the expression of TIMM50, a molecule involved in intramitochondrial protein transport. The mitochondrial import inner membrane translocase subunit TIM50 is an essential component of the TIM23 complex, the main entry gate for proteins of the matrix and the inner membrane. It has been reported that interactions of peptides with TIM50

are weakened in the presence of Ca^{2+}.[40] TIM50 is downregulated in both human dilated cardiomyopathy and murine hypertrophic hearts.[41] Consistent with this finding, we also found a downregulation of TIMM50 in cardiac rejection. This molecule could be an excellent candidate to detect cardiac rejection, as it showed a robust capability for detection that improves gradually with the severity of rejection.

RNAs are unstable molecules that are vulnerable to degradation by ribonucleases. Thus, circulating cell-free RNAs can be encapsulated within extracellular membrane to protect them from nuclease activity.[42,43] These membrane structures mediate intercellular communication. In fact, a significant inflammatory event such as allograft rejection would perturb the serum exosomal content.[44] However, although it could be the mRNA that we detect is protected in exosomes, we have not verified it and also the origin of these exosomes in rejection conditions remains to be determined. This approach is outside the scope of this study, but understanding these issues is important to providing insights into pathogenic mechanisms of cardiac rejection.

Our study has several limitations, and the results must be interpreted within this context. This investigation only involved a single center. In addition, it is focused on cellular rejection and has not specifically evaluated antibody-mediated rejection, a clinical entity of rejection associated with worse graft survival and characterized for intravascular macrophage accumulation.[45] However, we believe that our findings have provided substantial evidence and represent a necessary first step for future research in which these limiting factors could be overcome. The results of this study can be further validated in broad patient cohorts to contribute to a better analysis
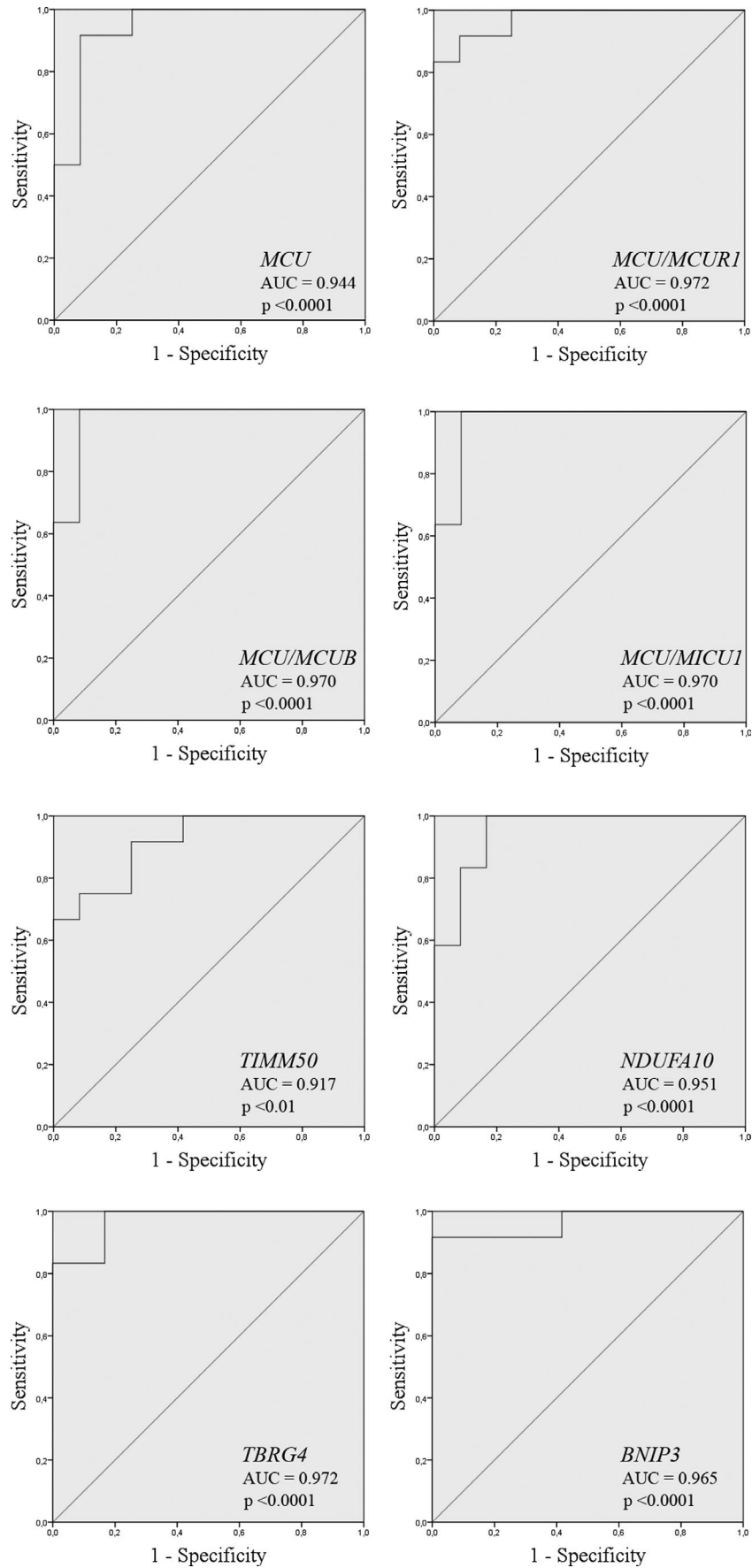
FIGURE 4 Receiver operating characteristic (ROC) curve of circulating mitochondrial genes for the detection of cardiac allograft rejection

of cardiac rejection and lead to the use of this relatively simple and noninvasive system as a complement to other analytical methods, as well as an alternative to EMB.

In summary, mitochondrial alterations are reflected in peripheral blood and are capable of discriminating between patients with allograft rejection and those not experiencing rejection with excellent accuracy. The dysregulation of the MCU complex was found to be the most relevant finding. We also found alterations in genes involved in intramitochondrial transport, respiratory chain and apoptosis. This mitochondrial dysfunction marker panel could be a novel noninvasive approach to identify acute transplant rejection.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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