Title

Rejection-associated Mitochondrial Impairment After Heart Transplantation.

Journal

Transplantation Direct, 6(11)

ISSN

2373-8731

Authors

Romero, Erick

Chang, Eleanor

Tabak, Esteban

et al.

Publication Date

2020-11-01

DOI

10.1097/TXD.0000000000001065

Peer reviewed

OPEN

Rejection-associated Mitochondrial Impairment After Heart Transplantation

Erick Romero, MD,[1] Eleanor Chang, BS,[2] Esteban Tabak, PhD,[3] Diego Pinheiro, PhD,[1] Jose Tallaj, MD,[4] Silvio Litovsky, MD,[4] Brendan Keating, PhD,[5] Mario Deng, MD,[2] and Martin Cadeiras, MD[1]

Background. Mitochondrial dysfunction is associated with poor allograft prognosis. Mitochondrial-related gene expression (GE) in endomyocardial biopsies (EMBs) could be useful as a nonimmune functional marker of rejection. We hypothesize that acute cardiac allograft rejection is associated with decreased mitochondrial-related GE in EMBs. **Methods.** We collected 64 routines or clinically indicated EMB from 47 patients after heart transplant. The EMBs were subjected to mRNA sequencing. We conducted weighted gene coexpression network analysis to construct module-derived eigengenes. The modules were assessed by gene ontology enrichment and hub gene analysis. Modules were correlated with the EMBs following the International Society of Heart and Lung Transplantation histology-based criteria and a classification based on GE alone; we also correlated with clinical parameters. **Results.** The modules enriched with mitochondria-related and immune-response genes showed the strongest correlation to the clinical traits. Compared with the no-rejection samples, rejection samples had a decreased activity of mitochondrial-related genes and an increased activity of immune-response genes. Biologic processes and hub genes in the mitochondria-related modules were primarily involved with energy generation, substrate metabolism, and regulation of oxidative stress. Compared with International Society of Heart and Lung Transplantation criteria, GE-based classification had stronger correlation to the weighted gene coexpression network analysis–derived functional modules. The brain natriuretic peptide level, ImmuKnow, and Allomap scores had negative relationships with the expression of mitochondria-related modules and positive relationships with immune-response modules. **Conclusions.** During acute cardiac allograft rejection, there was a decreased activity of mitochondrial-related genes, related to an increased activity of immune-response genes, and depressed allograft function manifested by brain natriuretic peptide elevation. This suggests a rejection-associated mitochondrial impairment.

(*Transplantation Direct* 2020;6: e616; doi: 10.1097/TXD.0000000000001065. Published online 19 October, 2020.)

INTRODUCTION

Improvements in immunosuppressive therapies have increased survival rates after heart transplant (HTx).[1] Nevertheless, acute allograft rejection remains a leading cause of allograft failure, death, and adverse long-term outcomes.[1,2] Endomyocardial biopsy (EMB) is the gold standard method for surveillance in HTx rejection. The grading system is based on the International Society of Heart and Lung Transplantation (ISHLT) criteria.[3-5] ISHLT grading is associated with a wide variation in the interpretation of EMB.[6] This lack of precision has motivated the identification of additional biomarkers and methodologies to support accurate diagnostic evaluation. Gene expression (GE) profiling is a valuable tool for monitoring allograft rejection, and it has been incorporated into clinical practice in the United States.[7-15] Gene-based classifications of EMBs have been suggested to improve accuracy in comparison to the ISHLT classification.[16] Although alloimmune response is the central focus of evaluation in allograft rejection, the evaluation of nonimmune-related biomarkers can also provide valuable information about the graft function.

Received 30 June 2020. Revision received 12 August 2020.

Accepted 1 September 2020.

[1] Division of Cardiovascular Medicine, UC Davis Medical Center, Sacramento, CA.

[2] Division of Cardiology, UCLA Medical Center, Los Angeles, CA.

[3] Courant Institute of Mathematical Sciences, New York University, New York, NY.

[4] UAB School of Medicine, University of Alabama at Birmingham, Birmingham, AL.

[5] Cardiovascular Institute, University of Pennsylvania School of Medicine, Philadelphia, PA.

This study was funded by American Heart Association grant no. 11GRNT7990092.

The authors declare no conflicts of interest.

E.R., E.C., and M.C. participated in the design of the work and acquisition of data. E.R., E.C., E.T., D.P., J.T., S.L., B.K., M.D., and M.C. participated in the analysis of the data, drafted the article, and authorized the final approval of the article. E.R. and E.C. have contributed equally to this work.

Correspondence: Martin Cadeiras, MD, Division of Cardiovascular Medicine, UC Davis Medical Center, 2335 Stockton Blvd, Sacramento, CA 95817. (mcadeiras@ucdavis.edu, mcadeiras@gmail.com).

ISSN: 2373-8731

DOI: 10.1097/TXD.0000000000001065

The mitochondrion is a crucial organelle involved in biosynthetic reactions, such as ATP synthesis, and it also participates in proinflammatory molecular signaling.[17,18] Additionally, GE signatures of mitochondrial impairment in allograft biopsies are associated with poor prognosis in kidney transplant recipients.[19]

We sought to evaluate if mitochondrial-related GEs in heart tissue specimens, obtained during rejection surveillance, provide useful information to improve the evaluation of cardiac transplant biopsies. The overarching hypothesis is that decreased GE of mitochondrial function–related genes in EMB correlates with cardiac allograft rejection.

MATERIALS AND METHODS

Ethics Compliance

The procedures in this study followed strict compliance with the ethical standards set forth by the World Medical Association. This study was approved by the University of California Los Angeles (UCLA) Office of Human Research Protection Program IRB (UCLA No. 12-001164) and the University of Alabama at Birmingham (UAB) (IRB protocol No. 080207014). All patients signed informed consent forms.

Study Sample

Cardiac allograft tissue samples were collected from 47 HTx patients at the time of EMB from 2 institutions. The tissue specimens (~1.5 mm in diameter) obtained during EMB were immersed in TRIzol, snap-frozen in liquid nitrogen immediately postprocedure, and stored in a −80°C freezer. Samples were subjected to next-generation mRNA transcriptome sequencing at the Universities' genomic core facilities. Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used to test the quality of the total mRNA present. Illumina HSeq2000 TruSeq library generation platforms were used for whole genome next-generation mRNA sequencing (Illumina, San Diego, CA). The genome library consisted of random fragmentation of the poly(A) mRNA, followed by cDNA production by random polymers. The cDNA libraries were then subjected to quantification using qPC Clusters to yield approximately 725K–825K clusters/mm^2. After the first base addition, parameters were assessed and the cluster density and quality were determined. Single-end sequencing runs were conducted to align the cDNA sequences to the reference genome. FASTQ files obtained from mRNA sequencing were then imported into Avadis NGS 1.5 (Agilent, Palo Alto, CA, and Strand Scientific, Santa Barbara, CA) for alignment of the raw reads to the reference genome. All RNA-seq data were DESeq normalized and quality assessed using Avadis NGS v1.5.

Clinical Information

Hemodynamic information was available at the time of biopsy. For a subset of 30 samples, we also had the white blood cells (WBCs), brain natriuretic peptide (BNP), ImmuKnow, Allomap (CareDx, Brisbane, CA), and left ventricular ejection fraction (LVEF).

Coexpression Network Construction

Weighted gene coexpression network analysis (WGCNA) was used to construct gene modules based on gene-gene interconnectivity.[20] Gene network modules were constructed using biweight midcorrelation at a soft-thresholding power of 4. The weighted adjacency calculations were transformed into overlap dissimilarity measurements to minimize noise and provide more biologically meaningful clusters. WGCNA uses principal topological component analysis to compute a representative eigengene that summarizes the bulk expression of the entire module (see SDC, http://links.lww.com/TXD/A287, for further details; Figures S1 and S2, SDC, http://links.lww.com/TXD/A287). Cytoscape, plug-in ClueGO, was used to explore the gene ontology (GO) enrichment within each module using 2-sided hypergeometric test with Benjamini-Hochberg correction.[21] Cytoscape, plugin CytoHubba, was used to predict essential gene nodes within the modules by the Maximal Clique Centrality method.[22]

Unsupervised Classification of Cardiac Transplant Biopsies

We analyzed the data using 2 different classifications as references. In addition to the ISHLT, we used a 3-class system we developed following an unsupervised analysis described elsewhere.[23-25] Briefly, the output that the procedure provides is the matrix P0 (class, sample) containing the probability that each sample belongs to each class, and the subset of genes whose expression is most consistent with the classification found. To assign samples to classes, we evolve from a noninformative small random perturbation of the uniform assignment to its final value through a Bayesian procedure that uses the expression of each gene as new evidence to relax P0, thought of as a prior, toward the corresponding posterior. For each gene, we first used optimal transport to eliminate from its expression the effects of the outside confounding factors such as batch effect, age, gender, quilty lesion in EMB, and variations in prednisone dose.[25] The algorithm finds the Wasserstein barycenter among the generalized batches—that is, the confounding factors—and maps each sample's expression toward a convex combination of the barycenter, weighted by the corresponding values of P0 for that particular sample. Therefore, the unsupervised algorithm results in a class assignment of heart tissue samples, taking into account gene expression characteristics and filtering outside confounding factors.

Statistical Analysis

The gene modules were correlated to the clinical phenotypes; we calculated the correlation following ISHLT grades, unsupervised class, clinical variables, and module eigengenes. The distribution of clinical variables was compared using a general linear model. The statistical significance of each correlation was corrected using the Benjamini-Hochberg method.

RESULTS

Patient Characteristics and Samples

Tissue samples were collected at routine surveillance or clinically indicated cardiac biopsies from a total of 47 HTx patients; the mean age of the study population was 50.0 ± 14.7 y; 30% of the population were female, and 70% were of European ancestry (patient characteristics are provided in Table 1). Samples were categorized into groups based on the ISHLT grading system. The distribution of the groups within the sample population was 46.9% of the biopsies with grade 0R, 35.9% with grade 1R, 17.2% with grade 2R, and 6.3% antibody-mediated rejection (AMR) (Table 2).

Gene Coexpression Network Analysis

WGCNA resulted in groups of genes with related biologic functions summarized into 16 modules. Modules had

TABLE 1.

Baseline characteristics of the 47 heart transplant patients

Characteristics	Subgroup	Mean or no. (SD or %)
No. of samples per patient	1 Sample	35 (74.47%)
	2 Samples	10 (21.28%)
	3 Samples	1 (2.13%)
	6 Samples	1 (2.13%)
Age	Y	50.0 ± 14.7
Sex (%)	Female	14 (29.79%)
	Male	33 (70.21%)
Race (%)	Asian	1 (2.13%)
	African American	6 (12.77%)
	Filipino	2 (4.26%)
	Other	5 (10.64%)
	Caucasian	33 (70.21%)
Clinical characteristics	RA	4.86 (3.86)
	PAP mean	17.11(5.58)
	PA systole	25.60 (7.21)
	PA diastole	12.13 (5.16)
	PCPW	9.44 (4.85)
	CO	6.14 (1.87)
	CI	3.17 (0.79)
	WBC	7.66 (5.89)
	BNP	279.60 (332.48)
	ImmuKnow	301.39 (134.91)
	Allomap	25.33 (9.54)
	LVEF	54.41 (9.41)

BNP, brain natriuretic peptide; CI, cardiac index; CO, cardiac output; LVEF, left ventricular ejection fraction; PA, pulmonary artery; PAP, pulmonary artery pressure; PCPW, pulmonary-capillary wedge pressure; RA, right atrium; WBC, white blood cell.

TABLE 2.

Histopathology description of the 64 heart tissue samples

Characteristics	Subgroup	(N) Frequency
ACR (%)	0R	30 (46.88)
	1R	23 (35.94)
	2R	11 (17.19)
AMR (%)	0	60 (93.75)
	1	2 (3.13)
	2	2 (3.13)
Batches (%)	Batch 1 (UCLA)	30 (46.88)
	Batch 2 (UAB)	20 (31.25)
	Batch 3 (UAB)	14 (21.88)

ACR, acute cellular rejection; AMR, antibody-mediated rejection; UCLA, University of California Los Angeles.

a size ranging from 86 to 3348. The GO enrichment analysis revealed different functions within the modules. The gene modules with the highest module-trait correlation were immune function (3348 genes), mitochondria function I (2077 genes), and II (757 genes) (see Figure S3, SDC, http://links.lww.com/TXD/A287, for further details).

Analysis Following the ISHLT Classification

The gene module-trait correlation showed divergent interaction between mitochondria and immune function modules. The rejection trait (ISHLT 1R n = 23/35.9% and 2R n = 11/17.2%) had a decreased activity of genes related to mitochondrial function (corr. −0.38, $P = 0.002$ and −0.22, $P = 0.08$) and enrichment for expressed genes related to immune function (corr. 0.51, $P = 1.0 \times 10^{-05}$) compared with the no-rejection trait (ISHLT 0R, n = 30/46.9%) (Figure 1).

Analysis Following the Unsupervised Classification

The class assignment using gene expression and optimal transport transformation resulted in 3 EMB classes: Unsupervised class (UC) UC1, UC2, and UC3. The UC1 class, was closely related with the no-rejection group, and the UC2 class showed similarities to the rejection group. Compared with the ISHLT classification, gene-based classes had a stronger correlation with WGCNA-derived functional gene modules. For example, class 2 had a high activity of genes in the immune function module (corr. of 0.78, $P = 5.0 \times 10^{-14}$) and a low activity of genes related to mitochondrial function I module (corr. −0.56, $P = 1.0 \times 10^{-6}$). UC3 had intermediate characteristics of classes 1 and 2 (Figure 1).

GO Enrichment and Hub Genes of the Mitochondrial Function Modules

Genes enriching the mitochondria function module I were predominantly involved in energy generation, regulation of oxidative stress, and substrate metabolism. The mitochondria function module II was mainly involved in the regulation of mitochondrial translation. The GO biologic processes of the mitochondrial-related modules are summarized in Table 3. The top hub genes in mitochondria module I included *ATP5O*, *AURKAIP1*, *COX14*, *COX4I1*, and *MYEOV2*. In mitochondria module II, hub genes involved *ACO2*, *ALDH2*, *ATP5B*, *FH*, and *MAPKAPK3* among others. Table 4 summarizes the top Maximal Clique Centrality-based scored hub genes, and Figure 2 represents the role of the hub genes identified.

GO Enrichment and Hub Genes of the Immune Function Module

GO categories enriched by expressed genes in this module included T-cell costimulation, T-cell and B-cell proliferation, antigen processing and presentation, leukocyte migration, NK cell–mediated cytotoxicity, among others. Highest ranked Hub genes within this module included *CD53*, *CTSS*, *HLA-DRA*, *IRF8*, *PTPRC*, *RAC2*, *IL10RA*, *HLA-DPB1*, *CD84*, and *MS4A6A*. As we are focusing on nonimmune factors, the immune module is not discussed here.

Clinical Correlations

We found a negative correlation between expression of mitochondrial function genes and levels of BNP (corr. −0.35, $P = 0.005$ and −0.25, $P = 0.05$), and a positive correlation between activity of immune-response genes and levels of BNP (corr. 0.3, $P = 0.02$). Allomap scores presented a negative correlation to mitochondria function II and (−0.39, $P = 0.01$) and a positive correlation to immune module (corr. of 0.48, $P = 5.0 \times 10^{-5}$). ImmuKnow only presented significant negative correlation with the mitochondria function module II (−0.29, $P = 0.02$). The correlations between the gene modules and WBC, hemodynamic variables, and LVEF were weak and not statistically significant. Gene modules and clinical trait correlations are illustrated in Figure 1.

DISCUSSION

This study suggests that EMBs of patients with cardiac allograft rejection have decreased activity of mitochondrial-related genes and increased expression of genes involved in immune response. This molecular pattern is more evident by

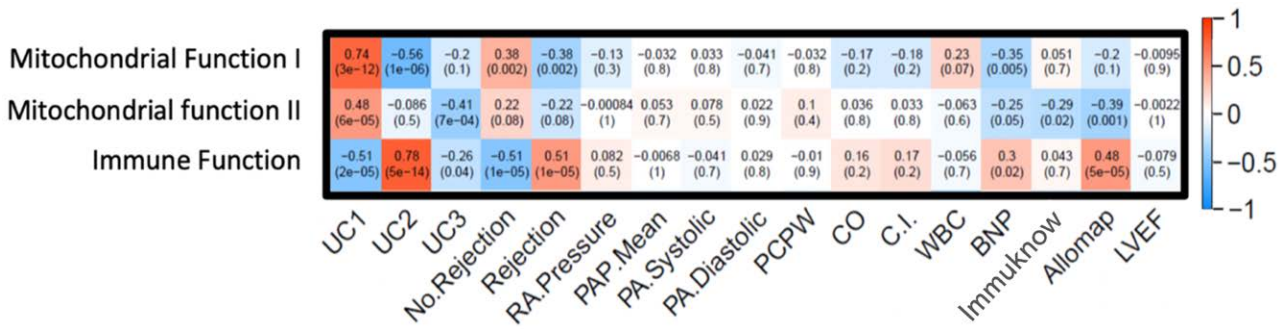

FIGURE 1. Gene module-clinical trait association. Each row corresponds to a module eigengene. The columns represent clinical traits of the 3 unsupervised classes: ISHLT rejection trait, RA pressure, PAP mean, PA systolic, PA diastolic, PCPW, CO, CI, WBC, BNP, ImmuKnow, Allomap, and LVEF. Within each cell, the correlation value (top) and *P* value (bottom) are depicted. BNP, brain natriuretic peptide; CI, cardiac index; CO, cardiac output; LVEF, left ventricular ejection fraction; PA, pulmonary artery; PAP, pulmonary artery pressure; PCPW, Pulmonary-Capillary Wedge Pressure; RA, right atrium; WBC, white blood cells.

TABLE 3.

Relevant biologic processes in mitochondria modules

Module	Term ID	Term name	Total genes	Genes	Enrichment *P*
Mitochondrial function I: Energy generation and regulation of oxidative stress	GO:0019752	Carboxylic acid metabolic process	458	158	3.82×10^{-13}
	GO:0006082	Organic acid metabolic process	530	170	5.85×10^{-11}
	GO:0044281	Small molecule metabolic process	1213	324	1.09×10^{-10}
	GO:0055114	Oxidation-reduction process	517	165	2.59×10^{-10}
	GO:0046395	Carboxylic acid catabolic process	109	54	1.85×10^{-09}
	GO:0051186	Cofactor metabolic process	175	73	6.85×10^{-09}
	GO:0006520	Cellular amino acid metabolic process	192	72	3.91×10^{-06}
	GO:0032787	Monocarboxylic acid metabolic process	272	92	8.29×10^{-06}
	GO:0009063	Cellular amino acid catabolic process	60	32	1.77×10^{-05}
	GO:0006631	Fatty acid metabolic process	162	62	2.74×10^{-05}
	GO:0006637	Acyl-CoA metabolic process	40	24	9.37×10^{-05}
Mitochondrial function II: Regulation of mitochondrial translation	GO:0006415	Translational termination	77	55	2.23×10^{-43}
	GO:0006414	Translational elongation	90	58	6.71×10^{-42}
	GO:0006413	Translational initiation	125	64	7.32×10^{-38}
	GO:0019083	Viral transcription	85	47	6.96×10^{-29}
	GO:0006613	Cotranslational protein targeting to membrane	45	35	1.43×10^{-28}
	GO:0006614	SRP-dependent cotranslational protein targeting to membrane	45	35	1.43×10^{-28}
	GO:0045047	Protein targeting to ER	48	36	1.77×10^{-28}
	GO:0000184	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	58	39	4.03×10^{-28}
	GO:0072599	Establishment of protein localization to endoplasmic reticulum	50	36	2.09×10^{-27}
	GO:0043624	Cellular protein complex disassembly	131	56	2.32×10^{-27}
	GO:0043043	Peptide biosynthetic process	271	80	3.95×10^{-27}
	GO:0000956	Nuclear-transcribed mRNA catabolic process	87	46	5.00×10^{-27}
	GO:0006412	Translation	259	78	5.23×10^{-27}

Significant gene GO Biologic Processes within the mitochondria function modules.
GO, gene ontology.

the molecular functional classification based on the GE (UC classification) in comparison to ISHLT, thus unveiling the underlying biology of the graft that is not evident under the microscope. Peripherally, elevated levels of BNP, ImmuKnow, and Allomap scores are associated with decreased mitochondrial-related gene activity and increased activity of immune-response genes. This likely represents a rejection-associated mitochondrial impairment.

Mitochondria Function and Transplantation

Studies of mitochondrial function in humans following HTx are very limited. In murine models, disturbances were found in mitochondrial oxidative pathways during the acute rejection process, and there was evidence of decreased glycolytic enzymes.[26-28] Studies in humans showed that disturbances in intracardiac mitochondrial bioenergetics have a role in cardiac allograft rejection.[29,30] Recently, a study evaluated intracardiac mitochondrial function by high-resolution respirometry after HTx; the results showed oxidative capacity declination along with an increasing number of CD3+ lymphocytes. The impaired mitochondrial respiration improved after steroid pulse therapy.[31] Another study in renal transplant recipients supports our findings, GE signatures of mitochondrial impairment were associated with allograft injury and worse long-term allograft survival.[19]

Mitochondrial Function in Heart Disease

The mitochondria module I was involved in energy generation, regulation of oxidative stress, and substrate metabolism.

TABLE 4.

Top hub genes in mitochondria function modules (I and II)

Module	Gene symbol	Gene name	Definition
Mitochondrial function I	ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	ATP synthase, H+ transporting
	AURKAIP1	Aurora kinase A interacting protein 1	Ribosomal subunit protein
	COX14	Cytochrome C oxidase assembly factor	Role in coordinating cytochrome C oxidase
	COX4I1	Cytochrome C oxidase subunit 4I1	Terminal enzyme of the mitochondrial respiratory chain
	MYEOV2	Myeloma-overexpressed gene 2 protein	Role in cell proliferation
	NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha, mitochondrial complex associated	NADH: transfers electrons from NADH to respiratory chain
	NDUFB8	NADH: ubiquinone oxidoreductase subunit B8	NADH: transfers electrons from NADH to respiratory chain
	NDUFV2	NADH: ubiquinone oxidoreductase core subunit V2	NADH: transfers electrons from NADH to respiratory chain
	RPL23A	Ribosomal protein L23a	Involved in mediating growth inhibition by interferon
	UQCR11	Ubiquinol-cytochrome C reductase, complex III subunit XI	Forms part of mitochondrial respiratory chain: Metabolism, electron transport, ATP synthesis, heat production by uncoupling proteins
Mitochondrial function II	ACO2	Aconitase 2	Enzyme involved in second step of TCA cycle
	ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	Oxidizes aldehydes to generate carboxylic acids for use in muscle and heart
	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	Subunit of mitochondrial ATP synthase (catalyzes ATP synthesis)
	FH	Fumarate hydratase	Component of TCA cycle
	MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase 3	Induced by growth inducers and stress stimulation of cells: involved in cytokines production, endocytosis, cell migration, chromatin remodeling, and transcriptional regulation
	NDUFV1	NADH: ubiquinone oxidoreductase core subunit V1	NADH subunit
	PDK2	Pyruvate dehydrogenase kinase 2	Regulates glucose/fatty acid metabolism through TCA cycle plays an important role in maintaining normal blood glucose levels. Plays a role in resistance to apoptosis under oxidative stress
	PEBP1	Phosphatidylethanolamine binding protein 1	Involved in modulating MAPK, NF-kappa B, GSK-3 signaling pathways
	SDHA	Succinate dehydrogenase complex flavoprotein subunit A	A complex of mitochondrial respiratory chain
	UQCRC1	Ubiquinol-cytochrome C reductase core protein I	A part of the mitochondrial respiratory chain

Highest ranked Hub genes within mitochondrial modules ranked by MCC score.
MCC, Maximal Clique Centrality; NADH, nicotinamide adenine dinucleotide + hydrogen.

The mitochondria module II was mainly involved in regulation of mitochondrial translation (see Table 3).

Energy Generation and Oxidative Stress

Impaired mitochondrial function is a consistent feature in the pathophysiology of heart failure, and its role as one of the key contributors in heart failure is increasingly recognized.[32,33] During oxidative stress, such an inflammatory state, reactive oxygen species (ROS) accumulate, which could potentially be a source of mitochondrial genomic instability. This leads to alterations of the mitochondrial bioenergetics and subsequently increases ROS. Excessive accumulation of ROS increases mitochondrial DNA damage, leading to altered mitochondrial function.[34] Disturbances in intracardiac mitochondrial bioenergetics and responses to oxidative stress were also found in allograft rejection, suggesting that those features can play a role in disease development. Antioxidant therapy with coenzyme Q10 was proposed to prevent rejection.[29,30] However, the concept is controversial.[35]

Substrate Metabolism

Glucose, fatty acids, and amino acids (minor role) are key substrates of cardiac metabolism. Through various enzymatic pathways, these substrates generate high levels of ATP, which is essential to fuel continuous cardiac function. Alterations in substrate metabolism could consequently contribute to cardiac energetic inefficiency and disease progression.[36-38]

Mitochondrial Translation

Nuclear encoded genes play an important role in mitochondrial maintenance, mitochondria translation, and transcription. Biogenesis of the mitochondrial machinery requires adequate translation and protein synthesis for the assembly and functioning of the oxidative phosphorylation complexes.[39] Defects in these processes are associated with cardiomyopathies and cardiovascular diseases.[40,41]

Mitochondria and Inflammatory Response

Mitochondrial dysfunction can contribute to the inflammatory response through both activation of the redox-sensitive inflammatory pathway and direct activation of the inflammasome.[17,18,42] Activation of these 2 systems could lead to an overstimulation of the inflammatory response, which increases mitochondrial oxidative stress and promotes a vicious inflammatory cycle.[43-45] The dysfunctional mitochondria need to be removed by mitophagy to keep cellular homeostasis. Deficient response to oxidative stress can lead to inadequate removal of dysfunctional mitochondria and mitochondrial DNA, contributing to the inflammatory process.[46,47] Figure 2 represents the role of the hub genes identified and their possible contribution to inflammatory response.

Molecular Classification of EMB

Few studies have explored the potential of cardiac gene expression in the diagnosis of HTx rejection.[14,16,48,49] In this study, we used an unsupervised classification based only

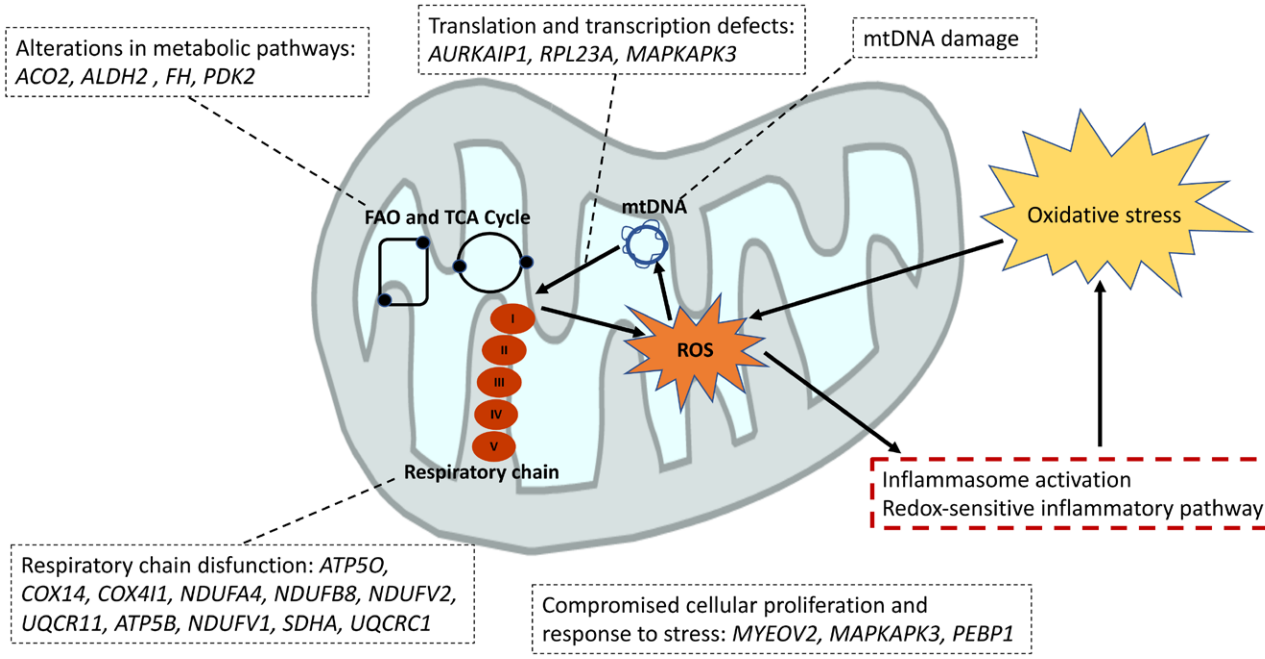

FIGURE 2. Hub mitochondria-related genes. The figure represents the hub genes identified in the mitochondria modules and their possible contribution to an inflammatory response. FAO, fatty acid β-oxidation; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle.

on GE to address the problem of wide variability in the classification of EMB and to improve evaluation accuracy. Genomic profile class 1 had characteristics of no-rejection, and class 2 had characteristics of rejection. The results showed a stronger gene module-trait correlation compared with the histology classification, thus, suggesting a possible improved representation of the underlying biologic process. Class 3 had intermediate characteristics of classes 1 and 2. The integrative analysis of transcriptomics and individual variability (confounding factors) show the potential of a precision medicine approach to refine diagnosis in HTx rejection.[50,51]

Clinical Correlations

Although limited by sample size, we found that elevated levels of BNP, ImmuKnow, and Allomap scores are associated with decreased mitochondrial-related gene activity and increased activity of immune-response genes (Figure 1). This possibly represents immune activation and cardiac dysfunction, whereas the intragraft mitochondrial function decreases. Hemodynamics, WBC, and LVEF correlations were not significant, possibly because of the fact that most cases of acute rejection are diagnosed when the patient is asymptomatic. In a typical surveillance population, severe hemodynamic compromise is present in <5% of the patients and echocardiography parameters have limited diagnostic performance.[52,53] If our findings are further confirmed, evaluation of mitochondrial function can potentially be used as a surrogate of allograft function in an early stage when no other diagnostic markers reveal abnormal allograft function.

Taken together, the mitochondrial function could serve to explore potential diagnostic markers and therapeutic strategies that could provide further insights into allograft function. Additionally, our unsupervised method offers opportunities to improve evaluation accuracy of the EMB in HTx rejection.

Limitations

We acknowledge that there are limitations to this study. The sample size was small, but it is consistent with sample sizes in gene expression profile studies.[13,48,49,54] Samples with well-defined AMR were limited, so reliable conclusions about AMR cannot be extracted. This study was not conducted longitudinally but highlights the importance of biobanking systematically to be able to study mitochondrial gene expression before moderate/severe rejection and after treatment. Tissue samples comprise several different cell types; bulk RNA-seq methods are not able to capture and define the cell type responsible for the gene expression; this is a universal problem of the RNA-seq methods. Further studies should be sought to confirm our findings and clarify the cell type responsible for the mitochondrial-related gene expression. The use of single-cell transcriptomic profiles and high-resolution respirometry could provide further insights about the cell type and mitochondrial function.[31,55] Additionally, as we are moving away from EMB as a surveillance test, with both peripheral GE and cell-free DNA, further research should correlate mitochondrial function to cell-free DNA. Also, exploratory research would be of interest in circulating mitochondria DNA and intragraft mitochondrial function.[56,57]

CONCLUSIONS

Our findings suggest that intragraft mitochondrial impairment is involved in acute cellular rejection. This highlights the role of mitochondrial function in cardiac allograft rejection and offers opportunities to explore diagnostic markers and therapeutic targets. The molecular classification of EMB based only on gene expression better represents the underlying biologic process in comparison to the ISHLT criteria. This illustrates the clinical potential of a precision medicine approach to refine evaluation of cardiac allograft rejection.

ACKNOWLEDGMENTS

The authors would like to acknowledge the assistance of Paulo Rocha and Lyrissa Leininger. This study was funded by American Heart Association Grant No. 11GRNT7990092.

REFERENCES

1. Lund LH, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: thirty-second official adult heart transplantation report–2015; focus theme: early graft failure. *J Heart Lung Transplant.* 2015;34:1244–1254.
2. Lund LH, Edwards LB, Kucheryavaya AY, et al; International Society of Heart and Lung Transplantation. The registry of the International Society for Heart and Lung Transplantation: thirty-first official adult heart transplant report–2014; focus theme: retransplantation. *J Heart Lung Transplant.* 2014;33:996–1008.
3. Billingham ME, Cary NR, Hammond ME, et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. *J Heart Transplant.* 1990;9:587–593.
4. 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;24:1710–1720.
5. Winters GL, Marboe CC, Billingham ME. The International Society for Heart and Lung Transplantation grading system for heart transplant biopsy specimens: clarification and commentary. *J Heart Lung Transplant.* 1998;17:754–760.
6. Crespo-Leiro MG, Zuckermann A, Bara C, et al. Concordance among pathologists in the second Cardiac Allograft Rejection Gene Expression Observational Study (CARGO II). *Transplantation.* 2012;94:1172–1177.
7. Pham MX, Teuteberg JJ, Kfoury AG, et al; IMAGE Study Group. Gene-expression profiling for rejection surveillance after cardiac transplantation. *N Engl J Med.* 2010;362:1890–1900.
8. Schoels M, Dengler TJ, Richter R, et al. Detection of cardiac allograft rejection by real-time PCR analysis of circulating mononuclear cells. *Clin Transplant.* 2004;18:513–517.
9. Starling RC, Pham M, Valantine H, et al; Working Group on Molecular Testing in Cardiac Transplantation. Molecular testing in the management of cardiac transplant recipients: initial clinical experience. *J Heart Lung Transplant.* 2006;25:1389–1395.
10. Bodez D, Hocini H, Tchitchek N, et al. Myocardial gene expression profiling to predict and identify cardiac allograft acute cellular rejection: The GET-Study. *PLoS One.* 2016;11:e0167213.
11. 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;37:2591–2601.
12. Deng MC, Eisen HJ, Mehra MR, et al; CARGO Investigators. Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. *Am J Transplant.* 2006;6:150–160.
13. Horwitz PA, Tsai EJ, Putt ME, et al. Detection of cardiac allograft rejection and response to immunosuppressive therapy with peripheral blood gene expression. *Circulation.* 2004;110:3815–3821.
14. Loupy A, Duong Van Huyen JP, Hidalgo L, et al. Gene expression profiling for the identification and classification of antibody-mediated heart rejection. *Circulation.* 2017;135:917–935.
15. Mehra MR. The emergence of genomic and proteomic biomarkers in heart transplantation. *J Heart Lung Transplant.* 2005;24(7 Suppl):S213–S218.
16. Halloran PF, Potena L, Van Huyen JD, et al. Building a tissue-based molecular diagnostic system in heart transplant rejection: The heart Molecular Microscope Diagnostic (MMDx) System. *J Heart Lung Transplant.* 2017;36:1192–1200.
17. Sandhir R, Halder A, Sunkaria A. Mitochondria as a centrally positioned hub in the innate immune response. *Biochim Biophys Acta Mol Basis Dis.* 2017;1863:1090–1097.
18. Angajala A, Lim S, Phillips JB, et al. Diverse roles of mitochondria in immune responses: novel insights into immuno-metabolism. *Front Immunol.* 2018;9:1605.
19. Zepeda-Orozco D, Kong M, Scheuermann RH. Molecular profile of mitochondrial dysfunction in kidney transplant biopsies is associated with poor allograft outcome. *Transplant Proc.* 2015;47:1675–1682.
20. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics.* 2008;9:559.
21. Bindea G, Mlecnik B, Hackl H, et al. ClueGO: a cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics.* 2009;25:1091–1093.
22. Chin CH, Chen SH, Wu HH, et al. cytoHubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst Biol.* 2014;8(Suppl 4):S11.
23. Tabak EG, Turner CV. A family of nonparametric density estimation algorithms. *Commun Pure Appl Math*. 2013;66:145–164.
24. Agnelli JP, Cadeiras M, Tabak EG, et al. Clustering and classification through normalizing flows in feature space. *Multiscale Modeling Simulation*. 2010;8:1784–1802.
25. Trigila G, Tabak EG. Data-driven optimal transport. *Commun Pure Appl Math*. 2016;69:613–648.
26. Duboc D, Abastado P, Muffat-Joly M, et al. Evidence of mitochondrial impairment during cardiac allograft rejection. *Transplantation.* 1990;50:751–755.
27. Abastado P, Duboc D, Marsac C, et al. Demonstration of abnormalities of myocardial mitochondrial oxygenation in cardiac graft rejection. *Arch Mal Coeur Vaiss.* 1991;84:855–859.
28. Maneus EB, Pomar-Moya JL, Climent F, et al. Glycolytic enzyme activities are decreased during acute rejection in transplanted rat hearts. *Transplant Proc.* 2005;37:4122–4123.
29. Gvozdjáková A, Kucharská J, Mizera S, et al. Coenzyme Q10 depletion and mitochondrial energy disturbances in rejection development in patients after heart transplantation. *Biofactors.* 1999;9:301–306.
30. Kucharská J, Gvozdjáková A, Mizera S, et al. Participation of coenzyme Q10 in the rejection development of the transplanted heart: a clinical study. *Physiol Res.* 1998;47:399–404.
31. Scheiber D, Jelenik T, Horn P, et al. P6033 Impaired myocardial mitochondrial function correlates with inflammatory cell burden in humans following heart transplantation. *Eur Heart J*. 2017;38(suppl_1):1268.
32. Brown DA, Perry JB, Allen ME, et al. Expert consensus document: Mitochondrial function as a therapeutic target in heart failure. *Nat Rev Cardiol.* 2017;14:238–250.
33. Murphy E, Ardehali H, Balaban RS, et al; American Heart Association Council on Basic Cardiovascular Sciences, Council on Clinical Cardiology, and Council on Functional Genomics and Translational Biology. Mitochondrial function, biology, and role in disease: a scientific statement from the American Heart Association. *Circ Res.* 2016;118:1960–1991.
34. Ayoub KF, Pothineni NVK, Rutland J, et al. Immunity, inflammation, and oxidative stress in heart failure: emerging molecular targets. *Cardiovasc Drugs Ther.* 2017;31:593–608.
35. Ristow M. Unraveling the truth about antioxidants: mitohormesis explains ROS-induced health benefits. *Nat Med.* 2014;20:709–711.
36. Noordali H, Loudon BL, Frenneaux MP, et al. Cardiac metabolism—a promising therapeutic target for heart failure. *Pharmacol Ther.* 2018;182:95–114.
37. Fukushima A, Milner K, Gupta A, et al. Myocardial energy substrate metabolism in heart failure: from pathways to therapeutic targets. *Curr Pharm Des.* 2015;21:3654–3664.
38. Bertero E, Maack C. Metabolic remodelling in heart failure. *Nat Rev Cardiol.* 2018;15:457–470.
39. D'Souza AR, Minczuk M. Mitochondrial transcription and translation: overview. *Essays Biochem.* 2018;62:309–320.
40. Boczonadi V, Horvath R. Mitochondria: impaired mitochondrial translation in human disease. *Int J Biochem Cell Biol.* 2014;48:77–84.
41. Siasos G, Tsigkou V, Kosmopoulos M, et al. Mitochondria and cardiovascular diseases-from pathophysiology to treatment. *Ann Transl Med.* 2018;6:256.
42. Garg NJ. Inflammasomes in cardiovascular diseases. *Am J Cardiovasc Dis.* 2011;1:244–254.
43. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol.* 2016;16:407–420.
44. López-Armada MJ, Riveiro-Naveira RR, Vaamonde-García C, et al. Mitochondrial dysfunction and the inflammatory response. *Mitochondrion.* 2013;13:106–118.
45. Tait SW, Green DR. Mitochondria and cell signalling. *J Cell Sci.* 2012;125(Pt 4):807–815.
46. Oka T, Hikoso S, Yamaguchi O, et al. Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature.* 2012;485:251–255.
47. Gottlieb RA, Thomas A. Mitophagy and mitochondrial quality control mechanisms in the heart. *Curr Pathobiol Rep.* 2017;5:161–169.
48. Bodez D, Hocini H, Tchitchek N, et al. Myocardial gene expression profiling to predict and identify cardiac allograft acute cellular rejection: the GET-study. *PLoS One.* 2016;11:e0167213.

49. Holweg CT, Potena L, Luikart H, et al. Identification and classification of acute cardiac rejection by intragraft transcriptional profiling. *Circulation.* 2011;123:2236–2243.
50. Sirota M, Sarwal MM. Transplantomics: toward precision medicine in transplantation research. *Transplantation.* 2017;101:1777–1782.
51. Jameson JL, Longo DL. Precision medicine–personalized, problematic, and promising. *N Engl J Med.* 2015;372:2229–2234.
52. Lu W, Zheng J, Pan X, et al. Diagnostic performance of echocardiography for the detection of acute cardiac allograft rejection: a systematic review and meta-analysis. *PLoS One.* 2015;10:e0121228.
53. Mills RM, Naftel DC, Kirklin JK, et al. Heart transplant rejection with hemodynamic compromise: a multiinstitutional study of the role of endomyocardial cellular infiltrate. Cardiac Transplant Research Database. *J Heart Lung Transplant.* 1997;16:813–821.
54. Hollander Z, Lin D, Chen V, et al; NCE CECR PROOF Centre of Excellence. Whole blood biomarkers of acute cardiac allograft rejection: double-crossing the biopsy. *Transplantation.* 2010;90:1388–1393.
55. Kalisky T, Oriel S, Bar-Lev TH, et al. A brief review of single-cell transcriptomic technologies. *Brief Funct Genomics.* 2018;17:64–76.
56. An Q, Hu Y, Li Q, et al. The size of cell-free mitochondrial DNA in blood is inversely correlated with tumor burden in cancer patients. *Precis Clin Med.* 2019;2:131–139.
57. Liu J, Cai X, Xie L, et al. Circulating cell free mitochondrial DNA is a biomarker in the development of coronary heart disease in the patients with type 2 diabetes. *Clin Lab.* 2015;61:661–667.