Many heart transplant biopsies currently diagnosed as no rejection have mild molecular antibody-mediated rejection-related changes

Philip F. Halloran, MD, PhD,[a] Katelynn Madill-Thomsen, PhD,[a] Arezu Z. Aliabadi-Zuckermann, MD,[b] Martin Cadeiras, MD,[c] Marisa G. Crespo-Leiro, MD, PhD,[d] Eugene C. Depasquale, MD,[c] Mario Deng, MD,[c] Johannes G€okler, MD,[b] Daniel H. Kim, MD,[a] Jon Kobashigawa, MD,[e] Peter Macdonald, MD, PhD,[f] Luciano Potena, MD, PhD,[g] Keyur Shah, MD,[h] Josef Stehlik, MD, MPH,[i] and Andreas Zuckermann, MD[b]

From the [a]University of Alberta, Edmonton, Alberta, Canada; [b]Medical University of Vienna, Vienna, Austria; [c]Ronald Reagan UCLA Medical Center, Los Angeles, California; [d]Complexo Hospitalario Universitario A Coruña, A Coruña, Spain; [e]Cedars-Sinai Medical Center, Los Angeles, California; [f]The Victor Chang Cardiac Research Institute, Sydney, Australia; [g]University of Bologna, Bologna, Italy; [h]Virginia Commonwealth University, Richmond, Virginia; and the [i]University of Utah, Salt Lake City, Utah.

Reprint requests: Philip F. Halloran MD, PhD, Alberta Transplant Applied Genomics Centre, #250 Heritage Medical Research Centre, University of Alberta, Edmonton, AB T6G 2S2, Canada. Telephone: 780-4926160. Fax: 780-407-7450. E-mail address: phallora@ualberta.ca

Abstract

Background. The Molecular Microscope (MMDx) system classifies heart transplant endomyocardial biopsies as No-rejection (NR), Early-injury, T cell-mediated (TCMR), antibody-mediated (ABMR), mixed, and possible rejection (possible TCMR, possible ABMR). Rejection-like gene expression patterns in NR biopsies have not been described. We extended the MMDx methodology, using a larger data set, to define a new "Minor" category characterized by low-level inflammation in non-rejecting biopsies.

Methods. Using MMDx criteria from a previous study, molecular rejection was assessed in 1,320 biopsies (645 patients) using microarray expression of rejection-associated transcripts (RATs). Of these biopsies, 819 were NR. A new archetypal analysis model in the 1,320 data set split the NRs into NR-Normal (N = 462) and NR-Minor (N = 359).

Results. Compared to NR-Normal, NR-Minor were more often histologic TCMR1R, with a higher prevalence of donor-specific antibody (DSA). DSA positivity increased in a gradient: NR-Normal 24%; NR-Minor 34%; possible ABMR 42%; ABMR 66%. The top 20 transcripts distinguishing NR-Minor from NR-Normal were all ABMR-related and/or IFNG-inducible, and also exhibited a gradient of increasing expression from NR-Normal through ABMR. In random forest analysis, TCMR and Early-injury were associated with reduced LVEF and increased graft loss, but NR-Minor and ABMR scores were not. Surprisingly, hearts with MMDx ABMR showed comparatively little graft loss.

Conclusions. Many heart transplants currently diagnosed as NR by histologic or molecular assessment have minor increases in ABMR-related and IFNG-inducible transcripts, associated with DSA positivity and mild histologic inflammation. These results suggest that low-level ABMR-related molecular stress may be operating in many more hearts than previously estimated. (ClinicalTrials.gov #NCT02670408).

Keywords: Gene expression; Biopsy; Heart; transplantation; Rejection

List of non-standard abbreviations: ABMR, antibody-mediated rejection; DSA, donor-specific HLA antibody; EMB, endomyocardial biopsy; INTERHEART, Diagnostic and Therapeutic Applications of Microarrays in Heart Transplantation, a Multicenter Study (ClinicalTrials.gov NCT02670408); LVEF, left ventricular ejection fraction; MMDx, Molecular Microscope® Diagnostic System; $MMDx_{Mod}$, Molecular Microscope® Diagnostic System modified by new archetype clusters; NR, No-rejection; NR-Normal, No-rejection by MMDx diagnosis and Normal by 5 rejectionbased archetype groups; NR-Minor, No-rejection by MMDx diagnosis and Minor by 5 rejection-based archetype groups; Early-injury, No-rejection by MMDx diagnosis and Early-injury by 5 rejection-based archetype groups; PCA, principal component analysis; PC1/2/3, principal components 1/2/3; RATs, rejection-associated transcripts; SOC, standard-ofcare; TCMR, T cell-mediated rejection; TCMR1R, ISHLT class indicating mild TCMR-like inflammation

Medical diagnostics is moving toward adding precision measurements from molecular platforms to conventional assessments.[1] This development presents an opportunity to improve the assessment of heart transplant endomyocardial biopsies (EMBs) and add mechanistic insights. The Molecular Microscope Diagnostic System (MMDx)[2-7] was developed for heart transplant EMBs to diagnose molecular T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR) based on expression of rejection-associated transcripts (RATs)[5,6] while distinguishing TCMR and ABMR from other forms of inflammation.[7-11] MMDx measures gene expression in the biopsy using microarrays and applies an ensemble of machine-learning algorithms to assign diagnoses of TCMR, ABMR, and possible rejection (possible TCMR and possible ABMR). (For a glossary of abbreviations, see Table S1.) Because injury evokes inflammation that shares mechanisms with adaptive immunity (rejection), RAT-based analysis also identified an inflamed “Early-injury” state distinct from rejection, associated with macrophage transcripts and depressed left ventricular ejection fraction (LVEF).[3,5-7]

Molecular features in biopsies MMDx calls No-rejection (NR) are of interest because many EMBs manifest mild histologic inflammation (TCMR1R) that clinicians believe does not require treatment. In the INTERHEART study, histology reported TCMR1R in 31% of all biopsies.[7] Approximately 15% of TCMR1R biopsies have molecular rejection, usually ABMR.7 The present study in an expanded biopsy population was designed to examine minor rejection-like inflammation in biopsies currently classified as NR and determine relationships to DSA positivity, function, and outcome. We hypothesized that there may be subtle ABMR-related activity within some biopsies currently considered to have no rejection by histologic or molecular assessment.

Materials and methods

Population. We used microarrays to explore the molecular characteristics of 1320 EMBs from the prospective observational INTERHEART study, approved by ethics review boards at each center (ClinicalTrials.gov NCT02670408). EMBs for clinical indications or protocol from consenting patients at 13 centers throughout Canada, the USA, Australia, and Europe, were collected as standard-of-care (SOC). Samples were placed in RNA*later* and shipped to the Alberta Transplant Applied Genomics Centre.[12] Histology was per SOC by each center, following ISHLT guidelines.[13-15] Histologic diagnoses, available for 1,278 of the 1,320 biopsies, were simplified to permit histology-molecular comparisons.[3,6,7] Molecular and histology analyses were blinded to one another. Approximately 2% of biopsies were excluded due to inadequacy for molecular examination, with no other exclusions.

Microarray analysis and MMDx signouts. Total RNA was labelled as previously described[3,5,7] and hybridized to Prime-View microarrays (Affymetrix). CEL files are available on the Gene Expression Omnibus website (GSE150059). Differential expression was by Bayesian *t*-test using the "limma" package[16] in R.[17] MMDx reports were signed-out as TCMR, possible TCMR (possible TCMR), ABMR, possible ABMR (possible ABMR), Mixed, Early-injury, or NR using previously described methods.[3]

Rejection-associated transcripts (RATs). RATs were the union of the top 200 probe sets from 3 kidney transplant biopsy class comparisons: all rejection vs everything else, ABMR vs everything else, and TCMR vs everything else,[2,18] reduced by IQRf iltering (removing low-variance transcripts) to 437 probe sets. Though originally derived in

kidney transplant biopsies, RATs represent the same processes in heart, lung, and liver tissue.[4,7,19,20]

Transcript sets. Transcript sets (Table S2) were previously annotated in cell lines, experimental models, and human transplant biopsies (https://www.ualberta.ca/medicine/institutes-centresgroups/atagc/research/gene-lists). Transcript set scores are the means of fold changes compared to controls (371 biopsies with no molecular rejection >30 days post-transplant), calculated using the original log2 raw data.

Dimensionality reduction, clustering, and data visualization. Principal component analysis (PCA) and archetypal analysis were described previously.[3,7] PCA is used to simplify analysis of highdimensional data sets (e.g., the 437 RATs). Archetypal analysis defines a specified number "*n*" of idealized archetypes, then assigns each biopsy *n* scores that reflect their similarity to each archetype, the *n* scores summing to 1. The highest score defines the archetype group. Rejection archetypes differ slightly from MMDxsign-outs (hereafter called "MMDx"), which also consider classifier scores.[7]

The input for both analyses was a 1,320 (biopsy) x 437 (RAT expression values) data set. We used the "FactoMineR"[21] and "archetypes"[22] R packages.

Regression analyses. Multivariable Cox regression was performed assessing 3-year death-censored survival using "cph" from the R package "rms." The predictor variables were heavily rightskewed, and were therefore log-transformed. Logistic regression predicting LVEF≤55 was done using "lrm" from the "rms" package. Because a large number of archetype scores were 0.0, a small constant (0.001) was added to each score to allow for log-transformation as per standard procedure.

Random forest survival analyses. As detailed elsewhere,[23] analyses were based on survival to 3 years postbiopsy, with longer times censored at 3 years. Error rates refer to those calculated in out-of-bag samples from the bootstrapping process, which are analogous to test set statistics calculated in standard cross-validation.

Experimental design. A flowchart describing how the molecular diagnostic classes in this paper ($MMDx_{Mod}$) were modified from an earlier (published) version of MMDx is shown in Figure 1. (New diagnoses are not currently used for MMDx signouts pending peer review.)

Results

Biopsy and patient populations. We examined 1,320 EMB (including 889 previously studied[7]) from 645 patients, taken 4 days to 28 years post-transplant: 62% taken for protocol and 25% for indications, with 13% not designated as either. Table S3 shows population demographics, while Table 1 shows MMDxdiagnoses and center histologic diagnoses following ISHLT guidelines,[13,14] along with DSA assessments.

To study MMDx and histology in parallel, we grouped the biopsies similarly. There were seven MMDx classes: No-rejection (excluding Early-injury), Early-injury, possible TCMR, TCMR, possible ABMR, ABMR, and Mixed.

The histology categories were No-rejection, possible TCMR (TCMR1R), TCMR, posible ABMR, ABMR, and Mixed.

At the time of biopsy, donor-specific HLA antibody (DSA) by SOC testing was present in 37% of the 824 patients tested (Table1) and was present in all diagnostic groups. In 496 biopsies, DSA was not tested (not indicated by SOC). DSA was more common in indication biopsies vs protocol biopsies (Table1).

Characterizing minor rejection-related molecular changes in NR biopsies. As in previous analyses,[3,7] we visualized variation in RAT expresión using PCA (Figure2). Left panels (A,C, and E) show dimensión 1 (PC1, separating no rejection from rejection) and dimensión 2 (PC2, separating TCMR (red) form ABMR (blue)). The right-hand panels (B,D, and F) rotate the biopsy distribution 90 degrees to reveal dimensión 3 (PC3), which separates Early-injury (green) from the other biopsies. Figure 2ª and 2B show our published 4 archetype groups[3] used in the ensemble of algorithms for signout: TCMR (red), ABMR (blue), Early-injury (green), and No-rejection (and no Early-injury) (grey). To explore rejection-related heterogeneity within NR biopsies, we developed a new 5 archetype model (Figure 2C and 2D), distinguishing a new subgroup of biopsies with Minor rejection-related changes (pink). We then applied the new archetype scores exclusively to the biopsies that MMDx signed out as NR to créate a modified MMDx classification ($MMDx_{Mod}$) that retained all existing rejection diagnoses but Split No-rejection into NR-Normal and NR-Minor (Figure 2E and 2F). This created eight groups: NR-Normal, NR-Minor, Early-injury, and the 5 rejection groups.

Relating NR-Minor biopsies to histology. Table 2 shows the % DSA positivity in MMDx$_{Mod}$ rejection classes in 824 biopsies with DSA test results, high lighting the NR and Minor groups. DSA was significantly more prevalent in NR-Minor tan NR-Normal (p=0.02). Percent DSA positivity increased in a gradient, from NR-Normal 24% to NR-Minor 34% to posible ABMR 42% to ABMR 64% (Table2). Class II was distributed similarly, with a gradient of NR 15%, Minor 24%, posible ABMR 30%, and ABMR 56%.

Top genes with increased expresión in NR-Minor vs NR-Normal. Using 49,495 probe sets, we determined the top transcripts increased in NR-Minor biopsies versus NR-Normal biopsies (Table3). All of the top 20 genes by *p*-value were previously annotated as IFNG-inducible,[24] increased in ABMR,[25] or both.[24] Top transcripts included IFNG-inducible chemokines CXCL9 and CXCL11, 7 HLA genes (mostly class II), IFNG-inducible genes GBP1, GBP4, and GBP5, and NK chemokine CCL4. There was a gradient of increasing expresión from NR Normal to NR−Minor to posible ABMR to ABMR for every gene, similar to the gradient in % DSA positivity in Table 2. Thus, the transcripts increased in NR-Minor vs NR-Normal are part of a continuum from NR-Minor to ABMR.

Comparing rejection transcripts set scores in DSA positive and DSA No-rejection biopsies. To see whether similar ABMR-related molecular heterogeneity existed in histology, we divided both MMDx No-rejection and histology No-rejection into DSA negative and DSA positive groups (Table 4). Scores were elevated in DSA positive for both definitions of NR.

Thus, DSA positivity in biopsies designated No-rejection by MMDx or by histology is associated with subtle increases in ABMR-related transcripts and transcript sets.

Survival with in MMDx$_{Mod}$ rejection groups. In a subset of 543 biopsies (one random biopsy per transplant, median follow-up 343 days), 52 grafts failed within 3 years. None of the eight MMDx$_{Mod}$ classes differed significantly from any other class in pairwise comparisons, using post hoc tests (Table5), but the simple size and number of overall failures were small. Most failures were not related to rejection in the biopsy: 28 of 52 failures were in NR-Normal or NR-Minor biopsies. The histology classes also did not differ from each other−data not shown.

It was surprising that ABMR biopsies were seldom followed by failure. Figure 3[a] shows the failures as “x” on the rejection-based distribution: only 3 of the 52 failures followed biopsies with molecular ABMR.

Multivariable Cox regression análisis was done to assess 3-year death-censored survival. Three predictors were significant (<0.05): Early injury (Hazard ratio = 1.86, p = 0.0001), TCMR (HR = 1.71, p = 0.001), and TxBx (HR = 1.78, p = 0.002), that is, higher scores for each were associated with reduced survival. The overall p-value for the model was 0.00002. Relative variable importance assessed by proportion chi-square is shown in Figure 3B; TxBx>Minor>TCMR>Normal. The bootstrapped error estimate (1.0−the C-statistic, i.e., 1-AUC) was 0.30.

For LVEF≤55, 4 variables were significant in multivariable logistic regression: TxBx (Odd sratio = 1.6, p = 0.0002), Minor (OR = 0.74, p = 0.003), TCMR (OR = 1.31, p = 0.008), and Normal (OR = 0.8, p = 0.04). Early Injury was border line significant, at p = 0.06 (OR=1.19). Higher scores for TxBx, TCMR, and Early Injury were associated with low LVEF, and higher scores for Minor and Normal were associated with high LVEF. The variable importance plot for predicting LVEF≤55 is shown in Figure 3C (Early injury>TCMR>TxBx), and the overall model p-value <0.0001

Relative importance of molecular rejection scores for predicting survival and function. To explore further the paucity of graft los in biopsies called ABMR by MMDx, we compared the relative importance of the archetype scores for predicting 3-year survival and LVEF using random forest analysis. In Figure S1A, the 5 rejection archetype scores (from the archetype model in Figure 2C/D) plus time post-transplant were used to predict 3-year survival after biopsy. The important scores were TCMR and Early-injury. NR-Minor and ABMR scores had Little importance, consistent with our observation of few failures in ABMR.

In Figure S1B, we examined the same variables for their relative importance in predicting reduced LVEF. The most important variable was time post-transplant, followed by Early-injury and TCMR scores; NR-Minor and ABMR scores were again not important. Abnormal LVEF was defined as ≤55 for this analysis, but other cutoffs (e.g., LVEF<45) showed similar results (data not shown).

Discussion

We analyzed rejection-related gene expresión in heart transplant EMBs currently considered to have no rejection to see if minor rejection-related molecular changes were being missed. We applied new archetype algorithms to the NR group to distinguish an NR-Minor from an NR-Normal group. Compared to NR-Normal, NR-Minor biopsies more often had mild histologic inflammation (TCMR1R), and the prevalence of DSA-positivity was relatively greater in NR-Minor as compared to NR-Normal (although the majority of NR-Normal and NR-Minor were DSA-negative.).The most striking difference between NR-Minor and NR-Normal was a uniform increase in expresión of ABMR-related and IFNG-inducible genes, which increased from NR-Normal to NR-Minor to posible ABMR to ABMR. DSA and class II DSA also increased in prevalence from NR-Normal to NR-Minor to posible ABMR to ABMR. Thus the mild increases in ABMR-like transcript expresión may be induced by subtle ABMR mechanisms induced by DSA, raising the possibility that ABMR-related stress is much more common tan previously appreciated, either by molecular diagnosis or histology.(This does not exclude alternative interpretations for the increase in DSA, molecular ABMR-like changes, and mild histologic abnormalities in NR-Minor biopsies.) Transcript sets related to effector T cells and injury also showed higher scores in NR-Minor vs. NR-Normal. The Minor archetype score had no apparent impact on LVEF or survival, both of which were more related to the TCMR and Early-injury scores. We conclude that using the 5 archetype model to subclassify No-rejection into NR-Normal and NR-Minor presents a more complete description of the molecular state of heart transplants and raises the issue of the long-term impact of low-level ABMR-like changes for management and prognosis.

Although TCMR1R lesions are related to TCMR, when histology TCMR1R biopsies had molecular rejection, it was usually ABMR (N = 61) or possible ABMR (N = 62). In 411 histology TCMR1R, there were 123 ABMR-related molecular diagnoses and 30 TCMR-like diagnoses (16 TCMR and 14 possible TCMR). The association of mild molecular ABMR-related changes with mild TCMR-like lesions may be influenced by the difficulty in distinguishing intracapillary from interstitial cells by histology,[14] but it is also likely that ABMR can have a mild interstitial infiltrate as well as intracapillary infiltrate. In kidney transplants, the TCMR-related lesions are sometimes found in molecular ABMR with no apparent TCMR.[26] Thus TCMR1R should raise suspicions of mild molecular

ABMR, particularly if DSA is present. However, while 133 TCMR1R were NRMinor, 114 were NR-Normal.

The lack of effect of ABMR on outcome is noteworthyonly 3 failures were recorded by 3 years post-biopsy in 76 hearts and 149 biopsies with follow-up. In part, this must reflect the success of clinicians in avoiding severe early ABMR, nowvery uncommon in the centers participating in this study. The good survival in ABMR, possible ABMR, and NR-Minor, and the lack of association of the ABMR and Minor archetype scores on LVEF and graft loss in random forest analysis, probably reflects the lack of parenchymal (cardiac myocyte) injury in ABMR, which is a microcirculation disease, compared to TCMR, which is an interstitial process. Of interest, kidney transplants with early-stage ABMR also experience relatively little 3-year loss,2 but late deterioration often follows. ABMR in heart transplants emerges as a spectrum with a characteristic molecular phenotype, mild functional effects and a relatively benign early prognosis, making treatment decisions complex.

The interpretation of the increased prevalence of DSA and the mild ABMR-like characteristics of NR−Minor must acknowledge the complex relationship between DSA and ABMR, both in hearts and in kidneys. DSA can be associated with no changes or with the ABMR spectrum, but ABMR can also be DSA negative. In the present study, DSA was present only in about 65% to 75% of both histologic and molecular ABMR, and DSA was present in some NR-normal biopsies. As a predictor of clinical ABMR states in kidney and heart transplantation, DSA is often present with no ABMR, and often absent in ABMR, both by molecular or histologic assessment. These trends are illustrated here (DSA-positive NR-Normal 24% and DSA negative ABMR 34%) and in the kidney transplant molecular[23] and histologic[27] literature, and acknowledged by the Banff consensus.[28] DSA measurements vary among centers and within centers due to many factors and its pathogenicity depends on many factors such as titer, complement binding, IgG subclass, specificity, and de novo appearance.[29,30] Defining DSA with this level of granularity is technically demanding, expensive, and is not SOC among experienced centers. Other possible mediators of ABMR should also be studied, including non-HLA alloantibodies, autoantibodies, and “missing self” recognition by NK cells.[31]

As expected, this multicenter IRB-approved prospective cross-sectional biopsy study has limitations. The protocol specified that all center assessments and management be

standard-of-care, and the centers did not agree to share slides for central histology review. Follow-up times in a prospective IRB-approved ongoing biopsy study are of necessity relatively short, making the number of events small. Inclusion of indication biopsies is an important element, but as a result, the results are cross-section of the biopsied population but not the whole transplant population because the most stable patients never have indication biopsies. Nevertheless, the analyses within the study population are of interest, and the ongoing study will reveal more understanding of outcomes as it continues.

The association of DSA with NR-Minor biopsies supports the conclusion that the increased ABMR-associated transcript expression in NR-Minor biopsies reflects lowlevel ABMR mechanisms not recognized by previous molecular and histologic classifications. Rather than debating whether "Minor" ABMR-like changes should be called "rejection," we believe that that the results in the future will be expressed quantitatively and probabilistically, in relationship to the evidence concerning outcomes and management choices at each point along the gradient, and this will require ongoing study.

The disease mechanisms mediating this subtle "Minor" ABMR stress may involve NK cells, which are capable of IFNG release when triggered through their Fc receptors, and which are characteristic of kidney[25] and heart ABMR.[6] The top genes were all previously annotated in ABMR and recall the model we previously proposed in which NK cells triggered by CD16a Fc receptors engage the Fc portions of IGG bound to donor MHC, triggering release of IFNG (and probably other cytokines) and potentially release of granule-associated cytotoxic enzymes such as granulysin, with a corresponding change in the endothelium such as expression of IFNG-inducible genes CXCL11 and HLADRA.[6,24,25,32-34]

The long-term impact of NR-Minor changes for late heart transplant syndromes such as atrophy-fibrosis, systolic and diastolic dysfunction, and arterial disease (cardiac allograft vasculopathy) needs to be established. We are currently adding a new dimension of molecular measurements of parenchymal injury to complement the assessment of rejection-associated transcripts, and expect to map the disturbed late phenotypes that emerge over time and relate early molecular patterns to late phenotypes (PFH et al., manuscript in preparation). Late kidney transplant outcomes[23] are better predicted by parenchymal injury than by rejection activity, although the injury may often be the result of rejection, and we anticipate that heart and other transplants will be similar. These

relationships will ultimately help us to understand the implications of the subtle NR-Minor phenotype.

Data availability statement

CEL files are available on the Gene Expression Omnibus website (GSE150059).

Disclosure statement

P FHalloran holds shares in Transcriptome Sciences Inc., a University of Alberta research company with an interest in molecular diagnostics; has given lectures for Thermo Fisher and is a consultant for CSL Behring. The other authors have declared no conflict of interest exists.

Acknowledgments

We are grateful to Dr. Jeff Reeve for supervising all of the statistical analyses.
This research has been principally supported by grants from Genome Canada, Canada Foundation for Innovation, the University of Alberta Hospital Foundation, the Alberta Ministry of Advanced Education and Technology, the Mendez National Institute of Transplantation Foundation, and Industrial Research Assistance Program. Partial support was also provided by funding from a licensing agreement with the One Lambda division of Thermo Fisher. Dr. Halloran held a Canada Research Chair in Transplant Immunology until 2008 and currently holds the Muttart Chair in Clinical Immunology. Some biopsies were provided by Dr. Alexandre Loupy, Paris, France.
Author contributions: PFH − principal investigator, manuscript writing/reviewing
KMT-manuscript writing/reviewing
AZA-Z, MC, MGC-L, ECD, MD, JG, DHK, JK, PM, LP, KS, JS, AZ − biopsy collection, manuscript reviewing

References

1. Topol E. Deep Medicine: How Artificial Intelligence Can Make Healthcare Human Again. 1st ed New York, NY, USA: Basic Books; 2019:341.

2. Reeve J, Bohmig GA, Eskandary F, et al. Assessing rejection-related disease in kidney transplant biopsies based on archetypal analysis of molecular phenotypes. JCI Insight 2017;2:e94197.
3. Halloran PF, Reeve J, Aliabadi AZ, et al. Exploring the cardiac response to injury in heart transplant biopsies. JCI Insight 2018;3: e123674.
4. Halloran KM, Parkes MD, Chang J, et al. Molecular assessment of rejection and injury in lung transplant biopsies. J Heart Lung Transplant 2019;38:504-13.
5. 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;36:1192-200.
6. Loupy A, Duong Van Huyen JP, Hidalgo LG, et al. Gene expression profiling for the identification and classification of antibody-mediated heart rejection. Circulation 2017;135:917-35.
7. Parkes MD, Aliabadi AZ, Cadeiras M, et al. An integrated molecular diagnostic system for rejection and injury in heart transplant biopsies. J Heart Lung Transplant 2019;38:636-46.
8. Famulski KS, Broderick G, Einecke G, et al. Transcriptome analysis reveals heterogeneity in the injury response of kidney transplants. Am J Transplant 2007;7:2483-95.
9. Einecke G, Mengel M, Hidalgo LG, Allanach K, Famulski KS, Halloran PF. The early course of renal allograft rejection: defining the time when rejection begins. Am J Transplant 2009;9:483-93.
10. Einecke G, Kayser D, Vanslambrouck JM, et al. Loss of solute carriers in T cell-mediated rejection in mouse and human kidneys: an active epithelial injury-repair response. Am J Transplant 2010;10:2241-51.
11. Mengel M, Sis B, Kim D, et al. The molecular phenotype of heart transplant biopsies: relationship to histopathological and clinical variables. Am J Transplant 2010;10:2105-15.
12. Halloran PF, Reeve J, Akalin E, et al. Real time central assessment of kidney transplant indication biopsies by microarrays: the INTERCOMEXstudy. Am JTransplant 2017;17:2851-62.
13. Billingham M, Kobashigawa JA. The revised ISHLT heart biopsy grading scale. J Heart Lung Transplant 2005;24:1709.
14. Berry GJ, Burke MM, Andersen C, et al. The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. J Heart Lung Transplant 2013;32:1147-62.
15. Colvin MM, Cook JL, Chang P, et al. Antibody-mediated rejection in cardiac transplantation: emerging knowledge in diagnosis and management: a scientific statement from the American Heart Association. Circulation 2015;131:1608-39.
16. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015;43:e47.
17. (2019) RCT: R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for statistical Computing; 2019.

18. Halloran PF, Venner JM, Famulski KS. Comprehensive analysis of transcript changes associated with allograft rejection: combining universal and selective features. Am J Transplant 2017;17:1754-69.
19. Madill-Thomsen KS, Abouljoud M, Bhati C, et al. The molecular diagnosis of rejection in liver transplant biopsies: first results of the INTERLIVER study. Am J Transplant 2020. https://doi.org/10.1111/ ajt.15828:2156-72. Early View.
20. Halloran K, Parkes MD, Timofte IL, et al. Molecular phenotyping of rejection-related changes in mucosal biopsies from lung transplants. AmJTransplant 2020;20:954-66.
21. Lê S, Josse J, Husson F. FactoMineR: an RPackage for multivariate analysis. J Stat Softw 2008;25:18.
22. Eugster MJA, Leisch F. From spider-man to hero- archetypal analysis in R. J Stat Softw 2009;30:1-23.
23. Einecke G, Reeve J, Gupta G, et al. Factors associated with kidney graft survival in pure antibody-mediated rejection at the time of indication biopsy: importance of parenchymal injury but not disease activity. Am J Transplant 2020;21:1391-401.
24. Halloran PF, Venner JM, Madill-Thomsen KS, et al. Review: the transcripts associated with organ allograft rejection. Am J Transplant 2018;18:785-95.
25. Venner JM, Hidalgo LG, Famulski KS, Chang J, Halloran PF. The molecular landscape of antibody-mediated kidney transplant rejection: evidence for NK involvement through CD16a Fc receptors. Am J Transplant 2015;15:1336-48.
26. Madill-Thomsen K, Perkowska-Ptasinska A, Bohmig GA, et al. Discrepancy analysis comparing molecular and histology diagnoses in kidney transplant biopsies. Am J Transplant 2020;20:1341-50.
27. Chen M, Zoet Y, Roelen D, et al. Towards uniformity in the definition of acceptable mismatches for highly sensitized patients. HLA 2019;94:147-53.
28. Haas M, Loupy A, Lefaucheur C, et al. The Banff 2017 Kidney Meeting Report: revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. Am J Transplant 2018;18:293-307.
29. Reed EF, Rao P, Zhang Z, et al. Comprehensive assessment and standardization of solid phase multiplex-bead arrays for the detection of antibodies to HLA-drilling down on key sources of variation. Am J Transplant 2013;13:3050-1.
30. Tambur AR, Herrera ND, Haarberg KM, et al. Assessing antibody strength: comparison of MFI, C1q, and titer information. Am J Transplant 2015;15:2421-30.
31. Koenig A, Mezaache S, Callemeyn J, et al. Missing self-induced activation of NK cells combines with non-complement-fixing donor-specific antibodies to accelerate kidney transplant loss in chronic antibody-mediated rejection. J Am Soc Nephrol 2021;32:479-94.
32. Halloran PF, Famulski KS, Reeve J. Molecular assessment of disease states in kidney transplant biopsy samples. Nat Rev Nephrol 2016;12:534-48.

33. Parkes MD, Halloran PF, Hidalgo LG. Evidence for CD16a-mediated NK cell stimulation in antibody-mediated kidney transplant rejection. Transplantation 2017;101:e102-11.
34. Halloran PF, Merino Lopez M, Barreto Pereira A. Identifying subphenotypes of antibody-mediated rejection in kidney transplants. Am J Transplant 2016;16:908-20.

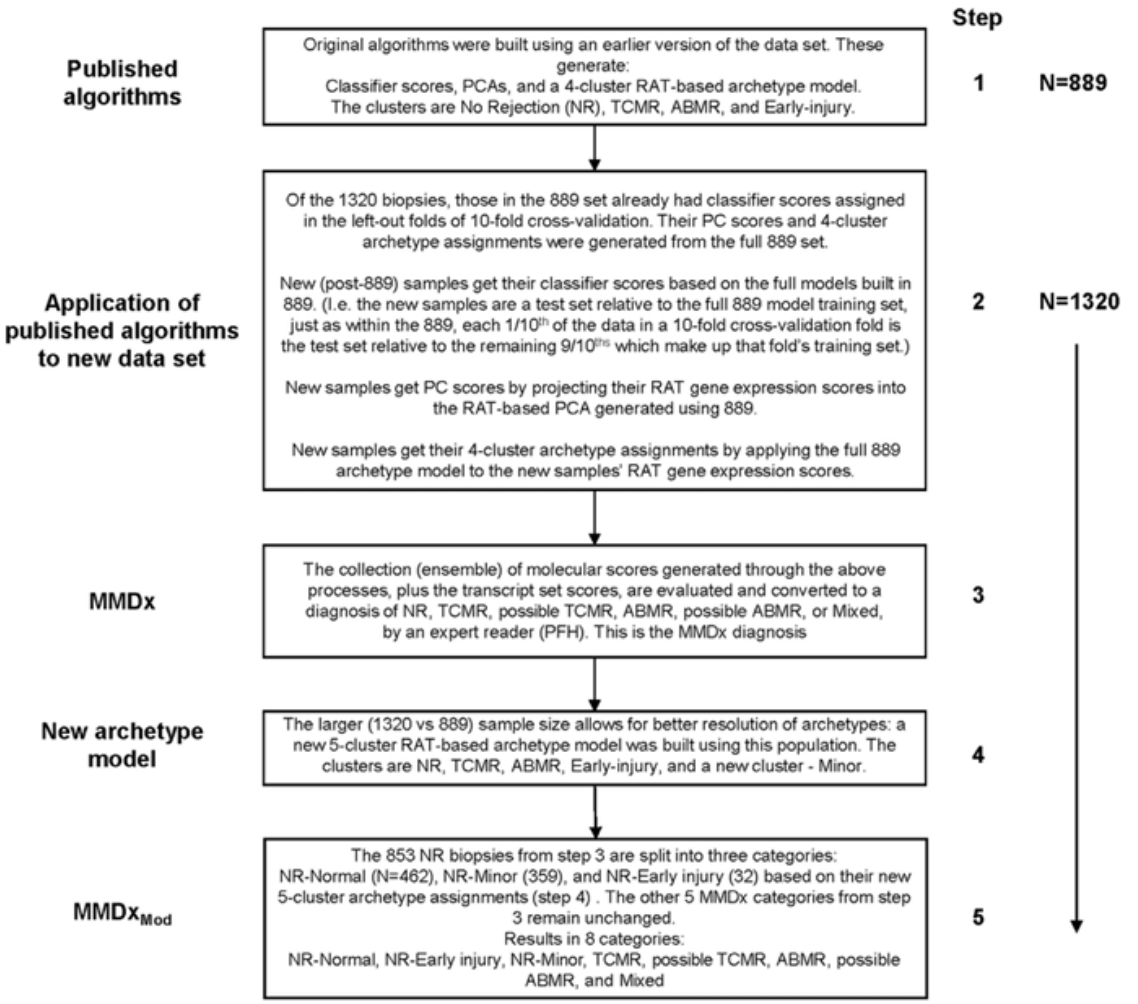

Figure 1 Strategy to assign supervised classifier and unsupervised archetype molecular scores to the 1320 biopsies (a previously published set of 889, plus 431 new samples) based on their microarray expression measurements, without "overfitting" when using supervised scores. Because class labels (e.g., Rejecting or non-rejecting) are used to learn a supervised classifier's rules, a basic principle of supervised analysis is that a new sample is never assigned a score by a classifier that used it for training. In unsupervised analysis, there are no labels used at any stage, so this restriction doesn't apply. In particular, step 2 outlines these details. Supervised (classifier) and unsupervised (PCA, archetypal analysis (AA)) models were built using the 889. All 889 were used to generate PCA/AA scores from a single PCA and single AA(4-archetype version). Classifier scores were assigned in 10 allotments via 10-fold cross-validation. The single models (classifier/PCA/ AA) from the 889 were then used to assign scores to the 431 new samples. An additional (5-archetype) model was also built using all 1,320 samples.

Table1 Histologic Diagnoses and DSA Status in 1,320 Endomyocardial Biopsies

Mean time of biopsy post-transplant (range) in days	759 (4-10150)			
Mean follow-up time (range) in days	644 (1-3854)			
	MMDx diagnoses		Histology diagnoses[a]	
Diagnoses	N (% of 1,320)	% DSA positive of those tested	N (% of 1,320)	% DSA positive of those tested
No rejection	831 (63%)	29%	519 (39%)	31%
Early injury	32 (2%)	41%	-	-
Possible TCMR	38 (3%)	29%	411 (31%)(TCMR1R)	34%
TCMR	79 (6%)	38%	113 (9%)	21%
Possible ABMR	161 (12%)	40%	150 (11%)	54%
ABMR	176 (13%)	65%	71 (5%)	68%
Mixed (ABMR+TCMR)	13 (1%)	91%	14 (1%)	82%
All ABMR (including Mixed)	189 (14%)	44%	85(6%)	71%
Incomplete	-	-	42(3%)	-
DSA status at biopsy (824 tested)			N (% of tested)	
Positive			307 (37%)	
Indication biopsies			(49%)	
Protocol biopsies			(30%)[b]	
Negative			517 (63%)	
Not tested			496	

[a]Biopsy histology diagnoses were converted as follows: pAMR0 No ABMRpAMR1, pAMR1I+, pAMR1H+ Possible ABMRpAMR2, pAMR3 ABMRTCMR0R No TCMRTCMR1R Possible TCMR (TCMR1R) TCMR2R, TCMR3R TCMR. [b]DSA positivity was significantly more frequent in indication vs protocol biopsies (Chi-square *p*-value ~ 1E-11).

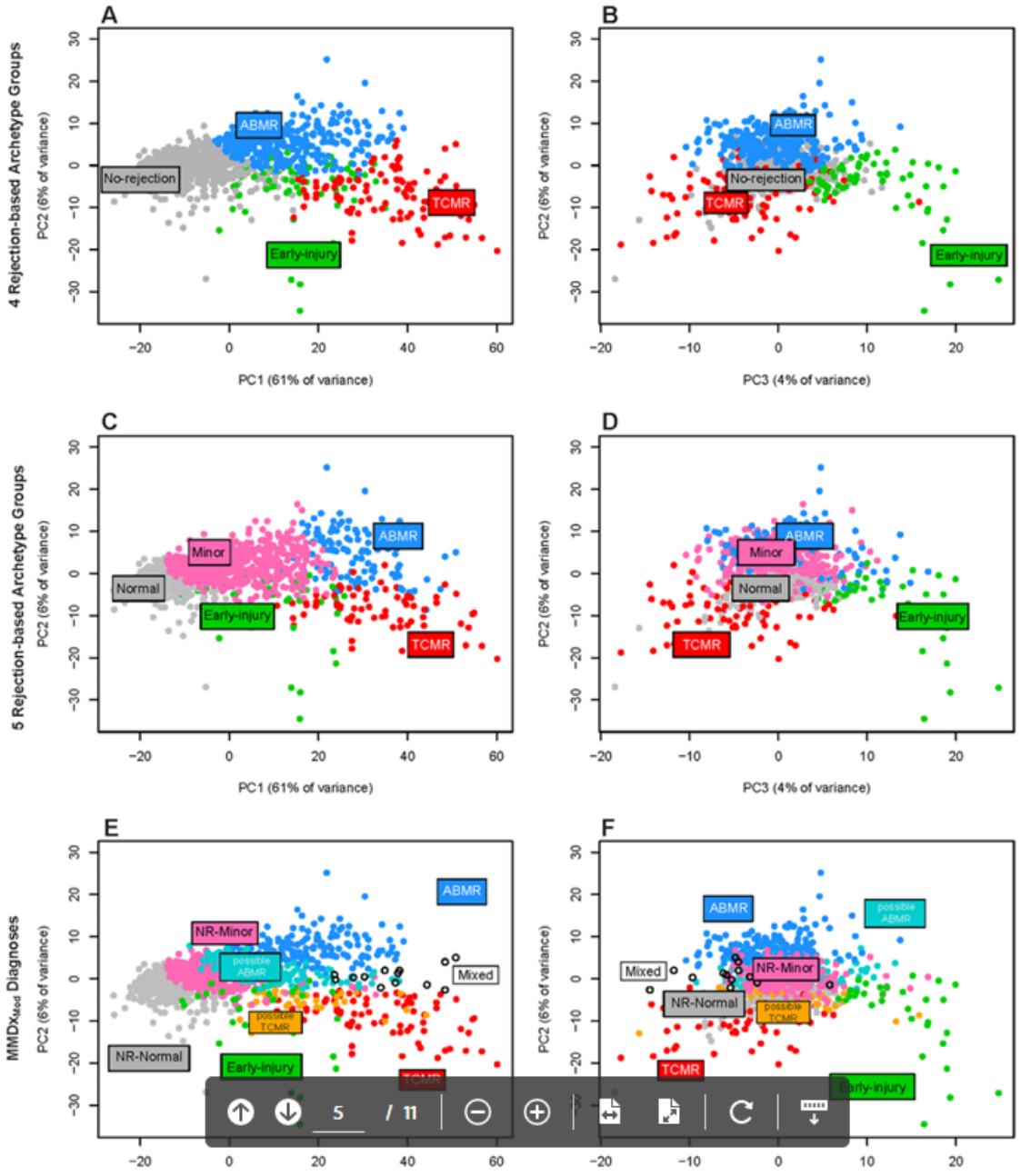

Figure 2 Visualizing the new division of NR into NR-Normal and NR-Minor. Biopsies distributed in principal component analysis (PCA) based on expression of rejection-associated transcripts (RATs) in 1,320 heart biopsies, with PC2 vs PC1 in the left panels and PC2 vs PC3 in the right. Each dot is a biopsy, colored by: (A and B) The 4 archetype model built using N = 889 biopsies; (C and D) The 5 archetype model built using N = 1,320 biopsies; (E and F) The $MMDx_{Mod}$ diagnoses in N = 1,320, which are the original MMDx diagnoses but subdividing NR into 3 subcategories based on the 5 archetype model.

Table 2 DSA Positivity in $MMDx_{Mod}$ Rejection Classes in 824 Biopsies With DSA Test Results

		Any DSA			Class II DSA status reported	
Biopsy class		DSA-negative	DSA-positive (% total tested per row)	Number tested	DSA class II-positive (% total tested perrow)	Number tested
Modified MMDx	NR-Normal	207	65 (24%)	272	38 (15%)	262
	NR-Minor	142	74 (34%)[a]	216	50 (24%)	208
	Early injury	10	7 (41%)	17	4 (24%)	17
	posible TCMR	21	9 (30%)	30	7 (24%)	29
	TCMR	33	20 (38%)	53	15 (29%)	52
	posible ABMR[b]	56	40 (42%)	96	28 (30%)	94
	ABMR	47	85 (64%)[c]	132	71 (56%)	126
	Mixed	1	7 (88%)	8	6 (86%)	7
Total tested		517	307 (37%)	824	219 (28%)	795[d]

[a] Chi-square *p*-value = 0.02 for NR-Normal vs.NR-Minor. [b]This includes biopsies called posible ABMR/posible TCMR. [c] Chi-square *p*-value <0.001 for NR-Normal vs NR-Minor. [d] 29 biopsies were reported with DSA without specifying class I or class II.

Table 3 Top 20 Differentially Expressed Genes From Class Comparison Between NR-Normal (N=462) and NR-Minor (N=359)

SYMB	Gene	Transcript set annotation[a]	Adjusted *p*-value[b]	Mean expresión			
				NR-Normal	NR-Minor	posible ABMR	ABMR
CXCL11	Chemokine (C-X-Cmotif) ligand 11	ABMR-RAT,GRIT	6E-106	25 (23.1-26.9)	118 (116.1-119.9)	368 (367.3-368.8)	965 (964.4-965.6)
HLA-DRA	major histocompatibility complex, classII, DR alpha	ABMR-RAT,GRIT	1E-105	2507 (2506.4-2507.6)	4665 (4664.3-4665.7)	6881 (6880.7-6881.3)	8753 (8752.7-8753.3)
CXCL 9	chemokine (C-X-Cmotif) ligand 9	ABMR-RAT,GRIT	3E-105	200 (198.2-201.8)	939 (937.1-940.9)	2681 (2680.3-2681.7)	5172 (5171.5-5172.5)
GBP1	guanylate binding protein 1, interferon-inducible	ABMR-RAT,GRIT	1E-103	84 (83.0-85.0)	213 (211.9-214.1)	455 (454.4-455.6)	843 (842.4-843.6)
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	ABMR-RAT,GRIT	1E-102	993 (992.4-993.6)	1729 (1728.3-1729.7)	2611 (2610.6-2611.4)	3444 (3443.7-3444.3)
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	ABMR-RAT,GRIT	6E-101	2278 (2277.3-2278.7)	4226 (4225.3-4226.7)	6478 (6477.6-6478.4)	8542 (8541.7-8542.3)
CD 74	CD 74 molecule, major histocompatibility complex, class II invariant chain	ABMR-RAT,GRIT	2E-98	773 (772.4-773.6)	1410 (1409.2-1410.8)	2227 (2226.6-2227.4)	2885 (2884.7-2885.3)
GABBR1	gamma-aminobutyric acid (GABA) B receptor, 1	ABMR-RAT	3E-95	48 (46.4-49.6)	141 (139.4-142.6)	433 (432.5-433.5)	1026 (1025.4-1026.6)

GBP4	guanylate binding protein 4	ABMR-RAT,GRIT	5E-95	50 (49.2-50.8)	97 (96.1-97.9)	178 (177.6-178.4)	292 (291.6-292.4)
NLRC5	NLR family, CARD domain containing 5	ABMR-RAT,GRIT	1E-92	169 (168.4-169.6)	261 (260.4-261.6)	402 (401.7-402.3)	560 (559.7-560.3)
CXCL 10	chemokine (C-X-Cmotif) ligand 10	ABMR-RAT,GRIT	2E-92	93 (91.6-94.4)	376 (374.4-377.6)	955 (954.3-955.7)	1922 (1921.5-1922.5)
IRF1	interferon regulatory factor 1	ABMR-RAT,GRIT	8E-92	162 (161.3-162.7)	283 (282.2-283.8)	496 (495.6-496.4)	739 (738.6-739.4)
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	GRIT	1E-91	117 (116.1-117.9)	261 (260.0-262.0)	494 (493.4-494.6)	748 (747.5-748.5)
HLA-DOA	major histocompatibility complex, class II, DO alpha	ABMR-RAT,GRIT	8E-90	96 (95.1-96.9)	198 (197.1-198.9)	345 (344.6-345.4)	509 (508.7-509.3)
HLA-DMA	major histocompatibility complex, class II, DM alpha	GRIT	3E-89	219 (218.4-219.6)	385 (384.3-385.7)	594 (593.6-594.5)	771 (770.6-771.4)
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8	GRIT	6E-89	230 (229.3-230.7)	369 (368.2-369.8)	599 (598.6-599.4)	852 (851.6-852.4)
HLA-F	major histocompatibility complex, class I, F	ABMR-RAT,GRIT	1E-88	457 (456.4-457.6)	705 (704.4-705.6)	1062 (1061.7-1062.3)	1491 (1490.7-1491.3)
CCL4	chemokine (C-Cmotif) ligand 4	ABMR-RAT	8E-86	39 (37.5-40.5)	129 (127.4-130.6)	378 (377.5-378.5)	901 (900.4-901.6)
GBP5	guanylate binding protein 5	ABMR-RAT,GRIT	3E-85	22 (20.6-23.3)	51 (49.6-52.4)	132 (131.3-132.7)	295 (294.2-295.8)
BTN3A3	butyrophilin, subfamily 3,member A3	ABMR-RAT,GRIT	1E-84	122 (121.5-122.5)	189 (188.5-189.5)	260 (259.7-260.3)	358 (357.7-358.3)

ABMR-RAT, antibody-mediated rejection-associated transcripts; GRIT, interferón gamma-inducible transcripts.

[a] False Discovery rate calculated using the Benjamini-Hochberg procedure after a Bayesiant-test. Adjusted p-values represent the comparison between NR and NR-Minor biopsy groups.

Table 4 Mean Transcript Set Scoresa for ABMR-Related Transcript Sets Split by DSA Status

		Expression in MMDx no rejection			Expression in histology no rejection		
		DSA negative (N = 342)	DSA positive (N = 142)	t-test*p*-value	DSA negative (N = 212)	DSA positive (N = 94)	t-test*p*-value
ABMR-related transcript sets	ABMR-RAT	1.16	1.26	0.005	1.16	1.40	0.0002
	Rejection-RAT	1.22	1.38	0.003	1.23	1.56	0.0003
	IFNG-inducible (GRIT3)	1.12	1.18	0.02	1.11	1.23	0.001

[a] Transcript set scores (defined in the Methods), are average fold changes of each transcript set's gene expression values compared to that of the same genes' expression values in the controls. As fold changes, they are unitless.

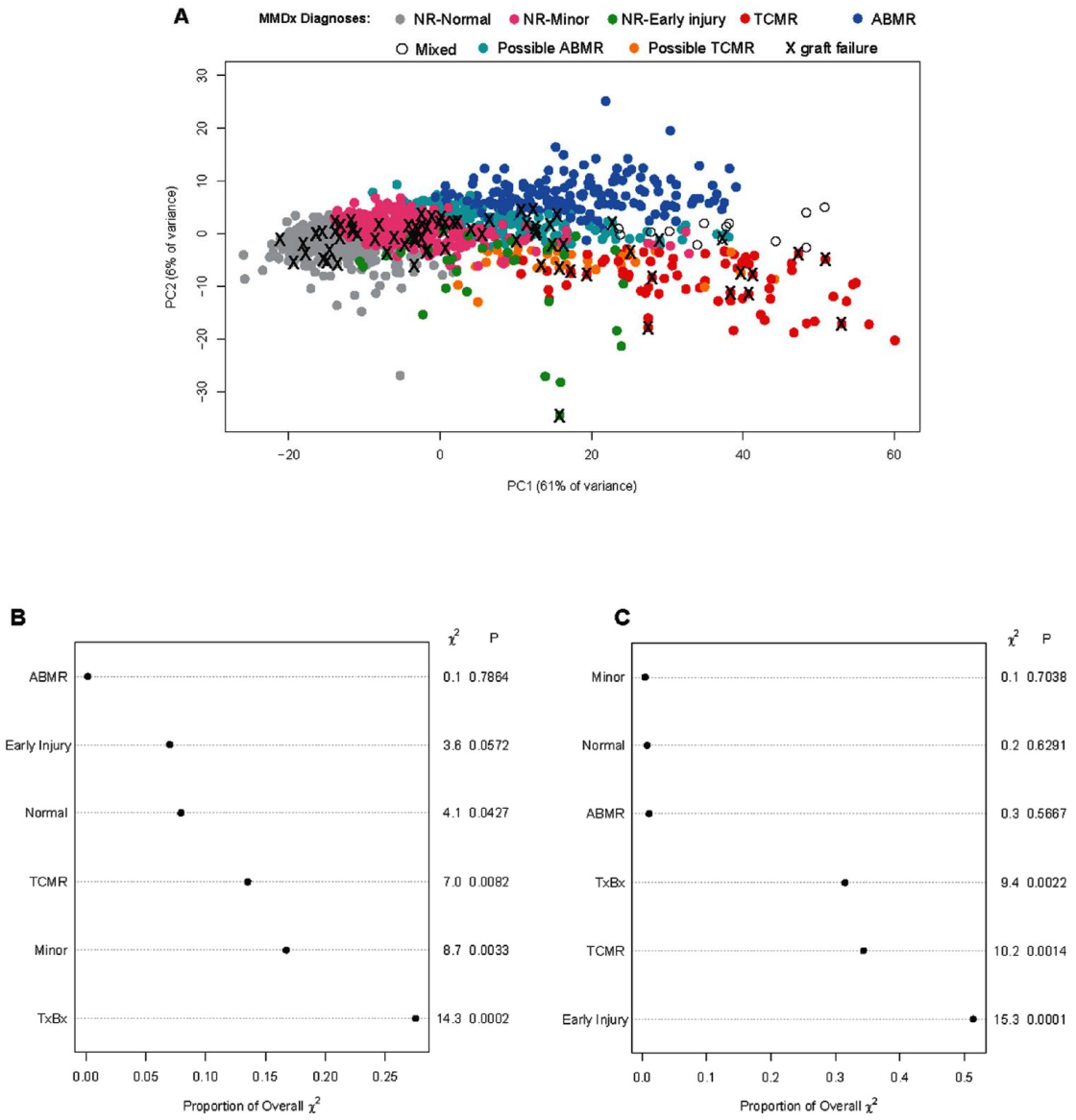

Figure 3 Relationships of the rejection classes to survival and LVEF. (A) The PC2 vs PC1 plot from Figure 1E, colored by $MMDx_{Mod}$ diagnosis using molecular results in N = 1,320 heart biopsies. X-marks indicate transplants that went on to fail at any time postbiopsy. Vari- able importance plots and *p*-values for predicting (B) 3-year postbiopsy survival using multivariable Cox regression. (C) LVEF ≤ 55 using multivariable logistic regression.

Table 5 Distribution of Failures Within 3 Years of Biopsy in $MMDx_{Mod}$ Diagnoses

$MMDx_{Mod}$ diagnoses		Failures (% of row total)	Probability of failure by 3 years	Total
One random biopsy per patient (N = 543, 52 failures (10%))	NR-Normal	16 (8%)	0.14	211
	NR-Minor	12 (10%)	0.15	124
Early injury	1 (11%)	0.25	9	
possible TCMR	3 (19%)	0.20	16	
TCMR	5 (16%)	0.18	31	
possible ABMR	11 (15%)	0.22	72	
ABMR	3 (4%)	0.06	76	
Mixed	1 (25%)	0.25	4	

No groups were significantly different from NR-Normal (likelihood ratio test).