Assessing the Relationship Between Molecular Rejection and Parenchymal Injury in Heart Transplant Biopsies

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Background. The INTERHEART study (ClinicalTrials.gov #NCT02670408) used genome-wide microarrays to detect rejection in endomyocardial biopsies; however, many heart transplants with no rejection have late dysfunction and impaired survival. We used the microarray measurements to develop a molecular classification of parenchymal injury. **Methods.** In 1320 endomyocardial biopsies from 645 patients previously studied for rejection-associated transcripts, we measured the expression of 10 injury-induced transcript sets: 5 induced by recent injury; 2 reflecting macrophage infiltration; 2 normal heart transcript sets; and immunoglobulin transcripts, which correlate with time. We used archetypal clustering to assign injury groups. **Results.** Injury transcript sets correlated with impaired function. Archetypal clustering based on the expres- sion of injury transcript sets assigned each biopsy to 1 of 5 injury groups: 87 Severe-injury, 221 Late-injury, and 3 with lesser degrees of injury, 376 No-injury, 526 Mild-injury, and 110 Moderate-injury. Severe-injury had extensive loss of normal tran- scripts (dedifferentiation) and increase in macrophage and injury-induced transcripts. Late-injury was characterized by high immunoglobulin transcript expression. In Severe- and Late-injury, function was depressed, and short-term graft failure was increased, even in hearts with no rejection. T cell–mediated rejection almost always had parenchymal injury, and 85% had Severe- or Late-injury. In contrast, early antibody-mediated rejection (AMR) had little injury, but late AMR often had the Late- injury state. **Conclusions.** Characterizing heart transplants for their injury state provides new understanding of dysfunction and outcomes and demonstrates the differential impact of T cell–mediated rejection versus AMR on the parenchyma. Slow deterioration from AMR emerges as a major contributor to late dysfunction.

INTRODUCTION

Parenchymal injury occurs in every heart transplant, and the quality of the heart parenchyma determines function and, ultimately, outcome. Transplantation subjects heart tissue to unique stresses, including brain death, preserva- tion-implantation, donor-derived changes,[1] rejection, and infection. Cardiac myocytes are particularly susceptible to injury, which triggers inflammation/innate immunity.[2] In addition, many late heart transplants show suboptimal function and outcomes[3-11] and have abnormalities such as interstitial fibrosis and diastolic dysfunction reflecting parenchymal injury.[12,13] This may be associated with coronary artery abnormalities—cardiac allograft vasculopathy (CAV)[14]—which is not unexpected because arteries are donor tissue and subject to all of these unique stresses.

A molecular assessment of parenchymal injury is therefore of interest. The Molecular Microscope Diagnostic System (MMDx)[2,15-19] measures genome-wide gene expression from 49 495 probesets in endomyocardial biopsies (EMBs). MMDx previously used the expression of rejection-associated transcripts (RATs) plus archetypal analysis (AA) to molecularly define T cell–mediated rejection (TCMR) and antibody-mediated rejection (AMR). Because some transcripts are shared between rejection and innate immunity, this analysis also detected some early inflamed biopsies with injury but with no rejection (NR-Early injury)[20]; however, this analysis of RATs distin- guished "early injury-or-rejection" and therefore could not assess the extent of parenchymal injury in biopsies with rejection or explore the problem of late injury.

The present study of heart transplant EMBs aimed to define the extent of parenchymal injury in every biopsy, as well as its rejection state and to establish the relationships of TCMR and AMR with parenchymal injury. We aimed to measure the expression of previously defined injury-related transcript sets to classify injury states using strategies similar to those that defined rejection states using RATs. The injury-related transcript sets included those with increased expression in mouse heart isografts compared with normal hearts—cardiac injury-repair transcripts (cIRITs)[21]— and heart transcripts (HTs) with high expression in normal human hearts.[19] We included additional injury-induced transcript sets originally annotated in injured kidneys but which also increase in injured hearts with no rejection, correlating with the transcripts induced in injured hearts (cIRITs).[2,20] We included macrophage transcripts to reflect the innate immune response.

Finally, we included immunoglobulin transcripts as a potential marker for late parenchymal deterioration because they increase with time in other organ transplant biopsies[22-25] and correlate with atrophy-fibrosis in kidneys. We hypothesized that assessing the parenchymal injury state in the heart in addition to diagnosing the rejection state would help us to under- stand dysfunction and risk of graft loss in biopsies with no rejection, and allow us to compare the relative impact of TCMR and AMR on the heart parenchyma.

The research plan is summarized in **Figure 1A** and **B**. A summary of abbreviations is provided in **Table S1, SDC**, (http://links.lww.com/TP/C469).

MATERIALS AND METHODS

Population

We used microarrays to analyze gene expression in 1320 EMBs from the prospective INTERHEART study, approved by the ethics review board of each local center (ClinicalTrials.gov #NCT02670408).[17] Standard-of-care (SOC) biopsies for clinical indications or protocol from consenting patients at 13 centers were placed in RNA*later* and shipped to the Alberta Transplant Applied Genomics Centre.[26] Histology followed International Society for Heart and Lung Transplantation guidelines[27,28] per local SOC, interpreted to permit histology-molecular compari- sons as previously reported.[2,18,19] Molecular diagnoses were assigned without knowledge of histology or donor-specific antibody. All biopsies adequate for molecular examination were included (~98%).

Microarray Analysis

As described,[2,17,19] total RNA from EMBs was labeled with the 3′ IVT Plus kit (Affymetrix, SC) and hybridized to PrimeView microarrays (Affymetrix) using manufacturer protocols (www.affymetrix.com). CEL files are available on the Gene Expression Omnibus website (GSE150059).

Pathogenesis-based Transcript Sets

Transcript sets were previously annotated in cell lines, experimental models, and human transplant biopsies (https://www.ualberta.ca/medicine/institutes-centres-groups/atagc/research/gene-lists). Transcript set scores are the mean fold change across

all probes within the set, using log2 raw data, compared with controls (371 biopsies with no molecular rejection >30 d posttransplant). Statistics and calculations were performed using the log of the scores.

We used 10 transcript sets listed in Table 1:

1. 5 previously annotated as induced by recent injury[2,21];
2. 2 reflecting macrophage infiltration, which is triggered by heart injury[2];
3. 2 highly expressed in normal hearts[19];
4. the immunoglobulin transcripts, which increase with time in kidney and lung transplants and correlate with atrophy-fibrosis in kidney transplants.[22,23]

Dimensionality reduction, clustering, and data visualization. Principal component analysis (PCA) and AA were described previously.[2,19] PCA is used to reduce the dimensionality of datasets with large numbers of variables with minimal loss of information, facilitating analysis and visualization. AA assigns a user-defined number of archetypes (idealized extreme phenotypes) to a dataset. Each sample is assigned scores representing its propor- tional relationship to each archetype, summing to 1.0.

The highest score for each biopsy assigned the biopsy to etype group.

Rejection PCA and AA were based on RAT expression.[20] Injury PCA and AA were based on injury-related transcript set scores. We used the "FactoMineR"[29] and "archetypes"[30] packages in R, version 3.6.2.[31]

Rejection

Molecular rejection sign-out categories have been described.[20] Each biopsy was assigned to 1 of the 8 modified sign-out categories: AMR, pAMR, TCMR, pTCMR, mixed, and No rejection (NR), which was subdivided into NR-Minor, NR-Normal, and NR-Early injury.[20]

In addition, the published 5 rejection archetype model[20] was used for some analyses: NR, TCMR, AMR, Minor- injury, and Early-injury. Archetypes are automatically assigned and avoid subjectivity. Rejection PCA and AA used a 1320 (biopsy) × 437 (RATs) dataset as input.

Injury

PCA and AA for injury were based on the expression of 10 injury transcript sets (Table 1).

We used 1320 (biopsy) × 10 (injury transcript set scores) dataset as input. The injury class assignments were independent of the rejection class assignments.

A 5-archetype injury model was selected by inspecting models with 2to6 groups and choosing the one with the best trade-off between biological interpretability and diversity: No-injury, Minor-injury, Moderate-injury, Severe-injury, and Late-injury.

Rolling Averages

Data were first ordered by the variable on the x-axis. Then a sliding window of the size indicated was used to plot mean y versus mean x values, for example, with a window size of 200 the means of samples 1–200 is plotted, then samples 2–201, 3–202, etc.

Survival Analyses

Analyses were based on death-censored survival 3 y postbiopsy, using 1 random biopsy per transplant. Patients with grafts surviving longer were censored at 3 y. In this set, median follow-up was 343 d, and 52 transplants failed within 3 y of biopsy. Kaplan-Meier estimates and plots used the R "survival" package.[32]

Logistic Regression

The R "rms" package[33] was used for logistic regression. Because of collinearity, multivariable logistic regression using the rejection and injury archetype scores excluded the "normal" scores for No-rejection and No-injury.

Splines

Restricted cubic splines were used to show nonlinear relationships between variables. Three "knots" were selected and smooth curves fit based on within-knot data constrained so that curves between segments are joined. Overfitting is minimized in restricted cubic splines by using only linear trend lines for segments beyond the left- and right-most knots, reducing the influence of the tails of the distributions where fewer data points are available. Splines were generated using the R package "rms."[33] The threshold selected for

each plot was based on visual clarity (0.4 in the AMR biopsies, 0.35 in the TCMR biopsies).

Left Ventricular Ejection Fraction

We selected a cutoff of 55 for left ventricular ejection fraction (LVEF) when binary groups were needed for analyses (high LVEF as >55, low LVEF as ≤55). This was based on recommendations from the clinical investigators in the INTERHEART study group and on literature supporting this threshold.[34-36] We additionally used multiple cutoffs to show relationships with LVEF in more detail when needed (LVEF <30, 30–45, and >45).

RESULTS

Population and Demographics

To understand the relationships between rejection and parenchymal injury, we examined the same 1320 EMBs from 645 patients used for the previous rejection analysis[20] (Figure 1A). **Table S2, SDC,** (http://links.lww.com/TP/ C469), shows population demographics,[20] and **Table S3, SDC**, (http://links.lww.com/TP/C469), shows histologic and molecular diagnoses. Molecular rejection sign-outs[20] classified 853 biopsies as NR, with 3 "NR" subclasses: NR-Normal (N = 462), NR-Minor (N = 359), and NR-Early injury (N = 32). Rejection-related sign-out classes were assigned to 467 biopsies: AMR-related (AMR = 179 and possible AMR [pAMR] = 161), and TCMR-related including Mixed (TCMR = 76, pTCMR = 38, and Mixed = 13). We grouped Mixed with TCMR-related for some analyses because TCMR rapidly produces parenchymal injury.[2,21,37]

LVEF was decreased in many late hearts and slightly increased in many hearts early posttransplant (likely from donation-implantation injury; **Figure S1A** and **S1B, SDC**, http://links.lww.com/TP/C469). In a *t*test comparing biop- sies before and after 1-y posttransplant, mean LVEF was lower in later biopsies ($P = 0.02$).

Relationships Between Injury-induced Transcript Sets and LVEF

We examined the average expression of each injury- associated transcript set in EMBs from hearts with high LVEF >45, intermediate LVEF 30 to 45, or low LVEF ≤30 (Table 2). Except for IRITD5 and DAMPs, injury- related transcript sets were significantly different between LVEF groups. In hearts with low LVEF <30, expression of the injury-

increased transcript sets (cIRIT, IRRAT, and IRITD3) and the macrophage transcript sets was higher, and the normal HTs (HT1 and HT2) were lower than hearts with intermediate (30–45) or high LVEF (>45). This confirmed that the injury-associated transcript sets reflect the injury status of the heart parenchyma.

Visualizing Parenchymal Injury Groups (Archetypal Clustering)

Figure 2A shows the correlation of each of the 10 injury transcript sets with injury PC1 and PC2. PC1 accounted for 71% and PC2 for 12% of the variance in the data set (Figure 2B). PC1 correlated positively with all transcript set scores increased by injury and negatively with normal heart parenchymal transcripts. Thus, increasing PC1 indicates increasing parenchymal injury and dedifferentiation. Injury PC2 correlated positively with immunoglobulin transcript sets (IGTs), which strongly correlate with time-dependent parenchymal deterioration (atrophy-fibrosis) in kidneys.[22,25]

As shown by their vectors in Figure 2A, LVEF decreased as PC1 increased and as PC2 increased. Time posttransplant and immunoglobulin transcripts increased with PC2. Figure 2B shows the biopsies plotted by their injury PC scores. The location of each biopsy is determined by scores for the injury-related transcript sets and the vectors in Figure 2A. Each biopsy is colored by its injury archetype group assignment. We named the injury groups for the molecular and clinical features most characteristic of the group: No-injury, N = 376; Mild-injury, N = 526; Moderate- injury, N = 110; and Severe-injury, N = 87. The archetype group with high PC2 was called Late-injury (N = 221).

Figure 2B shows a progression of injury severity corresponding with increasing PC1: No-injury to Mild-injury to Moderate-injury to Severe-injury. The Late-injury group had high PC2 scores, which correlate with time.

Characteristics of the Injury Phenotype States

Table 3 shows the mean (median) time posttransplant (d) and injury transcript set scores across the 5 injury archetype states. Moderate-injury was earliest (mean 218 d), followed by Mild-injury (408 d) and then No-injury (1065 d), suggesting recovery from universal donation-implantation injury. Severe-injury was intermediate (548 d), perhaps because it sometimes reflects TCMR-induced injury—see below. Late-injury had the longest mean time posttransplant (1430 d).

Injury scores increased on a gradient from No- to Mild- to Moderate- to Severe-injury, with correspondingly progressively decreasing normal HT scores.

The main feature of Late-injury biopsies was high expression of the immunoglobulin-associated transcript set, compatible with a plasma cell infiltrate as described in late kidney transplants with atrophy-fibrosis,[22] in late lung transplants,[23] and in late failing heart allografts.[38,39]

Although the INTERHEART study only includes SOC biopsies and is not allowed to deliberately perform biopsies for time series analyses, there were some patients with multiple biopsies. Most Mild-injury phenotypes either stayed Mild-injury or became No-injury in later biopsies; most Moderate-injury phenotypes improved to Mild- or No-injury or stayed Moderate- injury (data not shown).

We previously showed that early cardiac injury was associated with loss of myosin and tropomyosin transcripts.[2] We confirmed that myosin and tropomyosin genes, which were highly expressed in normal EMBs, declined with increasing scores for PC1, Severe-injury, and Late-injury (Table 4), compatible with injury-induced myocyte dedifferentiation.

Figure 3 illustrates some trends in this biopsy population. A striking rise in the IGT and Late-injury scores over log time was accompanied by the decline of the Severe- injury score, the recent injury transcript set scores (cIRIT and IRRAT), and the macrophage transcript set scores (Figure 3A). The close relationship between the cIRIT and Severe-injury score is shown in Figure 3B, and likewise between Late-injury scores and IGT scores in Figure 3C.

Relating Injury Archetype Groups to Rejection

Table 5 distributes the biopsies called TCMR and AMR by molecular rejection sign-outs into their parenchymal injury groups. To avoid excessive subgroups, we grouped the TCMR-related biopsies (TCMR, pTCMR, and Mixed rejection) and the AMR-related biopsies (AMR and pAMR). Mixed was grouped with TCMR because TCMR rapidly induces parenchymal injury.[37]

Biopsies with TCMR almost always (85%) had exten- sive parenchymal damage (assigned to Severe- or Late- injury phenotypes) and virtually never had No-injury.

In contrast, AMR biopsies seldom had Severe-injury, and 19% had No-injury. Early AMR-related biopsies usually had the mild parenchymal injury characteristic of all biopsies in the early posttransplant period: Moderate- injury (350 d), Mild-injury (510 d), and No-injury (970 d); however, 30% of AMR-related biopsies were classified Late-injury at a much later time (1729 d). AMR was detected in many hearts (46%) with Late-injury.

Figure 4 visualizes these associations using splines. We plotted the Severe-injury archetype score against time post- transplant and colored each biopsy by its rejection sign-out category (Figure 4A). We summarized each rejection group by a spline line. TCMR biopsies (red) consistently had high Severe-injury scores, but AMR biopsies (blue) did not.

We similarly examined the Late-injury archetype scores versus time and colored the rejection groups (Figure 4B). TCMR was consistently associated with elevated Late-injury scores. At early times, AMR biopsies did not have elevated Late-injury scores; however, AMR at late times (>3 y) often had Late-injury (101 of 340 or 30%), much more than late biopsies with no rejection (15 of 462 or 3%). NR-Minor biopsies (which have mild AMR-like molecular changes despite being considered no rejection[20]) also showed rising Late-injury scores over time. Biopsies with NR (NR-Normal) showed no substantial increase in Late-injury scores at late times.

In summary, TCMR is strongly associated with exten- sive parenchymal injury, but AMR in the early years post-transplant is not. Nevertheless, AMR in the long term is highly associated with Late-injury.

Associations with Short-term Postbiopsy Graft Failure

As a prospective cross-sectional study, INTERHEART does not have extensive long-term follow-up but does permit estimates of short-term loss. We randomly selected 1 biopsy per patient with available follow-up information (543 hearts) and compared survival between groups of biopsies defined by their injury archetype groups.

Late- and Severe-injury biopsies showed increased risk for failure (Figure 5A, $P = 0.002$), even when all TCMR and AMR rejection sign-outs were excluded (Figure 5B, $P = 0.04$). We studied biopsies with TCMR or AMR by molecular rejection sign-outs. In biopsies with TCMR (Figure 5C), hearts with Severe- and Late-injury states showed increased

graft loss. In biopsies with AMR (Figure 5D), AMR with Severe- and Late-injury states showed increased short-term graft loss compared with AMR with less injury (P = 0.02ss).

Further Analyses

In multivariable Cox regression, both injury states and rejection contributed to predictions of short-term graft los within 3 y. Injury archetype scores added predictive value to rejection archetype scores alone ($P = 3.8 \times 10^{-5}$; **Table S4, SDC,** http://links.lww.com/TP/C469). Rejection arche- type scores also added predictive value to injury archetype scores alone ($P = 2.6 \times 10^{-5}$).

We assessed the injury states and LVEF within biopsies called AMR and TCMR by molecular rejection sign-outs in Table 6. Within both AMR and TCMR groups, LVEF was lower and the number of losses was increased when these hearts also had extensive injury.

DISCUSSION

This study used the existing genome-wide microarray measurements to assign parenchymal injury states to EMBs previously classified for rejection[20] and assessed the relationship between parenchymal injury and low LVEF, short- term graft loss, and rejection phenotypes. We previously found inflamed injured biopsies in our rejection analyses, but these "early injury-or-rejection" analyses could not assess the degree of parenchymal injury in biopsies with rejection or elucidate the common late dysfunction problem. The present study used archetype clustering methods based on expression of previously-annotated injury-related transcript sets. To study time-dependent late changes, we included immunoglobulin transcripts, which correlate with time-dependent parenchymal deterioration in kidney and lung transplants and which showed a striking increase with time in heart transplants. Injury transcript sets were significantly associated with low LVEF ≤30, validating their relationship to the state of the parenchyma. AA identified group of hearts with Severe-injury and a large group of hearts with Late-injury. TCMR was almost always accompanied by extensive parenchymal injury, even early posttransplant. In contrast, in the early years posttransplant, AMR had minimal parenchymal injury (No-injury or Mild-injury) beyond that expected in all early hearts and rarely displayed Severe-injury; however, in later years posttransplant, AMR was

associated with the Late-injury state, much more than in biopsies with no rejection. Injury states emerged as strong predictors of graft dysfunction (decreased LVEF) and graft loss within 3 y postbiopsy in TCMR and AMR groups, even in the many hearts that had no rejection. We conclude that assessing parenchymal injury states in addition to rejection states enhances our understanding of dysfunction and short-term outcomes, reveals the parenchymal impact of rejection, and highlights a large group of hearts with Late- injury, many associated with AMR.

These results underscore the major impact of TCMR on the parenchyma compared with AMR, explaining our earlier findings that TCMR is associated with impaired short-term survival, whereas AMR is not.[20] All failures in TCMR-related biopsies were those assigned to Severe- or Late-injury states. As clinicians, we often consider TCMR to be "treatable," but because it often has severe effects on the parenchyma, it adversely affects short-term survival, much more so than AMR, which appears to spare the parenchyma for prolonged periods of time.[20] A majority (85%) of TCMR biopsies have extensive parenchymal injury, which we suspect will persist even after TCMR activity has been sterilized by treatment. Moreover, because we do not actually know whether our usual treatments actually sterilize TCMR molecular activity, existing parenchymal injury at the time of diagnosis may be exacerbated by new injury induced by smoldering TCMR activity long term.

These results show that although in the early years AMR has minimal parenchymal injury beyond the uni- versal injury of donation-implantation and comparatively little short-term graft loss,[20] AMR is not benign and may slowly develop a progressive Late-injury state with its attendant dysfunction and graft loss. The Late-injury state in kidney transplants manifests as atrophy-fibrosis, and we are currently assessing the histology of late EMBs to characterize the corresponding state in hearts. Of interest, even the subtle "Minor" AMR-like molecular changes in biopsies usually considered to have no rejection are associated with the Late-injury state. AMR begins as a pure microcirculation disease that spares the parenchyma but becomes associated with Late-injury over time, and most short-term failures in AMR were in the Late-injury group (a concept supported by other recent studies[40,41]).

The contributions of TCMR and AMR to graft loss may be underestimated if hearts damaged by rejection are biopsied after rejection has abated. Extensive injury states can

persist even when the rejection state is sterilized by treatment or subsides spontaneously because of factors in the natural history of the immune response such as T cell checkpoints. Both TCMR and AMR impact heart transplant function and survival through the parenchymal injury states they induce, added to which is the possibility of new injury from persisting smoldering rejection after treatment. That is why both rejection and injury states contribute to predictions of future graft loss (The processes of brain death, preservation, and implantation are universal stresses that induce recent injury transcripts in every heart to various degrees and are the main driver of elevated means for the recent injury transcripts in the population, driving the recent injury scores. These scores regress over a long time[2] before reaching "normal" levels after 1 y; however, individual hearts also experience new injuries, eg, TCMR or virus infection.).

The Late-injury state is a major problem in heart transplants and is present in 17% of all biopsies and associated with impaired function and with impaired survival even in hearts with no rejection. AMR is present in 101 of 221 (46%) of Late-injury biopsies and is, therefore, an important contributor to this state. But even this may underestimate the impact of AMR because AMR activity can "burn out" as the parenchyma deteriorates. In kidney transplants, molecular AMR activity often becomes attenuated before graft failure (Late-stage AMR), with the persistence of atrophy-fibrosis and of the characteristic time-dependent histologic lesion, glomerular double-contours.[25] In hearts, it would be very useful to have a similar time-dependent feature like kidney double contours that identifies late-stage injury induced by earlier AMR. AMR activity may evolve over the years as the Late-injury state emerges—from AMR to pAMR to NR-Minor. It would also be useful to have long-term serial observations in individual patients, but this is impossible in INTERHEART, which cannot collect serial observations in patients without SOC indications. Institutional review board (IRBs) are understandably reluctant to approve time-series biopsies in patients without indications; however, using noninvasive methods such as donor-derived cell-free DNA measurements may be useful to understanding this natural history of AMR activity.

Future studies will be necessary to determine the extent to which Severe- or Late-injury without rejection represent an evolution from previously active TCMR or AMR, compared with the impact of factors such as stress from donation-implantation, virus infection, hypertension, and coronary artery disease. Heart transplant clinicians have long

been aware of a group of late heart transplants with compromised function and increased risk of failure without clear evidence of active rejection, and the MMDx Late- injury state provides an objective classification of this state and quantifies the molecular changes. The immunoglobulin transcripts suggest that Late-injury is characterized by increased expression of low-grade inflammatory infiltrate typical of atrophy-fibrosis in kidneys,[22] which also have impaired function[25,42] and increased risk of failure.

Determining the relationship between Late-injury and CAV[6,8,9,14] is of great interest for future studies but cannot be estimated in INTERHEART. As an IRB-approved SOC study, INTERHEART could not request coronary artery studies at the time of biopsy unless they were SOC, and indeed these were seldom done around the time of biopsy; however, we hypothesize that the Late-injury state is closely correlated to the CAV state as seen in the parenchyma. Both states are late and have impaired LVEF and survival, and the immunoglobulin transcripts in the Late- injury state recall the association of CAV with B cells.[43] Many in the Late-injury group had AMR, which has been associated with CAV.[44] But the overlap between CAV and Late-injury does not necessarily mean that arterial changes are always the "cause" of Late-injury parenchymal changes. The arteries and the parenchyma are subject to many of the same stresses and may simply deteriorate in parallel with the cumulative burden of shared injuries, for example, AMR. Some arterial narrowing in late organ transplants may reflect the loss of the parenchymal metabolic activity: in kidney transplants, fibrous intimal thickening of small arteries is a universal feature of advanced atrophy-scarring.[45]

Some additional limitations of this study are imposed by the IRB-approved prospective multicenter design, which in most centers limited the number of pieces available for molecular analysis to 1. The protocol for INTERHEART that the centers agreed to only allowed them to provide certain data and did not agree to share biopsy images for central histology review, and follow-up was relatively short (median 1 y). The study was based on intact RNA to avoid the irreversible damage from formalin fixation, which reduces the quality of the extracted RNA, but this does require additional tissue beyond that taken for histology. It would be useful to have the conclusions of the molecular injury studies validated outside of the INTERHEART study. The effect of treatment on molecular rejection and paren- chymal injury is of great interest in the ongoing MMDx studies, but

we get limited information because follow-up biopsies after treatment are not SOC. This issue will require a dedicated study with follow-up biopsies after treatment.

In conclusion, genome-wide microarray measurement of gene expression being performed to diagnose rejection also offers an opportunity for assessing parenchymal injury in heart transplant EMBs by analyzing injury-associated transcripts already measured by the microarray. This new information is “free”: no additional tissue or expense is required, only software. Expression of injury transcripts correlates with dysfunction and outcomes, and future studies can define the relationship between parenchymal injury states and CAV. The identification of injury-induced changes raises the hope that interventions can eventually be directed specifically at healing injury in damaged heart transplants to improve function and prevent graft loss.

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TABLE 1. The 10 injury-related pathogenesis-based transcript sets[a,b] used for the injury-based principal component and archetypal analyses

Biological processes	Transcript set	Description of transcript set	Detail
Expressed in macrophages	QCMAT	Quantitative constitutive macrophage associated	High expression in human primary macrophages, not inducible by IFNG[46]
	AMAT	Alternative macrophage activation	Alternative activation-induced in mouse macrophages[46]
Increased in recently injured hearts	cIRIT	Cardiac injury and repair induced	Injury—induced in mouse cardiac isografts[47] compared with normal hearts
Other injury-induced transcript sets that correlate with cIRITs in human hearts[48]	IRRAT	Injury-repair response associated	Induced in early human kidney transplant injury[49]
	IRITD3	Injury and repair induced	Induced in mouse kidney isografts, peaking at day 3 posttransplant[50]
	IRITD5	Injury and rejection induced	Induced in mouse kidney isografts, peaking at day 5 posttransplant[50]
	DAMP	Damage-associated molecular pattern	Literature-based damage-associated molecular pattern (DAMP)[51,52]
Highly expressed in normal heart ("Normalness")	HT1	Normal heart transcripts—set 1 (heart-selective compared with kidney)	High expression in normal mouse heart compared with kidney (no solute carriers)[53]
	HT2	Normal heart transcripts—set 2 (heart-selective compared with kidney)	High expression in normal mouse heart compared with kidney (solute carriers)[53]
Increased in late transplants	IGT	Immunoglobulin transcripts	Increased by time and associated with atrophy-fibrosis, reflecting plasma cells[54]

[a]https://www.ualberta.ca/medicine/institutes-centres-groups/atagc/research/gene-lists.

[b]The transcript sets were empirically derived in human cell lines, human transplants, and mouse models. They reflect biological processes relevant to rejection and injury.

AMAT, alternative macrophage-associated transcripts; cIRIT, cardiac injury-repair induced transcripts; DAMP, damage-associated molecular pattern transcripts; HT1, heart transcripts set 1; HT2, heart transcripts 2; IGT, immunoglobulin transcripts; IRITD3, injury-repair induced transcripts day 3; IRITD5, injury-repair induced transcripts day 5; IRRAT, AKI transcripts; QCMAT, quantitative constitutive acrophage-associated transcripts.

TABLE 2. Expression of injury-related pathogenesis-based transcript sets[a,b] in hearts with LVEF>55 vs LVEF≤55

Biological processes	Injury-related transcript set	LVEF >55	LVEF ≤55	*P* value for LVEF >55 vs LVEF ≤55
Expressed in macrophages	QCMAT	0.30	0.54	1.8×10^{-07}
	AMAT	0.40	0.68	1.5×10^{-07}
Increased in recently injured hearts	cIRIT	0.10	0.17	2.4×10^{-05}
Other injury-induced transcript sets that correlate with cIRITs	IRRAT	0.21	0.32	0.001
	IRITD3	0.07	0.10	0.007
	IRITD5	0.11	0.10	0.83
	DAMP	0.06	0.08	0.28
Highly expressed in normal heart	HT1	−0.08	−0.18	1.2×10^{-09}
	HT2	−0.12	−0.29	1.1×10^{-10}
Increased in time	IGT	0.30	0.80	5.8×10^{-09}

[a]https://www.ualberta.ca/medicine/institutes-centres-groups/atagc/research/gene-lists

[b]The transcript sets were empirically derived in human cell lines, human transplants, and mouse models. They reflect biological processes relevant to rejection and injury.

AMAT, alternative macrophage associated transcripts; cIRIT, cardiac injury-repair induced transcripts; DAMP, damage-associated molecular pattern transcripts; HT1, heart transcripts set 1; HT2, heart transcripts 2; IGT, immunoglobulin transcripts; IRITD3, injury-repair induced transcripts day 3; IRITD5, injury-repair induced transcripts day 5; IRRAT, AKI transcripts; LVEF, left ventricular fraction; QCMAT, quantitative constitutive macrophage-associated transcripts.

TABLE 3. Mean time posttransplant and transcript set scores in the 5 parenchymal injury states

Injury variables assessed		Parenchymal injury states				
		No-injury (N = 376)	Mild-injury (N = 526)	Moderate-injury (N = 110)	Severe-injury (N = 87)	Late-injury (N = 221)
Mean days posttransplant (median)		1065 (329)	408 (126)	218 (65)	548 (85)	1430 (712)
Biological processes	**Transcript sets**	**Mean transcript set scores in each parenchymal injury state**				
Expressed in macrophages	**QCMAT[a]**	**1.05**	**1.17**	**1.45**	**2.80**	**1.54**
	AMAT[a]	**1.08**	**1.24**	**1.67**	**3.28**	**1.78**
Increased in recently injured hearts	**cIRIT[a]**	**1.00**	**1.05**	**1.22**	**1.47**	**1.15**
Other injury-induced transcript sets that correlate with cIRITs	IRRAT[a]	0.99	1.15	1.61	**2.16**	**1.26**
	IRITD3[a]	0.99	1.04	1.19	**1.26**	**1.08**
	IRITD5[a]	0.99	1.07	1.35	**1.40**	**1.10**
	DAMP[a]	0.92	1.13	1.02	**1.41**	**1.03**
Highly expressed in normal heart	**HT1[a]**	**0.98**	**0.98**	**0.86**	**0.68**	**0.88**
	HT2a	**0.97**	**0.99**	**0.79**	**0.54**	**0.83**
Increased in late transplants	**IGT[a]**	**1.03**	**0.99**	**1.03**	**1.79**	**3.19**

Bold mark the highest expression groups in each row; underlining indicates the lowest.

[a]These were the 10 transcript sets used in the principal component and archetypal analyses. AMAT, alternative macrophage associated transcripts; cIRIT, cardiac injury-repair induced transcripts; DAMP, damage-associated molecular pattern transcripts; HT1, heart transcripts set 1; HT2, heart transcripts 2; IGT, immunoglobulin transcripts; IRITD3, injury-repair induced transcripts day 3; IRITD5, injury-repair induced transcripts day 5; IRRAT, AKI transcripts; QCMAT, quantitative constitutive macrophage-associated transcripts.

TABLE 4. Relationship of myosin and tropomyosin expression to parenchymal injurys[a]

			Spearman correlation of gene expression with PC1 and Severe-/Late-injury archetype scores in 1320 biopsies		
Affymetrix designation	Gene symbol	Gene name	Injury PC1 score	Severe-injury score	Late-injury score
11718277_a_at	MYL2	Myosin light chain 2	−0.28	−0.30	−0.14
11719790_a_at	MYL3	Myosin light chain 3	−0.51	−0.54	−0.87
AVERAGE			**−0.40**	**−0.42**	**−1.01**
11740313_s_at	MYH6, MYH7	Myosin, heavy chain 6, alpha; myosin, heavy chain 7, beta	−0.41	−0.48	−0.15
11717570_s_at	MYH7	Myosin, heavy chain 7, beta	−0.42	−0.43	−0.17
AVERAGE			**−0.415**	**−0.46**	**−0.16**
11738892_a_at	TPM1	Tropomyosin 1 (alpha)	−0.22	−0.26	−0.07
11738893_s_at	TPM1	Tropomyosin 1 (alpha)	−0.36	−0.40	−0.10
11742308_s_at	TPM1	Tropomyosin 1 (alpha)	−0.34	−0.39	−0.09
11742309_x_at	TPM1	Tropomyosin 1 (alpha)	−0.25	−0.35	−0.11
AVERAGE			**−0.29**	**−0.35**	**−0.09**

[a]Probesets for myosin light chains and heavy chains and for tropomyosin genes were selected only on the bases of high expression values (>10 000) in normal biopsies.

TABLE 5. Parenchymal injury states in hearts with TCMR and AMR

Modified rejection sign-outs	Parenchymal injury states					
	No-injury (N = 376)	Mild-injury (N = 526)	Moderate-injury (N = 110)	Severe-injury (N = 87)	Late-injury (N = 221)	Row Total
TCMR-related, including mixed and pTCMR 1	(1%) 14	(11%)	4 (3%)	**44 (35%)**	**64 (50%)**	**127**
Mean days posttransplant	164 d	134 d	144 d	**885 d**	**1064 d**	
AMR-related including pAMR (excluding mixed)	**66 (19%)**	**105 (31%)**	47 (14%)	21 (6%)	**101 (30%)**	**340**
Mean days posttransplant	**970 d**	**510 d**	350 d	342 d	**1729 d**	

Principal injury groups in each RAT sign-out group (>15% of row total) are bolded and shaded. AMR, antibody-mediated rejection; RAT, rejection-associated transcript; pAMR, possible AMR; TCMR, T cell–mediated rejection.

TABLE 6. Relating parenchymal injury state to LVEF and graft loss in AMR and TCMR

Modified rejection sign-outs	Injury archetype group assignment	N per group	Mean time posttransplant	Median time posttransplant	Mean LVEF	Number of graft failures within 3 y
AMR+pAMR[a]	No-injury	66	970	366.5	62.79	3
	Mild-injury	105	510	179.5	66.38	3
	Moderate-injury	47	350	95	61.96	0
	Severe-injury	**21**	**342**	**144**	**56.86**	**2**
	Late-injury	**101**	**1729**	**927**	**55.85**	**12**
TCMR+pTCMR+Mixed[b]	No-injury	1	164	164	55	0
	Mild-injury	14	134	115	63.71	0
	Moderate-injury	4	144	92	55.00	0
	Severe-injury	**44**	**885**	**123**	**53.11**	**8**
	Late-injury	**64**	**1064**	**702.5**	**52.48**	**8**

[a]Within AMR biopsies, a ttest comparing LVEF in biopsies with Late-injury+Severe-injury vs those with No-injury+Mild-injury+Moderate-injury was significantly different ($P = 1.43 \times 10^{-7}$).

[b]Within TCMR biopsies, a ttest comparing LVEF in biopsies with Late-injury+Severe-injury vs those with No-injury+Mild-injury+Moderate-injury was significantly different ($P = 0.007$). AMR, antibodymediated rejection; LVEF, left ventricular ejection fraction; TCMR, T cell–mediated rejection.

Bold highlights the Severe- and Late-injury rows for comparison.