Caveats in Interpretation of Molecular Diagnostics in Heart Allografts

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Abstract. Histologic separation of injury, T cell–mediated rejection, or antibody-mediated rejection in allograft heart biopsies is difficult. A critical review of publications was performed to evaluate the caveats of using molecular diagnostics (MDX) to distinguish between these entities. Typically, only 1 to 2 fragments of unknown histologic appearance are evaluated. Archetype and molecular classifier analyses use gene lists derived from histologic labels and associated reproducibility issues influence the accuracy of the derived MDX classes. Archetypes A1, A2, and A3 archetypes created by bioinformatics were renamed no rejection, T cell–mediated rejection, and antibody-mediated rejection despite as little as 40% concordance with histologic diagnoses and overlapping archetype scores. Additional archetypes S4 and minor injury were created using arbitrary cutoffs based on visual examination of principal component analysis plots. Therapeutic implications of the numerous discrepancies with histology remain unexplored. Many MDX-derived observations are ambiguous and open to alternate logical explanations. Better molecular methods and more rigorous validation studies are needed to advance the field. Ideally, these methods should analyze all available biopsy fragments to minimize sampling issues. It is also desirable to incorporate spatial transcriptomics into the workflow, so that gene expression data can be directly compared with the underlying histology lesions.

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THE CHALLENGING NEED FOR BETTER DIAGNOSTICS

The role of biopsies in optimizing patient management has been established over several decades. Interobserver variability is cited as its major limitation. However, the more severe forms of rejection are readily recognized, and boundary zone cases can be adjudicated with input from clinical parameters. T cell–mediated rejection (TCMR) in allograft heart biopsies (AHBs) is graded as 0 (no rejection [NR]), 1R, 2R, and 3R in the ISHLT 2005 system.[1] Antibody-mediated rejection (AMR) is graded according to ISHLT 2013 system into categories probable AMR (pAMR) (0) with NR, pAMR (1 h+) with microvascular inflammation (MVI) only, pAMR (1i+) with c4d staining only, and pAMR (2) with both MVI and C4d staining.[2]

Histologic separation of TCMR from AMR is difficult. TCMR-associated interstitial infiltrates include some MVI, which reflects diapedesis. Conversely, following tissue injury, AMR MVI spreads to the interstitium. If CD3 staining is not done, CD68-positive macrophages responding nonspecifically to any injury can be misconstrued as evidence of AMR.[3] Hence, histology-independent methods of diagnosis are needed, but all currently available MDX is tied to gene lists ultimately derived from tissues with histologic labels. Nevertheless, quantitation of MVI and tissue injury are areas in which MDX could complement histopathologic evaluation.[4,5]

ARCHETYPE ANALYSIS OF TISSUE TRANSCRIPTS AS A POTENTIAL SOLUTION

Initial molecular diagnostic (MDX) testing for AHB used rejection-associated transcripts (RATs) established in the kidney.[6-10] In the first study, RATs were used to divide 331 heart biopsies into 3 archetypes, namely NR, AMR, and TCMR. A key potential advantage of archetype analysis is the ability to assign relative proportions of multiple different pathologic processes in the same biopsy. However, the correspondence of the RAT-based archetypes to the relevant histologic diagnoses was quite imperfect. A1, A2, and A3 archetypes contained only 100 of 223 (44.8%), 18 of 45 (40.0%), and 42 of 62 (67.7%) biopsies with NR, TCMR>1R, and AMR, respectively. Yet, these bioinformatics-derived clusters have been renamed as archetypes NR, TCMR, and AMR. In histologic AMR, the mean AMR archetype score of 0.39 was barely higher than the NR Archetype score of 0.36 and likely not statistically significant.[6] Cutoffs proposed to

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TABLE 1.

Critical evaluation of published observations pertinent to molecular evaluation of heart allograft biopsies

	Molecular diagnostic assertion	Caveat/alternative interpretation of data
General	Kidney-derived gene lists can be used without modification for heart biopsies	1. MVI in the kidney has many causes other than AMR 2. False-positive TCMR will result in inflammation because of drugs, bacterial infection, and viral disease 3. Kidney-based algorithms need stress testing for heart-specific diseases (endocarditis, myocarditis, pericarditis, coronary ischemia, hypertensive injury)
Archetype analysis	It is an unsupervised analysis independent of histology	It uses gene lists that were derived from tissue specimens with histology labels
	It has allowed an objective classification of biopsies into diagnostic categories	Molecular scores used to separate diagnostic categories were arbitrarily chosen by visual inspection of PCA plots The initial goal was to maximize agreement with histology, but that was not very successful. There was no attempt at clinical validation in terms of a superior label with respect to treatment
	Archetype scores can be used to monitor response to treatment	1. Despite claims of 99% precision, statistical measures of precision on replicate samples are not available. 2. It is not clear, eg, if a score of 0.25 is believably different from 0.45 3. Replicate measurements are known to show 2–5 decile variations in molecular scores
TCMR	Most histologic grade 1R rejection shows no molecular rejection	This is a thresholding and semantic issue. Molecular-level changes are indeed found in these biopsies
	Many histologic grade 2R TCMRs cannot be confirmed by MDX	1. TCMR is focal: if 1 fragment taken for molecular analysis shows NR, then there is a 68% chance that rejection is present in the remaining biopsy fragments examined by histology 2. Molecular diffusion cannot correct for focal lesions because MDX is based on the presence as well as concentration of key mRNAs 3. Archetype and classifier cutoffs are arbitrary and not based on validation in terms of the need for treatment 4. MDX fragment can be a biopsy site, scar, pericardium, endocardium, Quilty lesion valve, or chordae tendineae 5. INTERHEART has NOT shown that histologic grade 2R or 3R TCMR not confirmed by MMDx can be left untreated. Many clinicians are appropriately very reluctant to do so
	MDX can diagnose TCMR in biopsies labeled as NR	The clinical significance of subpathologic (changes not even qualifying for grade 1R) is likely minimal
AMR	Molecular AMR is more common than TCMR in histologic grade 1R TCMR	Molecular classifiers over-call antibody-mediated damage (see the text)
	Molecular classifiers can diagnose C4d-negative, DSA-negative AMR	The classifiers can also mislabel MVI not mediated by AMR (T cells, macrophages, PIRAs, SIRPA, NKC)
	MDX recognizes rejection in biopsies labeled as NR.	1. NR is a category created by classifiers labeling subgrade 1R/pAMR (1) inflammation as not being rejected 2. It is not surprising that molecular changes are detected in biopsies that contain histologic inflammation 3. It is unclear whether such patients need more immunosuppression, IVIG, or rituximab
Injury	MDX can recognize injury not recognized by histology	1. The extent to which prior biopsy sites and Quilty lesions confound MDX is unknown 2. We have seen biopsies with macrophage-rich histologic AMR mislabeled as molecular injury
	S4 scores reflect donation implantation injury	1. S4 scores can be elevated in rejecting biopsies beyond the time frame of implantation injury 2. It is not known how often S4 score elevation may reflect fat necrosis, or prior biopsy sites, and infiltrative Quilty lesions
	S4 scores can identify cases misclassified by histology as rejection	Absence of molecular rejection in 1–2 fragments taken for transcriptomics cannot negate the presence of histologic rejection in 3–4 different fragments taken for histology
	MDX can recognize late injury representing atrophy/fibrosis	1. Atrophy-fibrosis will be missed if present only in the tissue fragments taken for histology 2. False positives can conceivably result if a prior biopsy site is analyzed
	MDX can recognize 5 different injury phenotypes	1. The reliability of biopsy assignment across closely related categories needs to be determined

mRNA is intracellular & diffusion in ecf another issue

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TABLE 1. (Continued)

Molecular diagnostic assertion	Caveat/alternative interpretation of data
	2. Interobserver agreement between closely related MDX categories in the kidney is only 38%–53% 3. Effect of sampling biopsy sites, fat necrosis, and infiltrative Quilty lesions on MDX labels of injury is unknown 4. Biopsy sites are commonly sampled and histologic pattern as well as MDX signature would vary with time because of fibrin thrombi, endothelial proliferation, neutrophils, or myofibroblasts being present in different phases of injury The corresponding range of MDX signatures and their effect on molecular diagnostic labels remains to be investigated

AMR, antibody-mediated rejection; DSA, donor-specific antibody; MDX, molecular diagnostics; MMDx, molecular microscope diagnostic; MVI, microvascular injury; NR, no rejection; NKC, natural killer-cell activation by missing-self; PCA, principal component analysis; PIRA, A-type paired immunoglobulin-like receptor; SIRPA, signal regulatory protein alpha; TCMR, T cell–mediated rejection.

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separate probable TCMR and pAMR from TCMR and AMR were arbitrary and based on visual examination of PCA plots. This was done with the intent to achieve reasonable agreement with the corresponding histologic categories.

THE UTILITY OF MOLECULAR BINARY CLASSIFIERS

The groundbreaking article in this area constructed classifiers using linear discriminant analysis on the top 20 diagnosis-specific transcripts in the RATs gene list. The training sets used in model development contained MDX labels derived from archetype and PCA scores.[9] Molecular scores derived in this manner do offer the opportunity to quantify inflammation and rejection activity in biopsy material. Unfortunately, numerical measures of the reproducibility of these scores in replicate analyses of AHB are not available, although graphical illustrations of wide interpatient SD are documented.[3] In the kidney transplant literature, a close examination of publications citing 99% precision of MDX actually shows many biopsies with 2 to 5 decile variations in TCMR and AMR scores on replicate analyses.[11] For samples like probable TCMR and pAMR that are difficult to classify, differences in opinion between 3 MDX experts can be seen to range from 38% to 53%,[12] although this limitation is only presented in Supplementary Tables.

THE PROBLEM OF DIAGNOSING AND QUANTIFYING MYOCYTE INJURY

Histology cannot reliably identify this pattern of injury that can be seen in the setting of (1) early posttransplant ischemia, (2) late graft dysfunction associated with chronic allograft arteriopathy, and (3) stress factors such as hypertension. In an effort to fill this unmet need, a molecular "S4" score for "early injury" was derived by archetype analysis.[10] However, S4 transcripts showed up to 2.27-fold rise in the TCMR archetype and 1.54-fold rise in the AMR archetype.

In biopsies sent for MDX from Pittsburgh, the S4 injury scores were elevated in 46.3% of grade 1R TCMRs and 52.2% of pAMR (1 h+) biopsies.[13] These observations limit the value of using injury scores to distinguish between rejection and injury in individual biopsy fragments. This problem has not been solved by subsequent refinements of molecular tests in which 5 different grades of injury have been formulated because 35% of TCMR-related biopsies are still found to have severe injury and 50% show late injury[7] (Table 5 and Table S3 in this reference).

The high incidence of molecular injury in biopsies labeled as TCMR has been used to argue that T cells rather than AMR are the major driver of injury in AHB. However, the apparent relative contributions of TCMR and AMR to tissue damage in any study would be a function of the stage of disease being analyzed. For instance, TCMR grade 1A is known to be a very benign and nonprogressive disease with some biopsies showing minimal inflammation. Likewise, antibodies would not be an important contributor to injury in biopsies consisting primarily of very early AMRs that are detectable only by molecular methods.[8] In contrast, late AMR would have substantial injury attributable directly to antibodies.

The problem of clarifying the pathogenesis of injury in AHB is further confounded by unresolved discrepancies in histologic versus molecular diagnosis of rejection.[14-16] Although confusion matrixes are not always presented, TCMR grade 1R biopsies are frequently labeled as AMR by MDX. Due to a lack of clinical validation, these MDX assertions should not necessarily be accepted as the ground truth. Indeed, increased effector T-cell transcripts can be seen in biopsies with molecular AMR and minor injury. Immunohistochemistry demonstrates the inflammation in AMR to include significant numbers of T cells that accompany macrophages and NK cells. Whether T-cell infiltrates precede or follow antibody injury is unknown, and anti–T-cell therapy may be indicated, irrespective of the actual sequence of events. There are also significant conceptual issues with the definition of AMR by archetype analysis (vide supra).

THE PATH FORWARD

The use of molecular AMR classifiers has an important caveat in that it analyzes *heart* tissue using genes from an AMR versus everything else comparison in *kidney* biopsies with *histology labels*. Importantly, AMR mimics of MVI were not included in the training set of kidney biopsies. As stressed in the forthcoming Banff 2022 Kidney report, the resulting molecular signature essentially reflects of MVI irrespective of C4d and donor-specific antibody status. It is agnostic to a growing list of potential causes of non-AMR MVI and other lesions that include TCMR, Quilty effect, ischemia–reperfusion, viral infection, missing-self–mediated activation of NK cells, $CD47^{-}$ macrophage-axis recognition of paired immunoglobulin-like receptors, and signal regulatory protein alpha.[17-22] Hence, an argument

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can be made that MDX overdiagnoses AMR, the incidence of which was 106 of 259 (41%) in one AMR-enriched study, which also diagnosed pAMR in an additional 106 of 259, but TCMR in only 51 of 259, and mixed rejection in only 11 of 259 patients.[9]

Many MDX-derived observations are open to alternate logical explanations and the transplant community should keep this in mind (Table 1). Better molecular methods with expanded training sets are needed to advance the field of MDX in AHB. Ideally, these methods should analyze all available biopsy fragments to minimize sampling issues.[23] It is also desirable to incorporate spatial transcriptomics into the workflow so that gene expression data can be directly compared with the underlying histology lesions. A critical validation study that remains to be performed is whether subclinical, subhistologic AMR or TCMR detected only by MDX need any treatment other than close monitoring (like some borderline kidney rejections!). Unnecessary intervention could do more harm than good. Likewise, many histologic AMRs have significant T-cell infiltrates with the potential to benefit from anti–T-cell treatment. Should such treatment be reserved for the very small proportion that is recognized as mixed by MDX?

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