Review

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Diagnostic Potential of Minimally Invasive Biomarkers: A Biopsy-centered Viewpoint From the Banff Minimally Invasive Diagnostics Working Group

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Abstract. With recent advances and commercial implementation of minimally invasive biomarkers in kidney transplantation, new strategies for the surveillance of allograft health are emerging. Blood and urine-based biomarkers can be used to detect the presence of rejection, but their applicability as diagnostic tests has not been studied. A Banff working group was recently formed to consider the potential of minimally invasive biomarkers for integration into the Banff classification for kidney allograft pathology. We review the existing data on donor-derived cell-free DNA, blood and urine transcriptomics, urinary protein chemokines, and next-generation diagnostics and conclude that the available data do not support their use as stand-alone diagnostic tests at this point. Future studies assessing their ability to distinguish complex phenotypes, differentiate T cell–mediated rejection from antibody-mediated rejection, and function as an adjunct to histology are needed to elevate these minimally invasive biomarkers from surveillance tests to diagnostic tests.

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INTRODUCTION

With the increasing utilization of minimally invasive biomarkers in clinical transplantation, a Banff Minimally Invasive Biomarkers Working Group was formed in early 2021 to review their applicability to the diagnosis and classification of kidney transplant pathology. Creation of the working group follows the Banff 2017 classification, which acknowledged that validated gene transcripts or classifiers from biopsy tissue can meet diagnostic criteria for antibody-mediated rejection (AMR).[1] There is already precedence for biomarkers in the Banff criteria for kidney allograft pathology, where donor-specific antibody (DSA) is utilized for a diagnosis of AMR. With the introduction of newer minimally invasive biomarkers to the market, there is now an opportunity to consider biomarkers other than DSA for inclusion in the Banff classification.

Minimally invasive biomarkers are mostly used as screening tests to rule out rejection or prompt confirmatory evaluation with a biopsy. In contrast, the objective of this working group is to explore their role as diagnostic tests primarily for rejection, either alone or in combination with histology. We considered that a diagnostic biomarker should: (1) differentiate rejection from the absence of rejection; (2) be specific

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for rejection; (3) hold the potential to replace biopsies or, at minimum, have additive value over histology alone; and if possible (4) have prognostic value. Although not felt to be absolutely necessary for inclusion in the Banff classification, the biomarker should ideally be in the causal pathway for rejection and distinguish between rejection types, that is, AMR and T cell-mediated rejection (TCMR).

Although many biomarkers were considered potential candidates, our focus was on those already in commercial use or likely to enter clinical practice given their stage in development and approval. Given that the workgroup's intent was to assess whether minimally invasive biomarkers should be considered for the diagnostic classification, there is a practical need for the biomarker to be accessible to the general transplant community. Additionally, we focused only on biomarkers that can be measured with minimal invasiveness through blood or urine. Applying these criteria, we chose 3 broad categories for consideration: (1) donor-derived cell-free DNA (dd-cfDNA); (2) blood or urine transcriptomics, and (3) urinary protein chemokines. Herein, we assess the potential role of these biomarkers for the diagnosis of kidney allograft rejection and recommend how they might augment our ability to diagnose and classify rejection.

Rationale for Consideration of Noninvasive Biomarkers in the Banff Classification

The key histologic feature of rejection is inflammation in the glomeruli, tubules, interstitium, capillaries, or arteries. Inflammation is not specific to rejection and can be seen with infections, allergies, and autoimmune conditions and as a response to injury. Furthermore, inflammation, such as arterial inflammation and peritubular capillaritis, can be seen in both TCMR and AMR leading to equivocal diagnoses. Tissue transcriptomics has described discrepancies with histology, raising questions about the accuracy of the current classification system.[2] Therefore, biomarkers present an opportunity to clarify areas of uncertainty in the Banff classification, such as Banff category 2 (antibody-mediated changes), when only minimal histologic lesions are present, DSAs are lacking, or when there is concurrent TCMR and category 3 (borderline/suspicious for acute TCMR) when mild inflammation is inconclusive for TCMR.

For a given histologic lesion, the prognosis can vary and outcomes are better characterized when histology is considered alongside clinical and immunologic factors.[3] Among patients with histologic lesions of AMR, graft survival is lower when DSA is present rather than absent, demonstrating how a biomarker might distinguish between phenotypes of histologic AMR.[4] Machine learning and semi supervised clustering can characterize archetypes with distinct graft survival prognoses, indicating an opportunity to refine the diagnostic classification by interpreting biopsies in the context of non–histology-related variables.[5,6] The only noninvasive biomarker currently included in the Banff classification is DSA; however, it is plausible that other biomarkers could be incorporated if validated. This validation should be assessed by their association with outcomes with consideration to whether the biomarker can characterize prognosis better than histology and function as a stand-alone diagnostic or whether they are more appropriately utilized as an adjunct to histology.

dd-cfDNA

dd-cfDNA is being explored as a noninvasive measure of allograft injury to detect rejection at its earliest stages before clinical allograft dysfunction, currently ill-defined as a rise in creatinine. cfDNA are fragments of DNA released from cells and, due to rapid turnover, can detect allograft injury in real time. Importantly, not all injurious processes may elicit substantial cell lysis or dysregulation leading to an elevation in dd-cfDNA.[7,8] Therefore, interpreting dd-cfDNA measurements across all events that elicit allograft dysfunction such as inflammatory cell infiltration, infection, calcineurin inhibitor toxicity, and disease recurrence remains complex and problematic.

Prospective and retrospective case-control studies in kidney recipients show dd-cfDNA outperforms serum creatinine as an early biomarker of rejection and injury.[9-18] Rejection prevalence and type varied between studies and different dd-cfDNA positive thresholds (0.2%–1.0%) were used depending on the study endpoint. Positive predictive value (PPV) and negative predictive values (NPVs) are impacted by the rejection prevalence; increased rejection in higher risk cohorts will increase the PPV but decrease the NPV. Similarly, lower rejection prevalence decreases the PPV while increasing the NPV. In spite of these variations, some common patterns have emerged. AMR was more readily detected using a higher dd-cfDNA positive threshold (1%), whereas lower thresholds were needed to correlate with TCMR, indicating that dd-cfDNA has a lower sensitivity for TCMR. Although this lower sensitivity may reflect methodological issues in dd-cfDNA testing, the insensitivity of histopathology to detect all forms of rejection and injury must also be considered.

dd-cfDNA as an Additive Diagnostic Marker in the Banff Classification

The ability of dd-cfDNA tests to accurately diagnose rejection is highly dependent upon the positive threshold used, the type of rejection, and the composition of the study cohort. Current data suggest that the sensitivity of dd-cfDNA is too low to be considered as a stand-alone diagnostic test. Future studies should consider whether dd-cfDNA can be used as a diagnostic test in conjunction with histology. To date, few studies have been performed to determine whether dd-cfDNA augments rejection diagnoses over histopathology or aids in discriminating between complex phenotypes (eg, mixed AMR and TCMR).

Recent studies assessing the correlation between dd-cfDNA, biopsy transcriptomics (MMDx), and histology suggest that dd-cfDNA is more strongly correlated with AMR than TCMR.[19,20] A subsequent analysis of a Trifecta study cohort incorporated both dd-cfDNA fraction (%) and quantity thresholds and showed improved diagnostic correlations with both MMDx and histology.[21] Gupta et al reported that among DSA-negative patients with dd-cfDNA ≥1%, several cases of no rejection or TCMR by histology were reclassified as AMR or mixed rejection by MMDx. This raises the hypothesis that high levels of dd-cfDNA might indicate AMR even if histology suggests otherwise. Similarly, a recent study of dd-cfDNA in heart transplantation suggested that nearly two-thirds of "false positive" dd-cfDNA test results, that is, elevated dd-cfDNA in conjunction with normal surveillance endomyocardial biopsies, were associated with concurrent allograft

dysfunction, later dysfunction, or future rejection.[22] Additionally, reports in kidney and lung have shown elevations in dd-cfDNA can precede de novo DSA detection and this risk is increased with higher-cfDNA levels.[18,23] There are also reports that decreased dd-cfDNA levels correlate with improved function following rejection treatment, suggesting some prognostic potential.[14,24] The concept that elevated dd-cfDNA with negative histology might indicate injury will need to be explored in future studies, bearing in mind that the specificity of alternative reference standards, such as tissue transcriptomics, also needs to be validated (ie, to confirm whether other causes of endothelial injury mimic a molecular signature of AMR).

Another potential role for dd-cfDNA in the Banff classification may be to reclassify rejection severity grading in the diagnostic classification. Huang et al found that dd-cfDNA measurements combined with Banff chronicity scores (cg and ci + ct) were predictive of estimated glomerular filtration rate (eGFR) over time.[25] Stites et al found that among patients with TCMR 1A/borderline rejections, an elevated dd-cfDNA (≥0.5%) was associated with greater eGFR decline, an increased risk of de novo DSA, and future or persistent rejection.[16] These studies provide preliminary evidence that levels of dd-cfDNA observed in the context of a given histologic lesion are associated with subsequent outcomes. If this concept is validated across rejection types, there is potential that dd-cfDNA could help redefine rejection grading.

Minimally Invasive Transcriptomics

Although tissue-based transcriptomics has been developed and validated for commercial use (MMDx), the requirement for a kidney biopsy makes them less practical for routine surveillance of allograft health. Blood or urine transcriptomics offer practical advantages over tissue-based studies because of safety and ease of collection and, if validated, hold the potential to provide minimally invasive diagnostic value.

Different techniques are used to analyze molecular profiles in blood and urine. Real-time qPCR and probe techniques are used for small gene sets and microarray platforms such as Affymetrix and OpenArray are used for many genes. AlloMap Kidney and the Kidney Solid Organ Response Test utilize qPCR and can be performed using 8–10 mL of blood. Trugraf uses DNA microarrays analysis from blood and is authorized in the United States for assessment of rejection. NanoString technology has developed the B-HOT panel; however, there are no studies on its applicability to blood and urine samples.

Noninvasive Transcriptomics as an Additive Diagnostic Marker in the Banff Classification

Transcriptomics in blood has the potential for discrimination of rejection and also between rejection types.[26,27] Using qPCR with only 17 genes, Roedder et al differentiated between rejection and nonrejection with an area under the receiver operating characteristics curve (AUC) of 0.94, a sensitivity of 83.0%, and specificity of 72%. Using DNA microarrays, Kurian et al built a 200 gene set classifier differentiating between well-functioning grafts, acute rejection, and dysfunction not due to rejection and was associated with an AUC ranging from 0.817 to 0.933.[26] Minimizing the classifier to 25 genes, the sensitivity increased to 85%–95% and the specificity to 90%–96%. In contrast, a large retrospective study by van Loon et al including 1763 samples was unable to validate the feasibility of Kidney Solid Organ Response Test to detect TCMR and AMR in blood within the first posttransplant year.[28]

The use of RNA-sequencing has been investigated in blood using small cohorts of patients (<100).[29] These studies show the possibility of differentiating between AMR and TCMR or no rejection with only a minimal number of genes. Thorough validation is needed to investigate the additional diagnostic potential and feasibility of RNA-sequencing in diagnostic classification.

TruGraf is a minimally invasive tool approved by Medicare to detect subclinical rejection but has not been studied as a diagnostic test. This microarray-based technique is based on a molecular profile of rejection associated with a sensitivity of 64% and specificity of 87% for subclinical rejection.[30] Data published on TruGraf indicate that it is more sensitive for TCMR rather than AMR.[31] This property hints that blood transcriptomics might have value when histology is equivocal for TCMR, with the caveat being that it has only been evaluated in a subclinical setting. Another peripheral blood transcriptomic test called AlloMap Kidney is being developed. This is a 5-gene classifier trained on 56 peripheral blood samples and validated in a small cohort of 116 cases, which included only 18 cases of rejection.[32] The AUC for discriminating rejection from no rejection was 0.79. AlloMap Kidney is currently not validated for clinical use and is undergoing further evaluation in a larger multicenter study (NCT03326076).

Urinary transcriptomics has also led to noninvasive testing possibilities to screen for and diagnose rejection. The groundbreaking study investigating the possibility of using mRNA as a noninvasive tool to diagnose rejection showed that increased expression levels in urinary cells of perforin and granzyme B were associated with acute rejection.[33] Increase in FOXP3 transcripts in blood and urine has also been associated with rejection and may be predictive of treatment response.[34,35] CTOT-04 was the first large study and included 4300 samples, rendering 9 genes assessed in urine by real-time qPCR.[36] Evaluation and validation of this panel showed that TCMR could be diagnosed with an AUC of 0.78 using a 3-gene model of CD3 emRNA, IP-10 ribosomal RNA, and 18S ribosomal RNA. External validation using the CTOT-01 samples showed an AUC of 0.74 (95% CI, 0.61-0.86; $P < 0.001$) in discriminating between biopsies showing rejection and those not showing rejection.[36] This test is currently being developed for commercial use.

Whole transcriptome analysis using RNA-sequencing is also a method that allows for the discovery of potentially novel biomarkers. This has been performed on urinary cells to discriminate between AMR, TCMR, and no rejection and indeed showed different genetic signatures and biological pathways.[37] The study included only 57 urine samples, of which 8 were AMR and 22 TCMR, resulting in a possibility of overfitting the data and requiring further validation.[37] Additionally, studies to determine the additive value of blood and/or urine transcriptomics in conjunction with kidney histology to better classify disease have not been performed. Interestingly, there might be an unmet

need to incorporate this data just as electron microscopy, C4d immunohistochemistry, and DSA assessment are of additive value in fine-tuning a classifying diagnosis.

Urinary Chemokine Proteins

Investigators have studied inflammatory biomarkers as markers of rejection since the early 2000s. Among the most promising are assays for the CXCR3-binding chemokines, CXCL9 and CXCL10. These are interferon-gamma-induced chemokines secreted by inflammatory cells and kidney tubule and mesangial cells that contribute to rejection through leukocyte recruitment. Multiple investigators have studied their association with rejection and show both as nonspecific markers of inflammation in the kidney allograft.

Both urinary CXCL9 and CXCL10 are elevated in the presence of rejection and discriminate from nonrejection but cannot distinguish AMR from TCMR.[38-41] In one study, urinary CXCL10 was predictive of subsequent rejection and a decrease in urine CXCL10 has been described among rejection-treated patients predominantly affected by TCMR.[38,42] Combined measurements of CXCL9 and CXCL10 do not appear to increase discrimination for rejection compared with measurements of either chemokine separately.[39,41] Various methodologies and reporting strategies for urinary CXCL9 and CXCL10, including absolute levels and normalization to urinary creatinine, were used across studies with similar discrimination.[38-40]

The sensitivity and specificity of urinary CXCL9 for rejection range from 58% to 86% and 64% to 80%, respectively, whereas the sensitivity and specificity of CXCL10 range from 59% to 80% and 76% to 90%, respectively.[38-41] These performance characteristics were described across studies using differing positive test thresholds and were derived from populations with varying compositions of TCMR and AMR. Data suggest that urinary CXCL10 might point toward the origin of inflammation within biopsy compartments, with elevated levels seen with tubulointerstitial inflammation and peritubular capillaritis but not with pure glomerulitis or isolated vascular inflammation (isolated v lesions).[43] More recent studies have investigated the ability of urinary chemokines to discriminate rejection in prospective, unselected cohorts including protocol and indication biopsies with definitive rejection diagnoses in addition to borderline and indeterminate lesions.[38,42] Urinary CXCL10, but not urinary CXCL9, was elevated among patients with subclinical rejection compared with those without, although its discrimination was only modest (AUC 0.64; 95% CI, 0.55-0.73).[42] Both urinary CXCL9 and CXCL10 were better able to discriminate rejection from patients with dysfunction without rejection (AUC 0.72 and 0.74, respectively).[42] The discrimination of urinary CXCL10 for rejection was similar in a prospective pediatric cohort study of mostly TCMR cases (AUC 0.76; 95% CI, 0.66-0.86).[38] Further, higher urinary CXCL10 over time was associated with eGFR decline, suggesting that persistent inflammation is linked to progressive allograft dysfunction.[38]

Urinary Chemokine Proteins as an Additive Diagnostic Marker in the Banff Classification

Urinary CXCL10 appears to be best characterized as an indicator of inflammation that is not specific for rejection. Urinary CXCL10 is similarly elevated among patients with BK viremia compared with uninfected patients with tubulitis on biopsy.[38,39,43] In contrast, urinary CXCL10 was not elevated among patients with cytomegalovirus viremia.[43] The different patterns of urinary CXCL10 may be related to a higher predilection of BK virus for genitourinary tissue compared with cytomegalovirus. Elevated urinary CXCL9 and CXCL10 have been described among patients with isolated leukocyturia and urinary tract infections.[44] The elevated CXCL10 in patients with leukocyturia seems to be an indicator of graft inflammation, being observed only among patients with inflammation present on biopsy but not among those without inflammation.[38]

Studies are needed to assess whether urinary chemokines are more appropriately used to screen for rejection rather than as a diagnostic test. Urinary chemokines are reported to be elevated before rejection becomes clinically apparent, suggesting that they have a promising role as a screening test.[42,45] An ongoing multicenter trial will randomize patients with high urinary CXCL10 to undergo a biopsy or routine posttransplant monitoring (NCT 03206801). Clinical and histologic outcomes will be assessed at 12 mo to assess whether elevated urinary CXCL10 prompting allograft biopsies and treatment will lead to improved outcomes. This trial could be an opportunity to study whether urinary chemokine levels considered alongside histologic variables can improve the prediction of allograft outcomes and could be a starting point for the exploration of the diagnostic value of urinary CXCL10.

Other Noninvasive Biomarkers

There are also less well-evaluated noninvasive biomarkers available in a research setting. These include microRNAs (miRs), nucleosomes, and extracellular vesicles (EVs). MiRs are small noncoding RNAs that act as negative regulators of posttranscriptional gene expression. They can be released into the extracellular compartment where they can be detected as a biomarker. Only a handful of studies have been performed in urine and blood investigating the use of miRs as a minimal-invasive biomarker to diagnose rejection. Those biomarkers discovered are based on small numbers of samples and the findings are heterogeneous and not extensively validated externally. It is difficult to compare analyses as techniques to quantify miRs also differ; miRs can be assessed using qRT-PCR, microarray, and next-generation sequencing.

Nucleosomes represent the smallest structural unit of chromatin, consisting of approximately 146 base pairs of DNA wrapped around 8 histone proteins. Nucleosomal chromatin regulates multiple DNA processes and during cell death, nucleosomes are released into the blood where they can be characterized by histone modifications and the packaged DNA can be identified by using standard molecular assays. Verhoeven et al found that increased nucleosome levels containing activating histone modifications correlated with acute kidney transplant rejection.[46]

EVs are membrane-bound vesicles, and exosomes, released from the cell and contain lipids, nucleic acids, proteins, miRs, and cytosol. They can be detected in blood and urine by flow cytometry or electron microscopy, amongst other methods. Preliminary studies in heart have interrogated unique blood EV protein profiles and corresponding endomyocardial biopsies to differentiate TCMR, AMR, and no rejection.[47,48] Another investigation showed an

exosomal mRNA signature in urine that was able to detect the presence of rejection in kidney transplant patients and differentiate between TCMR and AMR.[49]

Combining Noninvasive Biomarkers as Companion Diagnostics

Given the complexities of rejection diagnoses and their differentials, combining biomarkers might achieve greater diagnostic accuracy and improved characterization of rejection type and severity. It has been shown that dd-cfDNA combined with DSA improved AMR diagnostic accuracy above DSA alone.[12,50] Emerging data indicate that other biomarker combinations may also assist the detection of rejection.

Park et al retrospectively examined 428 surveillance biopsies with paired blood transcriptomics (TruGraf) and dd-cfDNA tests (Viracor TRAC) and found that transcriptomics was better at detecting subclinical TCMR, whereas dd-cfDNA performed better at detecting subclinical AMR.[31] In combination, the specificity increased to 98% when both tests were negative, although requiring both to be positive dropped the sensitivity to 20%. Requiring only 1 test to be positive increased the sensitivity to 69%, highlighting the complementary relationship of these tests. It must be noted that biopsies containing other relevant differential diagnoses such as infection, chronic tubular injury or fibrosis, calcineurin inhibitor toxicity, and recurrent primary disease were excluded from the analysis. Re-analysis of Circulating dd-cfDNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients study samples, including 98 no rejection and 18 rejection samples (7 TCMR, 10 AMR, and 1 mixed rejection), was performed using a 5-gene classifier (AlloMap Kidney) and a dd-cfDNA threshold of 1%.[32] Direct comparison of these 2 biomarkers showed a weak correlation (R = 0.15); however, individual dd-cfDNA results of ≥1% did correspond to higher transcriptomic scores. Analysis of a combined score with equal weighting of dd-cfDNA and blood transcriptomic scores showed superior performance to discriminate rejection from no rejection with an AUC 0.894, improving over transcriptomics alone (AUC 0.768) or dd-cfDNA alone (AUC 0.85).

Nolan et al reported on a new urinary Q-Score based on 6 biomarkers (cell-free DNA, methylated-cell-free DNA, clusterin, CXCL10, creatinine, and total protein) to differentiate stable kidney allografts from rejection.[51] The composite Q-Score was able to detect rejection with an astounding AUC = 99.8%, where in a Q-score >32 discriminated cases of rejection from those without.

Therefore, there is support for combining biomarkers. However, the success of a combined screening approach may be impeded due to the cost of these assays performed longitudinally by centralized laboratories.

New-generation Diagnostics

The field of molecular diagnostics is moving at a rapid pace and as technologies become more efficient and gain broader utilization their application to clinical medicine is increasing and the cost is decreasing. Current cfDNA assays focus on genotypic polymorphisms to quantitate donor cell injury; however, interrogations of epigenetic marks such as DNA methylation patterns and nucleosome positioning can further elucidate cell-specific signatures within cfDNA to indicate which donor cells within the allograft are undergoing injury.[52-54] This type of epigenetic analysis of cfDNA fragments is being evaluated in clinical oncology and within transplant diagnostics, may define the specific rejection type as well as the extent of the injury with greater accuracy. A pilot heart study examined left ventricle DNA methylated patterns in cfDNA isolated from serial plasma samples (n = 24) taken at the time of surveillance biopsies and found increased levels of ventricle-specific cfDNA in the plasma of recipients diagnosed with 2R grade TCMR, but no correlation with less severe 1R grade rejection.[55] Gai et al provided a genetic-epigenetic tissue mapping strategy using tissue-specific DNA methylation patterns to determine the tissue composition of dd-cfDNA in 11 lung transplant recipients.[56] Interrogation of dd-cfDNA 72 h posttransplant found that 17% originated from lung tissue cells, whereas 78% derived from donor neutrophils and lymphocytes passively transferred within the allograft.

Similarly, transcriptomic assays in transplantation have also relied on "bulk or total" RNA analysis of circulating blood cells or biopsy tissue and have not fully addressed the heterogeneity of cell types, activation status, and effector functions involved in the rejection process.[57] Pineda et al performed single-cell RNA sequencing of blood samples taken at the time of kidney rejection and found greater resolution and dynamic range for discriminating AMR and TCMR specific signals as well as recognition of rejection processes that integrate both coding and noncoding genes.[29] Defining cell-specific signatures deriving from either recipient or donor may provide greater diagnostic accuracy in the assessment of rejection and allograft injury as well as fostering a greater understanding of the heterogeneity of rejection phenotypes.

Future Directions

Table 1 highlights areas for further investigation that are needed to broaden our understanding of how to interpret and use the minimally invasive biomarkers discussed in this paper. Key questions that should be addressed to support a biomarker for inclusion in the diagnostic classification include: (1) Can a biomarker characterize mechanisms of allograft injury, thereby elucidating processes that may help distinguish between T cell- or antibody-mediated mechanisms and nonalloimmune injury? (2) For a given histologic lesion, can a biomarker inform on rejection severity and prognosis? (3) Can a biomarker indicate the presence of rejection in cases with minimal lesions on histology? (4) How does the biomarker correlate with other molecular diagnostic tests performed on tissue? and (5) Can a biomarker help to differentiate between rejection and other forms of nonrejection injury?

The traditional paradigm for assessing a biomarker's diagnostic capacity is to compare its performance against the gold standard of histology. Under this paradigm, none of the tests assessed by this workgroup demonstrate sufficient correlation with histology to be considered diagnostic tests. Most of the validation data referenced in this review report low sensitivities, indicating high false negative rates that preclude them from being used as stand-alone diagnostic tests. However, given the limitations of histology, this

TABLE 1.

Next steps for a Banff diagnostic classifier incorporating new minimally invasive biomarkers

	Advantages	Challenges	Potential solutions
dd-cfDNA			
Provides information specific to donor allograft	Donor specific	% measurements can be influenced by elevated recipient cfDNA Potential interference/false positives from blood products or multiorgan transplants	Absolute quantity measurements with corrections for allograft size/quality. Mitigate with algorithms using donor-specific genotypes
Provides information specific to rejection	High NPV	Low PPV, elevations due to nonrejection injury/dd-cfDNA release	Combine with companion diagnostics to rule out infection, recurrent disease, drug toxicity, ischemic injury Interrogation of cfDNA epigenetics to identify which cells within the allograft are injured
Defines specific types of injury on histopathology	High sensitivity for cell lysis/DNA release	Low sensitivity for injuries not associated with cell lysis/DNA release	Correlate with well-annotated rejection and nonrejection lesions Correlation with sequential biopsies of rejection/ nonrejection lesions with and without interventions
Specificity: AMR/TCMR	Sensitive for injury	Not specific for AMR/TCMR	Improved PPV when combined with DSA/infection/transcriptomics/epigenetics
Severity, prognostic value AMR/TCMR	Sensitive for injury Potential association with eGFR decline	Potential low sensitivity for DSA-mediated lesions not associated with cell lysis/DNA release (vasculopathy) Low sensitivity for cellular infiltration/ inflammation without cell lysis/DNA release	Combine with companion diagnostics such as detailed DSA characteristics/ transcriptomics Correlate with granzyme+/perforin+ infiltrates
Blood and urine transcriptomics			
Technology	Multiple platforms are commercially available	Comparability between platforms is challenging	External validation of different platforms and gene panels
Provides information specific to donor allograft		Not donor-specific	Use single-cell RNA sequencing techniques
Provides information specific to rejection	High NPV has been observed in some studies	Variable performance between assays and studies	Additional validation of target markers and assays are needed
Defines specific types of injury on histopathology		No data available	Correlate with well-annotated rejection and nonrejection injury
Specificity: AMR/TCMR		Limited data to differentiate between rejection types	Correlate with well-annotated AMR and TCMR cases
Severity, prognostic value, AMR/TCMR		No data available	Correlate with severity and outcomes
Urinary chemokines			
Provides information specific to donor allograft	Donor specific	Cannot assume donor-specificity with significant residual native kidney function Requires adequate urine volume; may not be applicable in patients with advanced allograft dysfunction	Assess chemokine levels in transplanted patients with residual native kidney function, particularly in those with inflammatory causes of end-stage kidney failure Establish reference ranges across the continuum of eGFR
Provides information specific to rejection	High NPV	Low PPV; infections can lead to elevated chemokines	Define disease conditions that affect the reliability of urinary chemokines
Specificity	Sensitive for inflammation	Not specific for rejection type	Combine with sequential companion diagnostics (DSA/transcriptomics)
Severity, prognostic value	Associated with eGFR decline	Prognostic value in association with defined histologic lesions not defined	Compare prognosis between high vs low chemokine levels in specific histologic diagnoses to inform on severity grading

AMR, antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; eGFR, estimated glomerular filtration rate; NPV, negative predictive value; PPV, positive predictive value; TCMR, T cell–mediated rejection.

approach may limit their potential as diagnostic tests. It is well-recognized from the literature on tissue transcriptomics that molecular diagnoses rendered by classifiers or archetypes of gene expression can be discordant with histologic diagnoses, raising the possibility of diagnostic misclassification by histology.[58] Although it cannot be concluded from these analyses that molecular diagnoses are more accurate than histology, the potential of ancillary testing to prompt reconsideration of a histologic diagnosis should be investigated further. We suggest that studies directed at assessing the diagnostic capability of a biomarker should demonstrate that the biomarker is associated with outcomes, irrespective of their association with histology. Additionally, validation of a biomarker's mechanistic link to rejection should be demonstrated to warrant consideration as a diagnostic test.

Acknowledging the limitations of solely using histology for classifying rejection, this workgroup suggests that studies should first focus on how a biomarker can improve histologic interpretation rather than replace biopsies as the gold standard. The hypothesis that combining biomarkers representing different points along the pathway from inflammation to rejection to injury may improve diagnostic accuracy and clinical utility is supported by recent data.[31,51] Additional studies are needed to delineate which combinations are the most informative for the diagnosis of rejection while maintaining financial accountability. Nevertheless, there is now an opportunity to leverage the strengths of all the available diagnostic modalities together to improve the diagnostic classification. One proposal is to move toward a probabilistic model of diagnosis,[59] integrating clinical information (eGFR, proteinuria, sensitization status), immunologic variables (DSA, chemokines), and molecular and transcriptomic data (dd-cfDNA, transcriptomics) together with histology to define archetypes that resemble rejection. Although this paradigm might not be able to define rejection with certainty, it has the potential to identify situations in which histology alone may not be sufficient in providing an accurate or conclusive diagnosis.

Finally, one must consider issues related to the practical implementation of any diagnostic biomarker before inclusion in the Banff classification. The biomarker should be accessible worldwide and results should be reproducible, regardless of where the test is performed. Additionally, costs and turnaround time for results are pragmatic concerns that affect clinical applicability and ideally should be improved.

CONCLUSIONS

With the introduction of novel minimally invasive biomarkers, there is an opportunity to improve the diagnostic classification of kidney transplant rejection. Our intent is to stimulate interest in reshaping the paradigm of a predominantly histology-based classification to one that involves a combination of histology, DSA, and other minimally invasive biomarkers to more precisely define allograft pathologies.

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