Biopsy-based transcriptomics in the diagnosis of kidney transplant rejection

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Purpose of review
The last year has seen considerable progress in translational research exploring the clinical utility of biopsy-based transcriptomics of kidney transplant biopsies to enhance the diagnosis of rejection. This review will summarize recent findings with a focus on different platforms, potential clinical applications, and barriers to clinical adoption.

Recent findings
Recent literature has focussed on using biopsy-based transcriptomics to improve diagnosis of rejection, in particular antibody-mediated rejection. Different techniques of gene expression analysis (reverse transcriptase quantitative PCR, microarrays, probe-based techniques) have been used either on separate samples with ideally preserved RNA, or on left over tissue from routine biopsy processing. Despite remarkable consistency in overall patterns of gene expression, there is no consensus on acceptable indications, or whether biopsy-based transcriptomics adds significant value at reasonable cost to current diagnostic practice.

Summary
Access to biopsy-based transcriptomics will widen as regulatory approvals for platforms and gene expression models develop. Clinicians need more evidence and guidance to inform decisions on how to use precious biopsy samples for biopsy-based transcriptomics, and how to integrate results with standard histology-based diagnosis.

Keywords
biopsy, biopsy-based transcriptomics, immune rejection, kidney transplant

INTRODUCTION

Despite advances in kidney transplant immunology, kidney transplant immune rejection remains a leading cause of allograft dysfunction [1]. Noninvasive diagnostic tests for rejection, such as urine chemokines and donor-derived cell-free DNA, do not yet substitute for a biopsy, which determines the type of rejection and its activity, providing the diagnostic and prognostic information necessary for patient management decisions [2].

The Banff Classification for Allograft Pathology recognizes two main types of rejection. In T-cell mediated rejection (TCMR), activated T cells infiltrate the graft endothelium (intimal arteritis lesion "v"), interstitium (interstitial inflammation "i"), and tubules (tubulitis "t") [3,4▪]. In antibody-mediated rejection (AMR), donor-specific antibodies (DSA) target human leukocyte antigen (HLA) molecules on graft endothelium, recruit intravascular immune cells (microvascular inflammation, "MVI" corresponding to peritubular capillaritis "ptc" and glomerulitis "g"), with or without complement-dependant effects evidenced by C4d deposition, and cause endothelial cell injury [5].

Analysis of gene expression in a homogenised piece of kidney biopsy ("bulk" biopsy-based transcriptomics, BBT) has yielded considerable insights into the pathophysiology of rejection and shown

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KEY POINTS

- Biopsy-based transcriptomics have enhanced our understanding of immune-mediated rejection in transplanted organs.
- Bulk biopsy-based transcriptomics have potential to improve diagnosis of rejection, and not only in cases where standard histology yields ambiguous results.
- The main obstacles to routine diagnostic use are limited robust diagnostic models reproducible across centres and platforms, defined and tested cost-effective contexts of use, and guidelines for integrating information from standard histology and biopsy-based transcriptomics.
- Prospective clinical trials (observational and interventional) should include BBT as well as digitised histology to develop new gold standards for rejection diagnosis.

consistent gene expression profiles in TCMR and AMR. Both types of rejection are characterized by expression of interferon-gamma inducible transcripts, with TCMR also expressing signals from activated effector T cells, activated macrophages and dendritic cells, and AMR-expressing transcripts indicative of endothelial activation and natural killer (NK) cells and/or monocytes [6]. Although single-cell and spatial transcriptomics are important new areas of discovery research [7], this review will focus on current progress in the clinical application of bulk BBT, which are more immediately amenable to diagnostic use.

STANDARD HISTOLOGY AND BIOPSY-BASED TRANSCRIPTOMIC WORKFLOWS FOR THE DIAGNOSIS OF REJECTION

Table 1 provides a comparison of key aspects of histology and BBT with regards to the diagnostic workflow. These are important to consider, in view of the potential "competition" for precious biopsy tissue that using both techniques entails. Histology, the current "gold standard", is highly accessible and provides a wider range of diagnostic information than BBT, but has key weaknesses that BBT may be able to redress. Histology appears to be more vulnerable to limited tissue sampling than BBT for a diagnosis of rejection [8,9]. It also suffers from interobserver variability, partly because its semi-quantitative scoring is vulnerable at threshold boundaries [10], whereas data from BBT may better reflect the gradual nature of pathophysiological processes. Many histological features lack specificity, for example tubulitis "t" and interstitial inflammation "i" are seen in TCMR but also in polyoma virus nephropathy (PVN); MVI is typical for AMR but is also seen in patients without a DSA; isolated "cg", "g", "v" or thrombotic microangiopathy (TMA) can be seen in AMR, but also in glomerulonephritis, TCMR or ischaemic injury [11]. We review below whether BBT can help identify rejection in these circumstances.

TRANSLATION TO CLINICAL PRACTICE: WHICH PLATFORM?

Rejection-associated molecular signatures have been noted with BBT using reverse transcriptase quantitative PCR (qPCR) [12–16], microarrays [4▪,17], RNA sequencing [6,18,19▪,20], and probe-based techniques such as Nanostring [9,21▪▪,22▪▪–24▪▪,25] or multiplex ligand-dependant probe-based assay (MLPA) [26▪▪,27]. In general, differential gene expression patterns first discovered using microarrays have been validated by qPCR and probe-based techniques, which provides evidence of robust gene expression changes in both AMR and TCMR.

Table 2 provides an overview of the key aspects of each of the gene expression analysis technique. An important consideration with respect to biopsy sample workflow, is whether the technique generally requires a good quantity of high-quality RNA extracted from a separate piece of tissue handled to preserve RNA (snap frozen or in an RNA preservative), or can be performed on the limited amount of fragmented RNA that can be extracted from formalin-fixed and paraffin-embedded (FFPE) tissue left over after standard histological diagnosis is complete. Probe-based techniques are designed to detect these degraded RNA species, by using multiple short probes [28].

The Molecular Microscope Diagnostic System (MMDx; Thermo Fisher Scientific, Waltham, MA, USA) is based on a DNA chip assessing 19 462-genes on fresh or RNA-later stored tissue [4▪]. Classifiers were derived from molecular phenotyping of large cohorts and prospective evaluation of real-life feasibility investigations [29]. It has a validated accuracy for the diagnosis of AMR and a weaker relationship with TCMR. Technical validity is demonstrated, although statistical measures of variability have not yet been reported [30]. The MMDx Kidney platform is licensed for commercial use as a send out test in a CLIA-approved laboratory as a laboratory developed test not requiring FDA approval for use in the USA. In Europe, the software is IVD-CE certified as a medical device. In terms of availability, it is based on a central assessment in Portland (Oregon) or Prague (Czechia) and provides results in 48 h after the sample is received. A report is generated with a probability of rejection (TCMR,

Table 1. Comparison of histology and biopsy-based transcripts

		Histology	Biopsy-based transcripts
Accessibility	Laboratory equipment	- Usual equipment	- Limited availability in standard pathology laboartories - Significant financial investment depending on the molecular assay
	Price	- Cost-effective	- Substantial reagent-related expenses depending on the molecular assay
	Time to diagnosis	- < 24 h	- > 48 h: RNA extraction, molecular analysis, data interpretation
	Storage	- Physical (FFPE blocks and stained slides) - Secure data storage (digital pathology)	- Frozen storage of RNA - Secure data storage
Tissue sampling		- Minimum sample size required for adequacy - Cortex and medulla - Potential for sample bias	- Less tissue required - Cortex or medulla - Less susceptible to sampling variation - May require full or portion of core to be removed from standard histology tissue examination pathway for RNA preservative (depending on the molecular assay)
Data management	Interpretation	- Pathologist expertise	- Bioinformatic expertise required
	Reproducibility	- Technical variability (sample preparation) across centres - High inter-observer and intra-observer variability for scores, less so for diagnostic categories - Guidelines available (Banff Classification for Allograft Pathology)	- Calibrated biological assays - Several platforms and gene panels/models available with unknown equivalence - No guidelines
Information provided	What is identified	- Histological features both generic and Banff lesion scores (semi-quantitative scoring)	- RNA expression levels (potentially before visible lesions occur) - Variable number depending in the assay, from a few to ~20 000
	Diagnosis	- Histological diagnostic categories, including nonrejection diagnoses (infection, glomerular disease, malignancies (e.g. PTLD), etc.) - Histological semi-quantitative scores (activity and chronicity, for all renal compartments)	- Molecular rejection - above or below threshold for rejection (any, TCMR, AMR), or probability of rejection (any, TCMR, AMR) - Other molecular scores, e.g. degree of acute injury, degree of chronic injury
	Other information delivered	- Prognostic -- related either to semi-quantitative scores, or diagnoses	- Prognostic - New layer of potential information: molecular insights, e.g. potential therapeutic targets, identification of new pathways of rejections
Clinical impact	Impact on diagnosis	- Extensive literature on histological diagnosis using Banff Classification	- Relatively recent studies - Scarce studies on applicability in routine practice
	Impact on patient management	- Therapeutic management currently based on Banff Classification	- No evidence for an improvement in kidney transplant survival based on clinical decisions enhanced with molecular data
Gold-standard	What value is to data obtained?	- Current gold-standard, though known limitations	- Complexity of proving value against known but in some cases flawed gold standard of histology - New layer of information from molecular phenotyping of biopsies could lead to data-driven refinement of diagnosis system

FFPE, formalin-fixed paraffin-embedded, PTLD, posttransplant lymphoproliferative disorder.

Table 2. Comparison of gene expression analysis techniques used for biopsy-based transcriptomics

Technique	Type of tissue sample	Maximum number of transcripts	Shortest realistic turnaround time	Cost	Advantages	Disadvantages	Validated for diagnostic use in transplantation
RT-qPCR	Usually requires RNA-later or Snap Frozen Tissue	Limited; depends on quantity if RNA	<8 h	Low per sample; Depends on number of target transcripts; requires staff, equipment, and expertise	Cheap, Easily Customisable	Genes need to be preselected, requires amplification step, not suitable for degraded RNA	No
RT-MLPA	Suitable for FFPE	60	<24 h	Low per sample; Depends on number of target transcripts; requires staff, equipment, and expertise	Cheap, Easily Customisable	Genes need to be preselected	No
Microarray	Usually requires RNA-later or Snap Frozen Tissue	47 000	48 h	High; As a send away test, does not require staff, equipment, expertise	Large selection of genes, suitable for Discovery Studies.	Requires a separate core of tissues, Not Suitable for Archived FFPE, currently available solutions require shipping to Central Lab	Yes (MMDx)
Nanostring	Suitable for FFPE	800	24–48 h	Intermediate; Depends on number of genes; requires staff, equipment, expertise	Suitable for Samples after routine histological processing or archived FFPE	Genes need to be preselected, access to Nanostring platform Required	No for Transplant (Yes in Cancer field)
RNA-Seq	Usually requires RNA-later or Snap Frozen Tissue	n/a Coding and noncoding RNA	Up to a week	High; Depends on number of reads	Whole Genome Sequencing, High Dynamic Range; suitable for discovery studies	Expensive, requires good quality RNA and library prep, data analysis time-consuming	No

FFPE, formalin-fixed paraffin-embedded.

AMR, or no-rejection) along with other molecular data such as acute tubular injury or atrophy-fibrosis scores. Potential use cases are identified, for example, "subpathological" AMR, DSA-negative MVI, polyomavirus nephropathy (PVN), isolated v-lesions, and so on. Some centres have started reporting on the clinical use of the MMDx platform [31,32,33▪▪].

Nanostring analysis of transplant biopsies using a consensus "Banff Human Organ Transplant" (B-HOT) panel or subsets of this panel have indicated ability to classify AMR and TCMR, both in silico and in retrospective cohort analyses [21▪▪,22▪▪, 24▪▪, 34▪,35▪,36]. The use of material left in the paraffin block after histological diagnosis is complete is a key benefit of this technology. The Nanostring platform is approved by FDA (USA) and IVD-CE (EU) certified; however, consensus for normalization across runs, platforms, and centres is needed to enable comparison of classifiers and prospective multicentre clinical trials testing clinical utility.

qPCR and MLPA are simpler, less expensive techniques that have also been investigated for use in transplant diagnostics. The latter can be performed using FFPE samples. Both use a subset of the genes in the signatures of rejection identified using microarray or NanoString technology. Both techniques yield gene expression results that correlate with results using Nanostring, but also require further validation in prospective clinical trials.

In our view, diagnostic gene expression panels for rejection with either a limited number of genes or the full transcriptome are both likely to yield sufficient accuracy to reach a molecular diagnosis of rejection and its subtypes. However, a restricted gene panel may also limit the number of questions that can be answered in any one given assay (e.g. diagnosis, prognosis, and so on). A diagnostic test might use additive or weighted scores of a handful of genes, or more complex machine learning-derived algorithms. Some institutions may be set up to validate cost-effective laboratory developed tests, whereas others might prefer to send samples away. Availability of a range of validated technologies for local clinical teams to choose from, depending on local expertise, equipment, and funding will enable widespread clinical implementation. However, it will be important to agree on acceptable performance metrics and determine if results of different molecular assays for the same indications are comparable.

TRANSLATION TO CLINICAL PRACTICE: WHICH INDICATIONS?

The existence of molecular signatures for different types of rejection is insufficient on its own to justify clinical use of BBT. BBT must also show clinical validity (add diagnostic or prognostic information to current practice) and clinical utility (cost-benefit analysis) [11,37].

The Banff Classification currently supports use of BBT only as an adjunct technique in the diagnosis of AMR, where transcripts "if thoroughly validated as substitute for MVI and available" can substitute for C4d positivity or MVI above threshold.

Potential future applications range from comprehensive use of BBT in all biopsies alongside standard histological assessment, to use only in a defined set of circumstances where BBT adds information to histology (Fig. 1). It is important to note that the assumption that BBT will be redundant in cases with obvious histological rejection, and useful in cases where histology is ambiguous, is incorrect, as outlined below and in Table 3. Discrepancies between histological and molecular rejection occur in around 35% of cases, more in TCMR than AMR [31]. Histology may be better than molecular for some indications (e.g. diagnosis of infection or glomerulonephritis) whereas molecular may ultimately be proven better for others [33▪▪].

Biopsy-based transcriptomics for antibody-mediated rejection phenotypes

The histological diagnosis of AMR requires integration of several histological lesion scores (g, ptc, v, TMA, cg, PTCML), additional diagnostic parameters (e.g. "in the absence of glomerulonephritis"), C4d, and DSA data. Many biopsies can show some but not all of these features, leading to "incomplete" AMR phenotypes, the significance of which is not yet fully understood, but which were clearly defined in the most recent Banff Report.

The assumption that biopsies with complete histological AMR will have increased AMR BBT is untrue. A proportion of cases with histological AMR are negative for AMR BBT and a proportion of cases without rejection are positive for AMR BBT, with overall discrepancies reported around 20% [24▪▪,31,38▪▪]. The lack of good treatment options for AMR makes it hard to compare histological and molecular definitions of disease for their ability to best stratify patients for treatment, or to potentially integrate both modalities for best diagnostic ability. Clinical trials that include molecular analysis are limited, and more are needed [39–44]. Molecular scores of AMR probability or of injury-repair response (IRRAT) in patients with histological AMR predict future eGFR or graft loss, providing evidence for prognostic (if not diagnostic) superiority of molecular analysis in this context [45▪]. Many potential explanations have been put forward for discrepancies

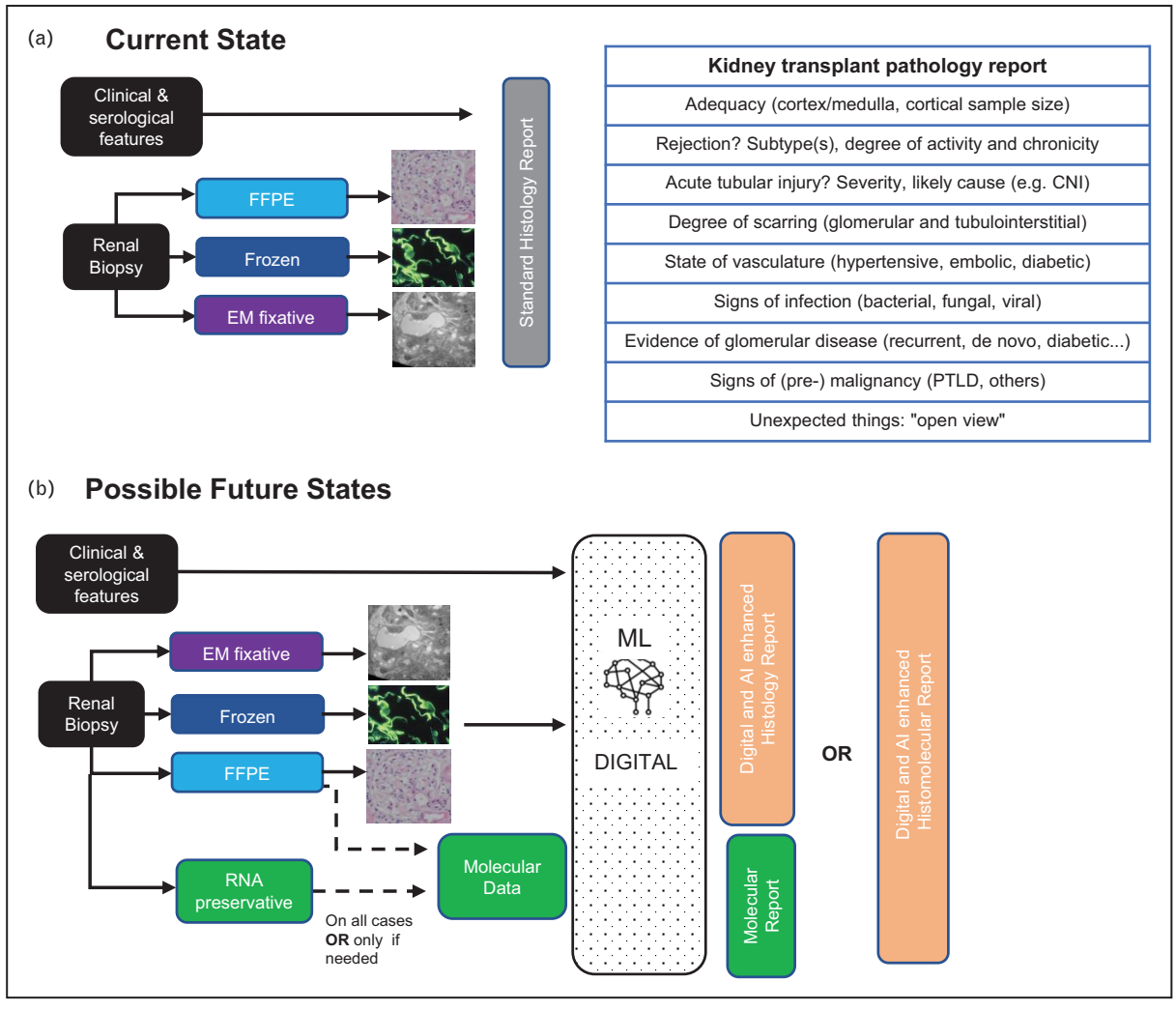

FIGURE 1. Potential clinical workflows integrating biopsy-based diagnostics. Current kidney transplant biopsy assessment is based on histological assessment including immunochemistry and electron microscopy (a). This open view approach enables the description of multiple parameters, including unexpected lesions, which are included in the pathology report. Future kidney transplant biopsy assessment may integrate biopsy-based transcriptomics systematically or in defined circumstances (b). We note that digital pathology and machine learning are likely to become part of the pathology workflow. CNI, calcineurin inhibitors; EM, electron microscopy; FFPE, formalin-fixed paraffin embedded; ML, machine learning; PTLD, posttransplant lymphoproliferative disorders.

between "histological AMR" and "molecular AMR", for example sampling of the biopsy tissue, poor application of Banff rules by pathologists [46], and inadequacy of current Banff rules. Interesting recent work suggests semi-supervised clustering of Banff lesion scores or logistic regression equations trained on molecular diagnoses of rejection might identify improved rules for histological diagnosis of rejection using Banff lesion scores [47▪▪,48,49▪▪].

BBT have also been investigated in incomplete AMR phenotypes, including cases that are "MVI-positive, C4d-negative, DSA-negative", and cases with histological lesions of AMR below threshold, either with a DSA ("probable AMR") or without a DSA (isolated v, g, cg, and TMA).

MVI-positive, C4d-negative, DSA-negative cases have been variably attributed to inability to detect circulating anti-HLA DSA, non-HLA DSA, other causes of NK-mediated rejection (including "missing self"), or ischemia-reperfusion. The hope is that BBT might enable classification of this phenotype according to pathophysiology, potentially facilitating treatment. In reality, studies examining differences in transcript expression between MVI-positive biopsies with and without detectable DSA have not shown any differences [5,17,50].

We used a 9-gene molecular AMR classifier to separate 50 biopsies with incomplete histological features of AMR into six biopsies with molecular

Table 3. Potential clinicopathological scenarios where biopsy-based transcriptomics might help with diagnosis

Banff Classification Category	Banff Classification sub-category	Histological challenge	How would biopsy-based transcriptomics help?	References
Normal biopsy or nonspecific changes		Banff lesion scores below t hreshold for rejection (TCMR or AMR)	Is there molecular TCMR? Is there molecular AMR? How severe is the injury-repair response?	[15,24▪▪,26▪▪]
		DSA-positive, negative histology	Is there molecular AMR?	[22▪▪,24▪▪,53]
		Acute tubular injury	How severe is the injury-repair response?	[45▪]
Antibody-mediated rejection	Active AMR	Probable AMR (DSA+ with g1, ptc1, v or TMA)	Is there molecular AMR?	[24▪▪]
		MVI+ DSA- C4d-	Is there molecular AMR? Can molecular findings distinguish causes of MVI (e.g. HLA antibody, non-HLA antibody, missing self, etc)?	[17,24▪▪,50]
	Chronic/chronic active AMR		Degree of injury-repair response? Degree of chronicity?	[45▪,61]
	C4d staining without evidence of rejection		Is there molecular AMR?	[13]
Borderline for TCMR			Is there molecular TCMR? Is there molecular AMR?	[15,31,62]
TCMR	TCMR grade II and III	Presence of endarteritis	Is there molecular TCMR? Is there molecular AMR?	[52]
	Chronic-active TCMR	i-IFTA lesion	Is there molecular TCMR? Is there molecular AMR? How severe is the injury-repair response?	[54]
Mixed AMR and TCMR			Is there molecular TCMR? Is there molecular AMR?	
IFTA NOS		Scarring? cause	Is there molecular TCMR? Is there molecular AMR? How severe is the injury-repair response?	[54]
Other findings	Polyomavirus nephropathy		Molecular identification of BK virus Is there molecular TCMR?	[56,57]
	Glomerulonephritis		Is there molecular AMR?	

AMR, antibody-mediated rejection; DSA, donor specific antibody; g, glomerulitis; MVI, microvascular inflammation; ptc, peritubular capillaritis; TCMR, T-cell mediated rejection; TMA, thrombotic microangiopathy; v, intimal arteritis.

AMR and 44 biopsies without molecular AMR. The six biopsies with molecular AMR had MVI of 1 or more and a worse outcome, similar to that of AMR. Cases with isolated v or TMA were negative for molecular AMR in our study. Other studies with isolated v-lesions have not consistently identified a molecular signature associated with TCMR or ABMR, and data are generally lacking for cases with TMA as the main diagnostic feature [24▪▪,51,52]. These data suggest that molecular AMR is mainly driven by MVI, which is not surprising, as it is the most frequent histological finding in AMR.

Finally, some patients with a DSA have no histological features of rejection, and BBT might provide an opportunity for earlier diagnosis in such patients with high immunological risk. Indeed, a proportion of these biopsies may have molecular AMR [53], but it remains unclear whether these patients are more at risk of developing histological rejection at a later stage, or if treatment of rejection would alter outcomes.

Biopsy-based transcriptomics for T-cell mediated rejection phenotypes

TCMR comprises active TCMR and chronic active TCMR (caTCMR) in the Banff Classification. As for AMR, discrepancies have been found between histological TCMR and molecular TCMR, with little evidence for which is superior to the other. Ideally, BBT might help in circumstances where histology is problematic, namely borderline for TCMR, PVN versus TCMR and caTCMR.

caTCMR refers to cases that where the interstitial inflammation component mainly affects the scarred areas of the biopsy. Whilst inflammation in areas of atrophy is associated with allograft loss, it can be due to TCMR or to other pathological processes such as infections [37]. BBT show that such biopsies show a variety of molecular signatures, and in fact have molecular AMR in 45% of cases and molecular TCMR in 16% of cases according to the MMDx system [54].

Similarly, biopsies that are borderline for TCMR have shown a range of molecular features. In a study using MMDx, 74% of biopsies borderline for TCMR had a no-rejection gene signature, 13% had a molecular signature of AMR, and only 9% had a molecular signature of TCMR [31]. In another study, a molecular tubulitis score in an early borderline biopsy predicted patients at risk for molecular rejection in a later follow-up biopsy [55]. Data (in particular, clinical trial data) are lacking on whether different molecular signatures correlate with different response to different treatments.

PVN and TCMR both show tubulitis and interstitial inflammation and are distinguished by looking for evidence for polyomavirus replication in urine, blood, or within the kidney tissue (nuclear inclusions, SV40 immunostaining). It has also been shown that BBT can identify polyomavirus-specific transcripts, supporting PVN diagnosis in contentious cases [56]. However, as TCMR and PVN share a common pathophysiological pathway of antigen-driven T cell activation, assessment of the co-occurrence of these two pathological processes using BBT is limited. Moreover, more data are needed to better understand the evolution of the molecular signal of polyomavirus in order not to misidentify a de-novo TCMR following PVN resolution [57].

CONCLUSION

Extensive investigations using bulk transcriptomics over the last few decades have enabled a better understanding of rejection, and future use of more powerful platforms for single cell and spatial transcriptomics will no doubt increase that output. Translational research has established that BBT can detect rejection with good accuracy compared to a histological "gold standard". Frequent discrepancies between histology and transcriptomics suggest potential for synergy between the two techniques, although to date, the indications where BBT adds value are unclear. Only a few centres use biopsy-based transcriptomics [58], and key barriers to wider use include comparability of data and models across centres and platforms; defined and tested cost-effective contexts of use, and guidelines for integrating information from standard histology and BBT. It is important to stress that there are many tasks for which BBT cannot replace histology, and this has implications for how we select portions of precious biopsy samples for best diagnostic yield. Digitization of standard histology enhanced by machine learning is likely to be synergistic with BBT, but has potential to supplant BBT as a diagnostic tool, as has already been noted in the field of cancer [59].

Panels and tools for molecular diagnosis have been defined and tested for AMR, although prospective clinical trials that investigate their clinical utility when used in addition to standard histology are needed. As effective AMR treatments are currently limited [60], BBT may not have that much impact on allograft survival. Nevertheless, BBT has potential to help define specific molecular pathways as therapeutic targets.

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Conflicts of interest

There are no conflicts of interest.

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