Beyond Histology: Novel Tools to Diagnose Allograft Dysfunction

Roslyn B. Mannon and Allan D. Kirk

Transplantation Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland

Kidney biopsy is the gold standard procedure for the assessment of allograft dysfunction. The differential diagnosis for both acute and chronic dysfunction can encompass a number of different causes, and a biopsy frequently can suggest a specific cause. However, many of these causes are difficult to distinguish on morphologic basis alone, and the information that is obtained from a biopsy is limited with regard to functional and prognostic importance. Additional methods therefore are needed to guide the diagnosis and the treatment of allograft dysfunction, and numerous methods have been studied. Potential markers include protein and gene expression profiles in the peripheral blood, the urine, and the graft itself, all compartments that are relevant to the alloimmune response. Recent comprehensive sequencing of the human genome has led to an unprecedented opportunity to develop these genetic and proteomic techniques, and ongoing evaluations of potential tests have led to an improved understanding of the complexity of immune responses. The future challenge for promising tests is validation in larger patient populations to facilitate their addition to the diagnostic armamentarium.

Determination of the cause for renal allograft dysfunction can involve a variety of diagnostic maneuvers and frequently necessitates an allograft biopsy. Histologic assessment of the graft is considered the gold standard for most diagnoses, and the criteria for diagnosis of transplant-specific graft pathologies have been established by consensus (1). These criteria have been revised with the recent appreciation of the impact of antidonor antibodies on graft injury (2), and studies suggest that humoral immunity may be a prevalent component in both acute and chronic graft injury (3).

As the transplant field has matured, it has become clear that the differential diagnosis for both acute and chronic dysfunction can encompass many causes (4), and biopsy can suggest specific causative agents such as hypertension, calcineurin inhibitor toxicity, recurrent disease, BK polyomavirus infection, and antibody- and cellular immune-mediated injury (5). Moreover, recent studies suggest that inflammation can exist in the graft without clinical evidence of disease, so called “subclinical rejection” (SCR), and this also may contribute to late graft loss (6,7). However, many of these causes are difficult to distinguish on morphologic basis alone, and the information that is obtained from a biopsy is limited with regard to functional correlation and prognostic importance. Therefore, identifying additional diagnostic methods is a critical task that the clinician faces.

An added clinical challenge is identifying the proper timing for graft biopsy, because the procedure is invasive and incurs expense. In many centers, a persistent 15% rise in serum creatinine or proteinuria that exceeds 0.5 g/d will trigger a biopsy. However, these reactive strategies may fail to detect injury at its inception. The relative lack of sensitivity of serum creatinine in detecting dysfunction has been discussed extensively (8), and there is incomplete information regarding the sensitivity and the impact of alternative prospective monitoring strategies on graft outcomes. Therefore, it is essential to identify additional tools to assess the state of the allograft in real time to prompt timely histologic evaluation.

This review highlights recent advances in the characterization and the detection of human renal allograft injury (Table 1). Most of these assays have yet to be validated and prospectively tested in large patient populations. However, they show promise and provide a platform for further clinical investigation.

Graft Injury: What Is Available for Evaluation?

Alloimmunity should be considered a systemic response that involves primary (thymus and spleen) and secondary (nodes) lymphoid tissues, peripheral and intragraft vasculature, parenchymal cells, and the inflammatory mediators that are required for cellular trafficking and communication (Figure 1). Rejection thus induces systemic inflammation that is proportional to the extent of the immune response. Despite the systemic nature of renal allograft rejection, relatively few sites are suitable for clinical evaluation, namely the peripheral circulation, the allograft, and the urine. The allograft is unique in its concentration of inciting and targeted components and provides a site that is rich for analysis. However, given the trafficking of immune cells to and from immune organs and the liberation of cytokines, chemokines, and other soluble factors that are associated with alloimmunity, the peripheral circulation remains attrac-
Table 1. Modalities to assess kidney allograft dysfunction

<table>
<thead>
<tr>
<th>Peripheral Serum</th>
<th>Peripheral Cells</th>
<th>Urine</th>
<th>Graft</th>
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<tr>
<td>Acute-phase reactants</td>
<td>LDA, MLR, CTL, ELISpot</td>
<td>PI-9, IP-10, CXCR3</td>
<td>immunophenotyping</td>
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<td>Cytokines</td>
<td>lymphocyte-stimulated ATP</td>
<td>proteomics</td>
<td>genomics: RT-PCR, microarrays</td>
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<td>flow cytometry: immunophenotyping, tetramer analysis</td>
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<td>Soluble CD30</td>
<td>genomics: RT-PCR, microarrays</td>
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*ELISpot, enzyme-linked immunospot; LDA, limiting dilutional analysis; MLR, mixed lymphocyte culture or response; RT-PCR, reverse transcription–PCR; CTL, cytotoxic T lymphocyte.

Assays of Lymphocyte Alloreactivity and Phenotype: Are They Helpful?

Nearly 30 yr ago, techniques were established to estimate the frequency of specific cytotoxic T lymphocyte precursors, including limiting dilutional analysis (16) and mixed lymphocyte culture or response (MLR). Early studies demonstrated hypersensitivity measured by MLR to donor HLA antigens in long-term surviving renal transplant recipients, but in general, the MLR has failed to yield clinically useful prognostic information (reviewed by Reinsmoen et al. [17]). The utility of these tests has focused on predicting graft outcomes by correctly surmising that elevated precursor frequencies increase the likelihood and import of an alloimmune response. Reinsmoen et al. (17) noted that reduced donor-specific precursor frequencies in kidney recipients correlates with lower rates of chronic allograft nephropathy (18). Patients with low limiting dilutional analysis values also enjoy improved long-term graft survival rates. However, although these assays may provide some prediction and risk stratification over a prolonged transplant course, they have not been used extensively in the setting of detecting the presence of allograft injury as a result of rejection, where in small study populations they seem to be elevated (19,20).

A less cumbersome and challenging method for precursor frequency analysis is the enzyme-linked immunospot (ELISpot) detection. ELISpot, a variation of the standard ELISA that detects cytokine that is produced by individual antigen-specific cells, was studied as a technique for immune monitoring after transplantation (21). This assay provides information on both cell frequency and cytokine function. Before transplantation, high frequencies of donor-reactive interferon-γ-producing cells have correlated with posttransplantation acute rejection (ACR) episodes (22). Serial monitoring after transplantation also has identified recipients who are at risk for ACR, with worse renal function in those with higher posttransplantation frequencies of both donor and third-party responses (23). Measuring indirect alloreactivity in this assay to donor HLA DR peptides also has
demonstrated a correlation between higher frequencies of IFN-γ-producing cells in patients with previous ACR episodes compared with clinically stable patients (24). Although this tool has not been implemented to measure the extent of alloreactivity at the time of acute graft dysfunction, it may be a useful adjunct to biopsy. Given the high individual variability in ELISpot readings, the test may be most useful for stratifying rejection risk preoperatively or in selecting patients who are in need of specific therapies such as depletional induction. Further testing in larger populations is under way to establish its role in immune therapy management.

Although flow cytometry also has been used to identify bulk lymphocyte subpopulations for decades, recent advances have made this an assay worth revisiting. The development of polychromatic flow cytometry (analysis with 10 to 20 distinct colors) now allows for the fine identification of cell subpopulations with known functional differences, e.g., naïve, regulatory, memory, effector (25). Moreover, this kind of analysis unambiguously identifies small subsets of rare lymphocytes, even when peripheral pools are depleted by induction therapy. As we become increasingly aware of the importance of regulation, memory, and heterogeneous activation in transplantation, fine phenotype analysis may provide clinically useful information. Indeed, this technique helped to identify a population of effector memory cells that is resistant to depletion after Alemtuzumab induction and is responsible for rejection events when this agent is used as monotherapy (26). Identifying a lymphocyte phenotype that is associated with rejection or tolerance...
using this technique could provide a relatively noninvasive method for identifying those who are at risk for developing rejection.

Even finer identification of cells with defined antigen specificities also may be possible using tetramer analysis. In this technique, the peptide-MHC complex ligand for a given population of T cells is multimerized to make soluble peptide-MHC tetramers that retain their tertiary structure and can bind specifically to T cell receptors that are specific for the given peptide-MHC or allo-MHC (27). When fluorochrome labeled, they bind in an alloantigen-specific manner and can be assessed by flow cytometry. This technique has been exploited in illnesses with limited and defined antigenic spectra such as autoimmune (28) and viral diseases (29). However, this has not been adopted for immune monitoring after solid-organ transplantation and may be somewhat cumbersome in that unique tetramers are needed for each donor/recipient combination and for direct versus indirect immune responses.

### Other Assays of Peripheral Cell Function: The More the Merrier?

Another technology that is being explored for immune monitoring is the detection of ATP levels in peripheral lymphocyte populations. In this assay, whole blood is incubated with specific or polyclonal stimulators, and the degree of cellular activation is measured by increases in intracellular ATP content detected by chemiluminescence. Cross-sectional analysis of kidney transplant recipients \( n = 127 \) from three US transplant centers has demonstrated that mean ATP production in CD4 T cells in healthy adults (432 ng/ml) is significantly higher compared with that in transplant recipients (282 ng/ml) (30). Zones of reactivity have been established in this relatively stable population, suggesting \( > 525 \) ng/ml as a “high responder” and \( \leq 225 \) ng/ml as a “low responder.” There was no significant effect of center, induction strategy, maintenance therapy, or age (31) on ATP measurements. This assay, if validated in prospective study, may provide a useful method for quantifying the functional extent of immunosuppression and may provide a regimen-insensitive indicator of global immunosuppression to guide therapeutic decision-making.

A number of investigators also have studied gene expression in peripheral blood mononuclear cells (PBMC) to identify a pro-rejection profile for use as a noninvasive diagnostic tool. Improvements in PCR techniques, including the advent of real-time PCR, have markedly improved the quantitative abilities of this sensitive assay. A number of different gene transcripts have been studied, including most often those that are related to cytotoxic T cells. In an early study, combined upregulated expression for perforin and FasL genes in peripheral blood lymphocytes that was detected by reverse transcriptase–PCR (RT-PCR) had high positive predictive value for clinically evident, biopsy-proven ACR (32). The absence of upregulation in at least one of these genes had a 95% negative predictive value of rejection. Using real-time PCR, Sabek \( et \ al. \) (33) similarly found elevated granzyme B transcripts in PBMC from a small group of recipients with biopsy-proven rejection compared with those with other causes of allograft dysfunction that necessitated a graft biopsy as well as HLA-DR, distinguishing rejection from chronic injury, ATN, and toxicity. Others have noted increased transcription of Th1 and Th2 cytokines associated with rejection (34). Potential predictors of rejection have included increased transcript levels of IL-18 (35), IL-13 and IL-5 (36), and IL-4 and TNF-α (37). Although these results are interesting and may have some biologic basis, this method has not been tested in large transplant populations on a variety of therapies or with clinically inapparent injury. The impact of depletion antibody agents, moreover, may have a deleterious impact on the ability to assay PBMC during periods of relative lymphopenia (38,39).

### What Can Urine Tell Us?

The urine seems to be an obvious choice for evaluating immune activity in the organ of its synthesis. A number of centers have explored RT-PCR detection of gene transcripts that are relevant to immune activation markers, using RNA from cells that are excreted into the urine. These observations include significantly increased levels of granzyme B and perforin, two cytotoxic effector molecules, in cells that were derived from rejection-associated urine compared with samples that were obtained in the absence of ACR, chronic allograft nephropathy, toxicity, or acute tubular necrosis (40). Other markers of the cytotoxic T cell pathway that are overexpressed in urine pellets during ACR episodes include the serine proteinase inhibitor-9 (PI-9), a natural antagonist of granzyme B (41), and CD103, expressed on alloreactive cytotoxic CD8+ T cells (42). Along this theme, protein and transcript expression of the IFN-γ-inducible chemokine IP-10 and the chemokine receptor CXCR3 are elevated in the urine sediments of recipients with ACR (43), with variable predictive characteristics depending on the cutoff values used.

Prospective real-time evaluation may be more promising as recently demonstrated by Kotsch \( et \ al. \) (44), who assessed multiple cytotoxic markers in urine samples simultaneously and prospectively in kidney transplant recipients who received anti-CD25 induction therapy. Enhanced urinary transcript expression for CD3, granulysin, and RANTES were significantly elevated during rejection episodes, and prospective sampling suggested that granulysin expression most frequently preceded the development of ACR.

Urinary FOXP3 also has been associated with biopsy-proven rejection (45). Given that this is a T cell activation marker that also is associated with regulatory T cells, its presence has been suggested to relate to both the presence and the control of rejection. FOXP3 transcripts indicate rejection, but as its presence increases, the rejection is proportionally easier to reverse. As with all other studies, these data require confirmation in larger patient populations on a variety of therapies, including lymphocyte-depleting agents. Moreover, the ability of these assays to predict rejection uniformly and the effects of antirejection therapies on these assays also are not known and require further investigation.
Urine Proteomic Approach

An alternative to the transcriptional approach is the use of proteomics. This entails separation of proteins into small fractions and, after peptic digestion, analyzing these fragments using chromatography and mass spectroscopy (reviewed by Traum and Schachter [46]). Technically more challenging than transcriptional assays, many proteins that are detected by proteomic screening are of unknown identity and function, and their mechanistic relationship to rejection can only be inferred. Whereas proteomic approaches have been used in other fields of human disease, it has only recently been considered in transplantation. In comparisons between 17 biopsy-proven rejection episodes and 15 biopsies from recipients with stable graft function, proteomic analysis has identified two protein peaks of 10 and 3.4 kd that could distinguish the two groups with specificity of 100% and sensitivity of 83% (47). However, the identity of these proteins has not published, and further confirmation using a larger patient data set has not been performed. Another comparison of stable function and rejection recipients used an algorithm of seven different protein masses with sensitivity of 91% and specificity of 83% (48).

Most recently, Nickerson and colleagues (49) carefully analyzed urine profiles on patients with biopsy-proven rejection, stable function, and acute tubular necrosis, as well as urine from healthy individuals. They identified three breakdown products of β₂-microglobulin that were present in all rejection samples but in only four of 22 stable function samples and were not present in ATN samples or samples from healthy individuals (50). Whereas serum β₂-microglobulin levels have been used as a more sensitive indicator of graft function for many years (51), they are increased in other settings of graft dysfunction and infection (52) and therefore may have limited clinical utility. It remains to be seen whether measuring urinary levels will have a diagnostic or prognostic impact in identifying the cause of graft dysfunction.

What More Can the Biopsy Tell Us?

Although allograft biopsy is the best current technique to identify the cause of renal dysfunction, there are circumstances in which it provides limited information about the clinical impact and prognosis of a process. These situations include the presence of inflammatory cell infiltrates in biopsies that are unassociated with tubulitis, distinguishing BK polyomavirus disease from acute cellular rejection, and distinguishing the causes of chronic allograft nephropathy. Similarly, no histologic assay has been able to distinguish functionally significant rejection from SCR. Finally, the contribution of antidonor antibody in both acute and chronic settings now is increasingly recognized as important in initiating and propagating graft injury, even in the absence of a concomitant cellular infiltrate. Whereas the presence of circulating alloantibody may be detected through a variety of newer in vitro techniques, staining of the graft endothelium for C4d has revolutionized the concept of antibody-mediated injury (reviewed by Colvin and Smith [53]). The histologic classification of these injuries also was revised to accept the notion that a number of different histologic lesions, in the proper context, indicate alloantibody participation (2).

The pathobiology of antibody-mediated injury remains poorly defined. Combining molecular techniques with prospective measures of alloantibody in concert with histology should lead to a new understanding of this entity.

One initial approach to fortifying the information that is available from biopsies has been to characterize the immune surface phenotype of graft-infiltrating cells by immunohistochemical staining. The predominance of activation markers such as CD25 and proliferation markers such as Ki-67 have been associated with rejection episodes (54) as have increases in CD8⁺ and CD45RO⁺ cells (55) and CD103 expression in infiltrating CD8⁺ T cells (56), but under closer scrutiny, particularly when evaluating repeated rejection episodes after therapy and viral infections, these markers may not be clinically reliable. The propensity of renal tubular epithelium to express HLA-DR when under immune insult has long been recognized as a means to identify immunologically active infiltrates (57). Indeed, this marker is regularly used to assess atypical infiltrates and is particularly useful in assessing lymphopenic rejections after depletional induction (38). There also has been a resurgence of interest in quantifying CD14-expressing monocytes as a relevant component of damaging infiltrates. These cells are associated with more aggressive infiltrates (58) but typically have been dismissed in favor of lymphocyte evaluation. However, with the recent increase in depletional induction trials, the role of the monocyte as an important mediator of injury has risen in importance (38) and is increasingly being recognized as relevant in nondepletional rejection (59). Similarly, the B cell was identified recently as playing a potential role in cellular rejection. Perhaps by serving as an antigen-presenting cell, aggregates of B cells have been shown to correlate with more severe cellular rejections (60).

The advent of PCR has allowed investigators to interrogate infiltrating cells in situ to assess their unique properties and exploit their potential diagnostic relevance. A number of groups have focused on identifying the unique molecular signatures of different forms of allograft dysfunction. Early efforts used non–real-time RT-PCR, demonstrating upregulated intra-grant levels IFN-γ, IL-7, IL-10, and IL-15 with acute cellular rejection, with a reduction in gene expression associated with successful treatment (61,62). Upregulation of intra-grant granzyme B and perforin also has been seen, again supporting the role of the cytotoxic T cell in allograft rejection (63). This observation also has been confirmed using real-time technology with high sensitivity (90%) and specificity (74%) for granzyme B as well as FasL (64). Moreover, the presence of enhanced expression of FasL more often was associated with therapeutic resistance to treatment.

With the recent sequencing of the human genome, there has been an explosion of transcriptional information in essentially every field of medicine. This has led to several exploratory studies that have analyzed rejection using microarray analyses. Using high-density microarray analysis, several groups have identified expression patterns that are associated with particular histologic types of rejection and varying outcomes (65,66). This method allows for the interrogation of several thousand genes simultaneously and is an increasingly robust and repro-
ducible technique. In contrast to real-time PCR, it is less sensitive, requiring higher quality RNA samples, and may not detect low-level responses. Recent investigations with this technique have demonstrated a molecular heterogeneity of allograft rejection, with differences detected by transcription that are not evident by light microscopy alone (66). These transcriptional differences were manifested by clinical differences of steroid responsiveness, success of return to baseline serum creatinine, and the presence of CD20 B cells, particularly problematic in steroid-resistant rejection episodes. Moreover, rejection profiles, despite their heterogeneity, still were distinguishable from chronic injury or toxicity. Therefore, the variable clinical behavior of histologically similar rejections is supported by the apparent transcriptional quantitative and qualitative differences.

Expanding on this work, Flechner and Salomon (65) examined transcripts using gene chips in both PBMC and biopsy tissue in a series of transplant recipients with stable function at 1 yr and ACR, as well as from living kidney donors. They demonstrated that despite normal histology, stable function grafts are transcriptionally unique to native kidney tissue at the time of donation. In addition, they identified a unique transcript set in ACR versus stable function and verified these findings using real-time PCR, and a number of these transcripts have not been reported on before. Expression in PBMC also paralleled that of biopsies, suggesting that a noninvasive but comprehensive assessment of gene expression might be a useful monitoring tool. Most important, these studies have provided a test set of targets to allow a number of laboratories to verify their technique. To date, a large volume of microarray data have been obtained on a relatively small number of patients, making it difficult to discern the reproducibility of the findings. Furthermore, microarrays have limited quantitative ability, particularly in low copy number transcripts, and remain relatively expensive. Therefore, it is not clear whether this approach will exit the realm of exploration to become a diagnostic tool.

Several groups now have combined the improved expense, speed, and quantitative capabilities of real-time PCR with the rapidly increasing availability of candidate transcripts to produce rapid-turnaround platforms for analysis of fixed sets of 20 to 100 genes. These low-density arrays specifically limit the assessment of expression to a set of gene transcripts as opposed to a more global interrogation. Such arrays are commercially available and may be customized to the setting under study. The isolation of RNA itself is a standard technique, and the method is clinically feasible to provide a high-quality template for analysis (67). The use of such expression data has demonstrated a number of unique findings in the understanding of the pathobiology of kidney transplantation in multiple settings, including ischemia reperfusion injury (68,69), stable function, ACR, SCR (70), and BK nephropathy (71). On the basis of these studies, specific transcriptional patterns that can be produced with a clinically applicable turnaround similar to that for standard histology have been identified. As such, it now is feasible to supplement biopsy information with contemporaneously available transcriptional analysis on a routine basis.

Focused PCR studies now have emphasized the marked alterations in transcription that can be detected despite minimal histologic change. For example, kidney grafts within 15 min of reperfusion demonstrate significantly increased expression of proinflammatory cytokines such as TNF-α and IL-10, and intercellular adhesion molecules ICAM-1 and A20, without significant morphologic changes (69). These molecular changes are associated with delayed graft function and ACR, suggesting a potential predictive strategy for recipient management. Similarly, 1 h after reperfusion, transcripts for chemokines, apoptosis, and monocyte markers are noted without evidence of T cell activation, suggesting a primary role for monocytes in ischemic injury (68). Therefore, histologically bland tissue can exhibit marked transcriptional activity and, under additional clinical scrutiny, may indicate new strategies for management of high-risk grafts.

Although the use of protocol kidney biopsies has been debated, SCR can be detected in anywhere from 4 to 51% of biopsies (reviewed by Bohmig et al. [72]). Therefore, while the typical histologic features of rejection are present, the functional features of acute cellular rejection are absent, and the impact and classification of these findings remain unclear. Sustained SCR in repeated protocol biopsies has been associated with long-term graft dysfunction (73). Moreover, corticosteroid treatment in the setting of SCR has been associated with a reduction in late rejection episodes and serum creatinine at 2 yr after transplantation (7). A functional clue to this has been demonstrated using low-density arrays to assay 72 immune-related transcripts in biopsies from recipients with stable function and clinical ACR and those with SCR (70). In SCR biopsies, there is marked transcriptional activity similar to that seen in ACR and elevated compared with stable function biopsies. Expression of the T cell transcription factor T-bet, FasL, and CD152 most prominently distinguish ACR from SCR. The molecular signatures of ACR and SCR are qualitatively similar but quantitatively very different, demonstrating that histology alone may be incomplete in assessing the clinical impact in the setting of SCR. Moreover, the infiltrates that are seen in SCR do not have a specific regulatory signature that is different from ACR. Finally, this study demonstrates that allograft rejection cannot be equated by immune cell infiltration alone, and the patient’s stability cannot be equated with immune inactivity.

Graft loss as a result of BK polyomavirus nephropathy (PVN) has been a considerable problem in the past decade. Although the characteristic viral cytopathic changes on graft biopsy are diagnostic of disease (74), these often are accompanied by an inflammatory cell infiltrate and tubulitis that are histologically indistinguishable from those seen in ACR. However, as Mannon et al. (71) recently demonstrated, gene expression in PVN graft biopsies has qualitative and quantitative differences as well as striking similarities compared with ACR. For example, the extent of CD8 cytotoxic T cell transcription such as perforin, IFN-γ, and CD8 were quantitatively more intense in BK PVN than in ACR biopsies. Moreover, transcripts that are associated with the induction of graft fibrosis and epithelial mesenchymal transformation are markedly upregulated in BK PVN compared with ACR. Therefore, the inflammatory immune response to BK has functional differences that are seen at the level
of transcription and are not appreciable using standard histologic techniques. Furthermore, the use of noninvasive urine studies to measure cytotoxic markers would not be particularly helpful in distinguishing BK PVN from ACR because of the intense cytotoxic response that is present within the graft. These transcriptional differences also may explain the substantial association of BK PVN with late graft loss.

Conclusion
Kidney biopsy with histologic evaluation remains the best readily available modality for the diagnostic evaluation of graft dysfunction. Identifying noninvasive monitoring tools and also indicators that can corroborate disease status are lacking but needed. Current noninvasive testing of urine and serum has generated interesting associations. Genetic approaches to graft biopsies also have demonstrated some unique insights into the immunobiology of graft inflammation and also have shown that clinical quiescence may not be matched by transcriptional inactivity. Universal utilization of such testing will require assessment of more patients in a prospective manner. Moreover, this may entail the use of surveillance biopsies after transplantation. Although noninvasive techniques may be more easily accepted, correlating these results in large patient populations is needed. Efforts should be made to pair novel tests with clinical trials that involve close patient monitoring and protocol biopsy so that these assays can be validated and become part of our diagnostic armamentarium.

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References


