New Approaches to the Diagnosis of Rejection and Prediction of Tolerance in Liver Transplantation

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Abstract. Immunosuppression after liver transplantation is essential for preventing allograft rejection. However, long-term drug toxicity and associated complications necessitate investigation of immunosuppression minimization and withdrawal protocols. Development of such protocols is hindered by reliance on current paradigms for monitoring allograft function and rejection status. The current standard of care for diagnosis of rejection is histopathologic assessment and grading of liver biopsies in accordance with the Banff Rejection Activity Index. However, this method is limited by cost, sampling variability, and interobserver variation. Moreover, the invasive nature of biopsy increases the risk of patient complications. Incorporating noninvasive techniques may supplement existing methods through improved understanding of rejection causes, hepatic spatial architecture, and the role of idiopathic fibroinflammatory regions. These techniques may also aid in quantification and help integrate emerging -omics analyses with current assessments. Alternatively, emerging noninvasive methods show potential to detect and distinguish between different types of rejection while minimizing risk of adverse advents. Although biomarkers have yet to replace biopsy, preliminary studies suggest that several classes of analytes may be used to detect rejection with greater sensitivity and in earlier stages than traditional methods, possibly when coupled with artificial intelligence. Here, we provide an overview of the latest efforts in optimizing the diagnosis of rejection in liver transplantation.

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INTRODUCTION

Excellent long-term outcomes can be achieved in liver transplant (LT) recipients, with approximately 75% graft and patient survival at 5 y after transplant depending on the underlying cause.[1] One of the key factors is the introduction of calcineurin inhibitor-based immunosuppression (IS) in the 1980s, which has become the standard of care for most transplant recipients.[2] However, acute rejection occurs in 15% to 35% of LT recipients in the first 2 y after transplant.[1,3,4] Typically, rejection episodes can be managed with increased IS, such as additional steroids or mammalian target of rapamycin inhibitors, higher calcineurin inhibitor or antimetabolite (eg, mycophenolate) doses, or lymphocyte depletion. Although adequately treated, early rejection episodes likely do not lead to long-term problems, repeat episodes of rejection are best avoided. As such, lifelong IS has become standard practice and is one of the main drivers of late morbidity and mortality after LT, including renal dysfunction, metabolic syndrome, neurotoxicity, infections, or posttransplant lymphoproliferative disease and other malignancies.[5]

Maintaining adequate graft function requires close surveillance and early intervention for acute rejection episodes. After ruling out anatomic or surgical causes of graft dysfunction, that is, biliary or vascular complications, evaluating causes of allograft injury generally requires a liver biopsy. In this article, the current standard-of-care approach to the diagnosis of acute cellular and antibody-mediated rejection after LT will be reviewed, and emerging noninvasive assays will be discussed further in detail (Table 1). As the utility of these new approaches in the diagnosis of chronic rejection after LT has not been studied in detail, the review will focus on acute rejection.

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TABLE 1.

Selected articles discussed in the review

Summary of new approaches to diagnosis of acute rejection in liver transplantation

Method	Reference	Findings
miRNA profiling	6	Identification of a profile of 31 miRNAs significantly associated with ACR and demonstrated that hsa-miR-483-3p and hsa-miR-885-5p-65 can be used to distinguish ACR with 0.72 PPV and 0.93 NPV.
	7	miR-122-5p, miR-194, miR-133a, and miR-148a-3p significantly elevated in bile of recipients who developed acute rejection. miR-122, miR-148, and miR-194 levels were demonstrative of rejection with 0.9, 0.53, and 0.84 PPV, respectively.
	8	miR-18b, miR-340, and miR-106b significantly upregulated in PBMCs of stable recipients relative to recipients with acute rejection.
Transcriptional profiling	9,10	Identification of a transcriptional profile significantly altered in recipients who developed acute rejection and downregulated in patients who never developed acute rejection. The 2020 study profile had PPV of 0.47 and NPV of 0.87, and 2021 study profile had PPV of 0.54 and NPV of 0.89.
Immunophenotyping	11	Peripheral natural killer cells and Treg elevated in stable recipients relative to recipients with acute rejection and healthy volunteers.
	12	Observed strong reexpression of naive T-cell phenotypes in recipients with acute rejection.
	13	Early reduction of Treg under tacrolimus-based immunosuppression with basiliximab induction associated with development of acute rejection.
Cytokine quantification	14	IL-10, IL-17, and CXCL10 measured 1–2 wk after transplant operation can be used to predict acute rejection.
Exosome analysis	15,16	Expression of exosome-derived galectin-9 protein was significantly higher in livers of recipients with ACR relative to a nonrejection group.
Microbiome characterization	17	Plasma microbiome *Enterobacteriaceae* was elevated, and microbial diversity was lower in recipients with ACR than in non-ACR patients.
	18	Intestinal microbial variation can be used to predict acute rejection in the early phase after LT in rats; proposed microbiome manipulation as a potential therapeutic target.

ACR, acute cellular rejection; CXCL10, C-X-C motif chemokine ligand 10; IL, interleukin; LT, liver transplant; miRNA, microRNA; NPV, negative predictive value; PBMC, peripheral blood mononuclear cell; PPV, positive predictive value; Treg, regulatory T cell.

Banff Histopathologic Criteria for Rejection

With standardization of IS regimens through the 1990s, post-LT outcomes became more predictable. At the same time, the drive to standardize biopsy interpretation in transplantation gained momentum, leading to the first multidisciplinary "Banff Consensus Conference on Allograft Pathology" in 1991.[19] Following the development of histologic criteria and scoring systems for kidney, heart, and lung allografts, the 3rd Banff Consensus Conference in 1995 set out to establish criteria specific to liver allograft rejection.[20] This international group of expert pathologists introduced the Rejection Activity Index (RAI) score for liver allografts comprising scores ranging from 0 to 3 for each of 3 subcategories: portal inflammation, bile duct inflammation/damage, and subendothelial inflammation of the portal veins or terminal hepatic venules. The cumulative RAI score enabled grading of rejection, with RAI 3 to 4 being categorized as "mild," 5 to 6 as "moderate," and 7 to 9 as "severe." Through subsequent Banff Consensus Conferences, the RAI has remained the gold standard for liver allograft biopsy interpretation, and additional criteria have since been established for acute antibody-mediated rejection (AMR).[21]

Validation of the Banff RAI for LT

Following the introduction of the Banff criteria for acute cellular rejection in liver allografts, the Pittsburgh group performed a large prospective analysis to validate the RAI and explore its relationships with clinical outcomes.[22] This study included >2000 sequential biopsies obtained from 901 adult LT recipients maintained on tacrolimus-based IS. Key findings from this study included the overall observation of acute rejection in 64% of biopsies, with 17% falling into the "moderate" or "severe" categories. Importantly, liver biochemistry values including alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, and gamma-glutamyl transferase did not correlate with histologic findings. Patients with moderate-to-severe rejection were more likely to develop perivenular fibrosis on follow-up biopsy, whereas patients with a bile duct subscore of 3/3 had higher total bilirubin, alkaline phosphatase, and gamma-glutamyl transferase values. At the same time, patients with subendothelial scores of 3/3 had lower bilirubin and alkaline phosphatase values, with an increased incidence of perivenular fibrosis on subsequent biopsy. Despite these findings, the overall rate of graft loss because rejection was only 1%.

A retrospective cohort study of 495 post-LT patients from the United Kingdom also maintained on tacrolimus-based IS analyzed potential relationships between Banff RAI and clinical outcomes.[23] In this study, 231 patients had histologic evidence of rejection, and there was no correlation between RAI and liver biochemistries, development of steroid-resistant rejection, or graft loss. A smaller study specifically evaluating correlation between liver biochemistries and RAI in 70 liver biopsies that either had no rejection (N = 35) or moderate-to-severe rejection (N = 35) was unable to establish relationships between these laboratory values and histologic rejection.[24] Finally, a systematic review was conducted to evaluate potential relationships between liver biochemistries and episodes of rejection in LT recipients who underwent protocol biopsy.[25] Among

>1500 patients, 1048 had histologic evidence of acute rejection, and among these, 32% had no evidence of elevated liver biochemistries. Taken together, these studies confirmed the value of the Banff RAI in establishing the diagnosis of rejection while demonstrating that the laboratory values do not correlate with histologic findings.

Antibody-mediated Rejection

Acute AMR in ABO-compatible LT is less common than T cell–mediated rejection (TCMR) but because of accumulating evidence, it was first included in the Banff Workgroup criteria for rejection in 2016.[21] The tissue diagnosis of acute AMR requires demonstration of diffuse microvascular complement component 4 deposition along with microvascular endothelial cell hypertrophy and microvasculitis, when detected concurrently with serum donor-specific HLA antibodies (DSAs). Although the diagnosis of AMR uniquely entails inclusion of DSAs as a biomarker, the association of DSA with AMR or unfavorable outcomes in LT remains controversial. This is mostly because of data from multiple centers that demonstrated that DSAs, either preformed or de novo, may persist in LT transplant recipients with no accompanying allograft dysfunction.[26-28] However, in patients with high titers of DSAs, specifically against HLA class II, the risk of rejection and graft failure seems to be higher.[29,30] Importantly, development of de novo DSA is common when IS withdrawal (ISW) is attempted,[31,32] and is associated with failure of withdrawal.[33]

Chronic AMR has been increasingly recognized in long-term liver allografts, particularly among pediatric recipients, patients receiving low-dose IS or participating in IS weaning protocols, and in patients with chronic TCMR that may exhibit some component of overlapping AMR. Further studies are required to understand the underlying pathophysiology, risk factors, and optimal IS management in the spectrum of patients with AMR post-LT.

Limitations of Histologic Assessment

Despite the standardization of the Banff RAI, differentiating specific causes of liver allograft injury can be difficult. The Banff scoring system is based on relative degree and localization of inflammatory infiltrates, regardless of cause, within the portal triad in a standard hematoxylin and eosin-stained tissue section. Conventional histology of liver allograft biopsies is typically supplemented by immunohistochemistry or immunofluorescence to increase diagnostic accuracy. Although these techniques help investigate markers of interest, they allow for simultaneous labeling of only a few biomarkers per tissue section. As such, the main limitations of the current standard-of-care histologic assessment of organ biopsies are the inability to evaluate spatial architecture, distribution of immune cells and their interaction with parenchymal cells, and expression of activation/deactivation markers. In addition, histologic assessment of allograft biopsies is open to interobserver variability. For example, before the development of direct-acting antiviral medications against hepatitis C virus (HCV) infection, differentiating early recurrent HCV from TCMR and the rare but serious complication of fibrosing cholestatic hepatitis (FCH) in the early post-LT period presented a clinical challenge. Dixon and Crawford[34] retrospectively reviewed 77 "for cause" liver biopsies, including 32 in patients with recurrent HCV, 6 in patients with TCMR, and 11 in patients with FCH and compared histologic features in detail. In this series, FCH was more likely to exhibit features of portal inflammation and less likely to demonstrate bile duct damage or venous endotheliitis when compared with TCMR. Conversely, biopsies with recurrent HCV or FCH were more likely to show evidence of fibrosis. Although this series is small, it highlights important similarities and differences between these overlapping histologic entities.

Although active HCV is less likely to confound biopsy interpretation in LT patients in the current era, de novo autoimmune hepatitis, also referred to as "plasma cell–rich rejection" (PCRR), continues to represent an important differential diagnosis in patients with late allograft dysfunction.[21] PCRR is characterized by plasma cell–rich necroinflammatory activity on biopsy, with additional criteria including the presence of autoantibodies and a clinical history of steroid responsiveness.[35] Histologically, PCRR is more likely to have a higher proportion of $CD3^+$ T cells, $CD20^+$ B cells, and plasma cells on biopsy when compared with patients with chronic rejection.[36] There are also data to suggest that immunoglobulin G4, which is associated with autoimmune diseases, is a marker of disease severity in PCRR.[35] However, these immunologic assessments are not routinely performed in standard-of-care biopsies, and thus diagnosis of PCRR versus TCMR is generally made by pathologists in conjunction with a careful review of the clinical history.

Invasive Assays (Alternative Analyses of Liver Biopsies)

Despite the above-mentioned limitations, liver biopsy remains the standard for diagnosis of rejection. Although the nomenclature and classification of the histological assessment of liver allograft biopsies have been standardized, the use of the Banff RAI for diagnosis of rejection is being reevaluated. The 2-dimensional histological assessment of liver allograft biopsies fails to provide a detailed spatial architecture and does not identify the molecular changes underlying the pathology. These limitations become more pronounced in patients with other potential causes of graft injury and in long-term recipients with idiopathic inflammatory lesions. To overcome these limitations, 2 new approaches have been introduced recently: *tissue transcripts* and *multiplex imaging*.

Tissue Transcripts

Molecular markers of alloimmune responses and alloimmune-mediated graft injury provide additional diagnostic opportunities in liver biopsies. mRNA transcripts have proven to be reliable markers in this capacity. Their use as diagnostic tools was initially performed in kidney transplantation.[37] Comparing kidney biopsies from individuals with known rejection (positive class) with biopsies with no rejection (negative class) led to the identification of a set of rejection-associated transcripts (RATs). These transcript sets were reproduced and verified in different cohorts, by various groups in kidney[38-42] and other organ transplants.[43] Subsequently, gene sets associated differentially and uniquely with TCMR and AMR were identified.[44] Given the reproducible results, transcript-based molecular

diagnostics were incorporated into the Banff classification for kidney transplant rejection in 2015.

In addition to aiding diagnosis, tissue transcripts in kidney transplants have helped identify underlying mechanisms. For example, differences in TCMR-associated versus AMR-associated transcripts sets can be ascribed to differences in the mechanism and pathophysiology of the 2. Kidney allografts with TCMR are enriched with effector T-cell and macrophage transcripts, whereas AMR kidneys have increased expression of natural killer cell and endothelial cell transcripts.[37]

Using a similar approach, the first molecular profiling of liver allografts was reported by Bonaccorsi-Riani et al.[45] RNA microarray analysis of liver biopsies obtained from both HCV^- and HCV^+ patients enrolled in an ISW trial revealed RATs that were found to be increased in patients with TCMR. Like gene sets found in kidney and heart transplantation patients, pathogenesis-based transcript (PBT) sets enriched in livers with TCMR were mostly related to macrophages, effector T cell, and interferon (IFN) signaling. Interestingly, there was no correlation between the histological RAI and gene expression. Also, the type of IS did not alter the gene expression within the liver allograft.

This and similar reports supporting the use of molecular profiling in liver allograft biopsies culminated in the multicenter, prospective Diagnostic and Therapeutic Applications of Microarrays in Liver Transplantation (INTERLIVER) study with the goal of developing a molecular diagnostic system for liver rejection.[46] Evaluating 235 liver biopsies with microarray analysis, the study used an RAT-based archetypal analysis, allowing investigators to divide the cohort into 4 groups: no injury, TCMR, early ischemia-reperfusion injury (IRI), and late fibrosis. The results demonstrated that biopsies with no histologic evidence of rejection did not express RATs or injury transcripts. Biopsies known to have TCMR were enriched with RATs, IFN-γ–inducible, effector T cell–related, rejection-related PBTs, and injury-related PBTs. The IRI group had higher expression of injury-related PBTs but no rejection-related PBTs. Biopsies of long-term LT recipients with known fibrosis were enriched in injury-related, endothelial-related, and atrophy/fibrosis-related PBTs.

When correlating molecular rejection scores with histological rejection scores, the INTERLIVER study found that with increasing histologic rejection lesions score cutoffs, the sensitivity of the molecular diagnosis tool increased (albeit from 0.36 to 0.46) but at the expense of decreasing specificity. Thus, it seems that the molecular rejections score could be used to complement the histologic analysis in cases with low RAI to bolster diagnostic accuracy. The INTERLIVER study is currently collecting a larger cohort, which will help establish the clinical impact of this approach in the future.

Molecular profiling has also been used to identify the pathophysiology of idiopathic fibroinflammatory lesions that are not uncommon in biopsies of long-term LT recipients. For example, portal inflammation was found in 67% of biopsies obtained >10 y post-LT in 49 patients with normal liver tests and no known recurrent disease or history of recent rejection.[47] In the same biopsies, gene expression patterns associated with portal inflammation overlapped with those of TCMR, including the transcript sets associated with cytotoxic T cells, macrophages, B cells, injury, and rejection, as well as IFN-γ–inducible genes. Thus, molecular profiling of these long-term recipients maintained on low IS helped link TCMR to idiopathic fibroinflammatory lesions.

Given the increasing incorporation of molecular diagnostics into organ transplant biopsies, a Molecular Diagnostics Working Group was formed as part of the 15th Banff Conference for Allograft Pathology.[48] By reviewing previous work on discovery and validation of all microarray studies performed in kidney, heart, lung, and liver biopsies, this group generated a panel that includes 758 genes covering multiple molecular pathways designed to include all organs. The so-called B-HOT (Banff Human Organ Transplant) is currently available for research use. It is likely that with accumulating experience, this panel will be simplified in the future. It is also possible that the panel is customized to each organ with continued discovery and validation of the transcripts.

As for the future, it is worthwhile remembering that the current knowledge of molecular diagnostics in liver allograft biopsies is based on whole transcriptome microarrays. Microarray studies are conducted on biopsies stored in RNAlater stabilization solution. Such samples are not available in most LT centers. Also, the storage of an additional core in RNAlater solution, as well as the microarray itself, is costly and resource intensive. Recent emergence of NanoString technology allows for molecular analysis of formalin-fixed, paraffin-embedded (FFPE) biopsies, with the advantage of not requiring an additional core, ability to do histology and molecular analysis in the same sample, and minimizing sampling errors. Also, NanoString-based molecular profiling could be performed retrospectively in stored FFPE biopsies more commonly available.

Multiplex Imaging

As discussed earlier, conventional histological evaluation of liver allograft biopsies has several limitations. Recently, several multiplexed tissue imaging techniques have emerged that allow for multiplexed quantitative molecular profiling, including colocalization, relative distribution, and spatial architecture.[49-51] Of several such techniques, quantum dot multiplex imaging and imaging mass cytometry have been used more frequently. In quantum dot multiplex imaging, semiconductor quantum dots, which are brightly luminescent nanoparticles, are conjugated to antibodies. The fluorescent properties of these dots help in the detection of multiple targets simultaneously using a fluorescence microscope. In imaging mass cytometry, antibodies are conjugated to laser-cleavable heavy metal ion tags. On laser irradiation, the beam ablates the tissue surface after a virtual raster inducing the cleavage of metal tags from the antibodies. Metal isotopes from a discrete position on tissue or pixel are then quantitatively analyzed by mass spectrometry and the isotope abundance of each position is mapped back to the original coordinates, producing a high-resolution image. Both techniques can be used on FFPE tissue sections using a cocktail of biomarker-specific conjugated antibodies, and quantitative digital analysis in both increases reproducibility of the results.

The investigators of the multicenter ISW for stable pediatric LT recipients have been successful in incorporating multiplex imaging in their studies. For example, in evaluating screening LT biopsies of stable pediatric patients with a combination of molecular profiling (microarray) and quantum dot multiplex imaging, 3 main clusters

were identified: portal inflammation with interface activity (cluster 1), significant fibrosis (cluster 2), and near-normal histology (cluster 3).[52] Multiplex imaging showed increased numbers of antigen-presenting cells (APCs), leukocytes, and APC/leukocyte pairings in cluster 1, suggesting an active alloimmune response within grafts. Not surprisingly, microarray analysis revealed that cluster 1 had more overlap with TCMR-associated gene sets than other clusters.

In a follow-up study of the same trial, the investigators compared eligibility biopsies of trial participants who achieved operational tolerance with those who did not.[33] Although portal and lobular inflammation grades of both groups were similar in routine histology, multiplex imaging revealed that operationally tolerant subjects had decreased numbers of leukocytes ($CD45^+$), APCs ($MHCII^+$), APC/leukocyte pairs, monocyte/macrophages ($MAC387^+$), and effector T cells ($CD8^+$). The combination of the 3 findings on multiplex imaging but not routine histology (APC/leukocyte pairs, monocyte/macrophages, and effector T cells) had a higher sensitivity and specificity than portal inflammation in predicting operational tolerance.

Considering the added information multiplex imaging provides in organ transplant biopsies and given their rapid emergence in other fields, it is likely that these techniques will increasingly be incorporated into practice.

Noninvasive Assays

Despite clinical dependence on graft biopsies, they possess an inherent risk of complications and are limited by sampling error, variability in grading technique, and cost.[53-55] Identification of noninvasive diagnostic methods, most commonly using biomarkers from blood samples, presents an opportunity to overcome such limitations and improve LT outcomes (Figure 1).

Liver Stiffness

Liver stiffness measurement (LSM) by transient elastography is commonly used to assess liver fibrosis. Because liver stiffness is not unique to fibrosis but may also reflect intrahepatic inflammation, the utility of LSM in detecting rejection after LT has been investigated by several groups.[56-58] Increased liver stiffness, indeed, is associated with the severity of TCMR.[56] Furthermore, treatment of TCMR seems to reduce stiffness. When tested specifically as a noninvasive tool to detect alloimmune liver injury in LT patients, LSM was found to be useful as an adjunctive assay rather than a stand-alone test.[58]

DNA-based Assays

Among emerging DNA-based assays, current research highlights the utility of donor-derived cell-free DNA (dd-cfDNA). These are short, nonencapsulated DNA fragments that are released into the bloodstream in association with cell death and turnover processes. After organ transplantation, total cfDNA in circulation also includes cfDNA released from the donor tissue. Release of dd-cfDNA occurs because of allograft injury; hence recipient serum dd-cfDNA can serve as biomarkers of allograft health and rejection status.

Immediately after LT, allograft IRI causes the dd-cfDNA fraction to rise relative to total cell-free DNA (cfDNA). In the absence of complications, the dd-cfDNA fraction then declines to a baseline level, reflecting routine cell turnover rather than ongoing injury. During rejection episodes, the

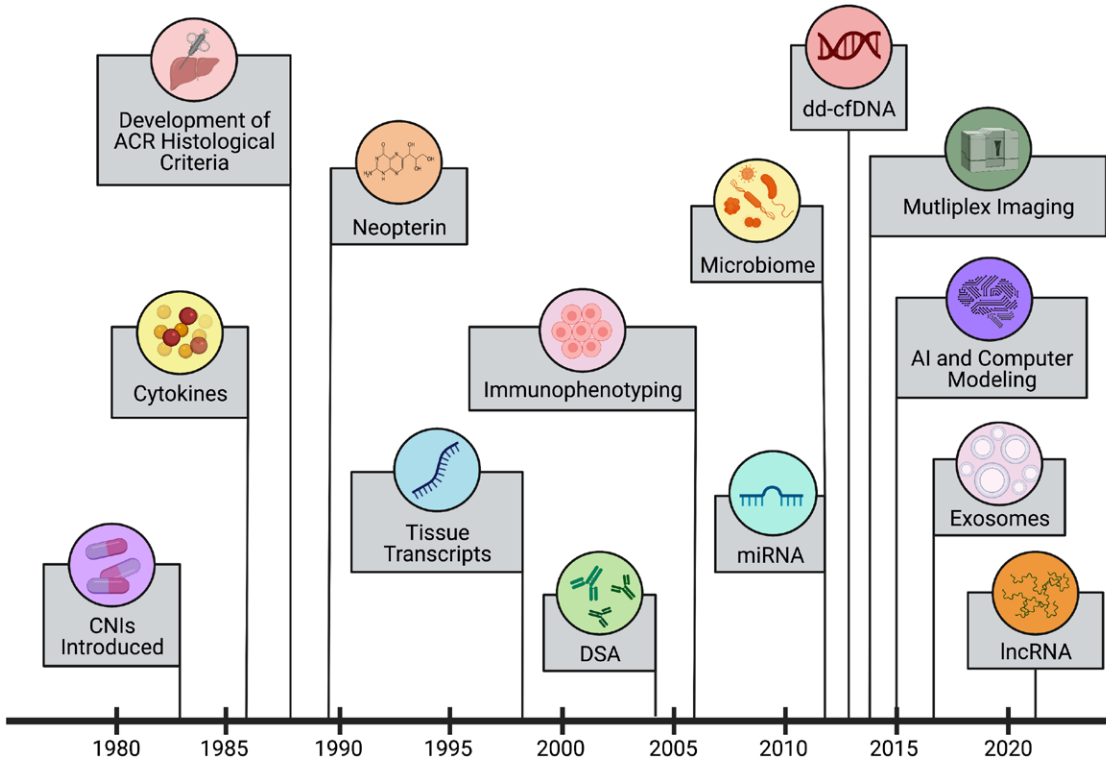

FIGURE 1. Evolution of therapeutic and diagnostic techniques and their application to liver transplantation over the years. ACR, acute cellular rejection; AI, artificial intelligence; CNI, calcineurin inhibitor; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; lncRNA, long noncoding RNA; miRNA, microRNA.

fraction of dd-cfDNA is elevated. This was demonstrated first in a 2013 digital droplet quantification study, in which dd-cfDNA was measured at 90% of total cfDNA on the day of LT. In patients without complications, dd-cfDNA dropped to <15% 10 d post-LT while remaining between 55% and 60% in 2 patients with biopsy-confirmed rejection.[59] In 2016, Grskovic et al[60] expanded on this methodology, creating a next-generation sequencing–based assay for quantification with clinical-grade limits of blank, limits of detection, and limits of quantitation. Likewise, a later study revealed significant differences in measured dd-cfDNA concentrations between kidney transplant recipients with and without biopsy-confirmed active rejection.[61] In LT, 2 more recent studies have indicated that dd-cfDNA may be even more sensitive in detecting acute rejection than conventional measurement of transaminases.[62,63]

Aside from applications for monitoring rejection status, dd-cfDNA shows potential to inform IS therapy modulation. In 2014, Oellerich et al[64] demonstrated a significantly negative correlation between dd-cfDNA and tacrolimus dosage. Hence, dd-cfDNA may provide a valuable assessment of allograft health during IS optimization. With respect to the known toxicities of IS medications, individualized modulation represents another important avenue for improving LT outcomes.[65]

RNA-based Assays

Other promising techniques center on the use of RNA analytes including mRNA, microRNAs (miRNA), and long noncoding RNA. Although miRNA and long noncoding RNA, along with other noncoding RNAs, were once thought to have little utility, new sequencing technologies and assay methods have revealed evidence of broad regulatory, immunological, and epigenetic functionality.[66-68] As mechanisms and functions of classes of noncoding RNAs are better understood, their capacity for clinical application will become clearer.

Initial investigations of miRNAs show potential for use as both diagnostic and prognostic biomarkers. Several studies have correlated specific hepatocyte-derived miRNAs, including miR-122, miR-148a, miR-192, and miR-194, with rejection and allograft injury.[69-72] Additionally, upregulation of miR-122, miR-148a, and miR-194 is observable in serum before aminotransferase levels are affected by rejection.[72] Hence, miRNA biomarkers could provide a temporal advantage compared with traditional liver function tests (LFTs). In 2017, Schmuck et al[7] further confirmed the prognostic capacity of miR-122-5p and miR-194, demonstrating that these miRNAs, in addition to miR-133a and miR-148a-3p, were significantly elevated in the bile of liver recipients during rejection episodes. A similar study found that preoperative plasma values of miR-155-5p were significantly higher in LT patients who developed TCMR or subclinical rejection within 3 mo of transplantation.[72] Most recently, Afshari et al profiled miRNA expression in LT recipients with hepatocellular carcinoma. They found that miR-194-5p and miR-548as-3p are significantly upregulated, whereas miR-3158-5p and miR-4449 are significantly downregulated in patients experiencing rejection when compared with stable adults.[73] Conversely, Zhang et al[8] revealed that miR-18b-340 and miR-106b were upregulated in peripheral blood mononuclear cells of long-term, stable LT recipients relative to both healthy adults without LT and recipients experiencing rejection. Early successes with miRNAs may stem from their roles in allograft injury pathways, including causative and signaling functions. This makes them ideal candidates for the development of new biomarker assays.

Protein-based Assays

Early exploration of potential biomarkers centered mainly on a catabolite of the guanosine triosephosphate pathway called neopterin. Released by activated macrophages and monocytes during T cell–dependent interactions, neopterin was identified as marker for proinflammatory immune status.[74] Multiple initial studies demonstrated success using this molecule to differentiate between liver allograft rejection and viral infections.[75,76] However, a 2015 study demonstrated that early post-LT measurements of serum neopterin were significantly correlated with 1-y survival in LT recipients.[77] Despite this, the pleiotropic actions of neopterin make achieving diagnostic specificity difficult. Hence, development of biomarkers has shifted focus to cytokines that mediate immune involvement in liver allograft rejection with greater specificity.

Unlike neopterin, cytokines may cause pro- or anti-inflammatory responses. In 2006, Hassan et al[78] profiled the early postoperative cytokine responses of LT recipients, characterizing plasma interleukins (ILs), tumor necrosis factor-α, and IFN-γ. This study revealed that elevated IL-6 levels were significantly associated with a subsequent rise in other proinflammatory cytokines, including IL-2, IL-4, tumor necrosis factor--α, and IFN-γ, and 1 anti-inflammatory cytokine, IL-10. In addition, in recipients experiencing complications, IL-6 was significantly associated with total bilirubin suggesting that IL-6, IL-10, and serum bilirubin may be predictive of common LT complications, including rejection. The utility of IL-10 as a biomarker was further supported by multiplex cytokine assays that demonstrated elevated IL-17 and IL-10 levels during rejection episodes 1 and 3 wk post-LT, respectively.[14]

Cell-based Assays

Cell-based assays have also gained recognition, with efforts to characterize LT recipients' peripheral blood immune cells using flow cytometry and, more recently, mass cytometry. An early study defined one such profile specific to rejection, in which $CD4^+$ lymphocytes showed increased T-cell activation and CD8 lymphocytes showed higher cytolytic potential.[11] Phenotypic shifts in memory T cells have also been significantly associated with rejection events.[12,13] In 2020, Han et al characterized regulatory T cells (Treg) response in LT recipients receiving basiliximab induction plus tacrolimus IS. They concluded that both total and activated Treg were significantly lower in patients with biopsy-confirmed rejection 7 d post-LT.[79] Furthermore, several studies have established immunophenotypes for operational tolerance in LT recipients, demonstrating elevated $CD4^+$ $CD25^+$ T-cell counts, as well as increased Vδ1/Vδ2 γδ T-cell ratios.[80-84]

Exosomes

Another growing area of investigation centers on the potential of exosomes and their contents to inform on allograft status and immune responses to transplantation. Exosomes are small extracellular vesicles of endocytic

origin that participate in cell-to-cell communication as carriers of bioactive molecules.[85,86] Because they are critical to crosstalk and immune pathways, exosomes are attractive targets for development of both diagnostic tools and therapies for allograft rejection. In a 2019 study, exosome-derived protein galectin-9 was significantly elevated in LT recipients experiencing rejection relative to stable LT recipients. This was consistent with immunohistochemical analyses of allograft tissue microarrays, which demonstrated that LT recipients with rejection had higher galectin-9 expression levels than recipients without rejection.[15,16] Another preliminary study explored possible therapeutic applications. Using a rat model, researchers identified a combination of immature dendritic cell exosomes and donor antigen-specific Treg capable of inducing liver allograft tolerance. The synergistic dose was administered 3 times—before, during, and after transplantation—and achieved long-term survival without the need for IS.[87]

Microbiome

The role of the microbiome in affecting transplant outcomes and marking rejection has also come into recent focus. A 2021 study revealed that after LT, the relative abundance of *Anelloviridae, Nocardiaceae, Microbacteriaceae*, and *Enterobacteriaceae* in the plasma microbiome was significantly altered. They also demonstrated that recipients with rejection experienced both elevated *Enterobacteriaceae* proportions and depressed microbial diversity compared with stable recipients.[17] These findings are mirrored in studies of the fecal and intestinal microbiome. Multiple studies have found a significant association of reduced diversity with allograft dysfunction and postoperative infection in LT recipients.[88,89] Moreover, specific profiles of the intestinal microbiome have been developed for the successful prediction of LT rejection in animal models.[18] However, researchers have yet to identify such a profile characterizing the intestinal or fecal microbiome for clinical diagnostic use.

CLINICAL IMPLICATIONS

Liver Allograft Surveillance and Implications for Tolerance Protocols

The liver is uniquely more tolerogenic than other commonly transplanted organs, and LT recipients require lower doses of IS drugs than recipients of other organs. In addition, some selected liver recipients can be completely weaned off from the IS treatment without alloimmune damage to the liver grafts achieving the so-called spontaneous operational tolerance status. The liver is the only solid organ with a propensity toward operational tolerance. This has been observed in retrospective studies in which IS nonadherence or intentional withdrawal for side effects has resulted in unexpectedly stable allograft function in 10% to 70% of patients, with >500 "tolerant" LT recipients reported in the literature to date.[90] This is in contrast to renal transplantation, in which only a small number of operationally tolerant patients have ever been reported, with a high failure rate among intentional ISW protocols.[91] This has prompted multiple prospective ISW studies in both select adult and pediatric LT recipients, resulting in varying levels of operational tolerance ranging from 30% to 60%.[31,92,93] In general, patient inclusion criteria involved patients >2 to 3 y post-LT, having nonautoimmune liver disease as an indication for transplantation, absence of recent episodes of acute cellular rejection, without significant histological alterations, and with normal LFTs before the start of the ISW protocol. Those studies reported a variable success rate of patients who achieved operational tolerance ranging from 16% to 60%, higher with increasing adherence to these inclusion criteria.[94] Clinical characteristics associated with the success of ISW included the time elapsed from transplantation to the beginning of ISW and the age of the patient at the time of ISW.[95] In the context of precision medicine, we expect that similar attempts at minimizing or withdrawing IS after LT will continue to become more common. Thus, techniques that bolster the assessment of liver biopsies and noninvasive biomarkers discussed previously will likely be incorporated into surveillance protocols of such patients in the future.

The advent of new biomolecular technologies, such as more accurate multiparameter flow cytometry and high-throughput gene expression analysis associated with the development of complex statistical methods, prompted the investigation of predictive biomarkers of tolerance. Such biomarkers would be useful to identify patients with a high probability of achieving operational tolerance and to exclude patients unlikely to tolerate ISW. For example, gene signatures or the presence of specific immune cell populations identified in operational tolerant liver recipients in cross-sectional studies have been used to select future candidates to be enrolled in prospective ISW clinical trials.[84,96,97] Unfortunately, none of the tolerance biomarkers used resulted in a better selection of potential tolerant liver recipients.[33,98]

Typically, in many of the studies, the weaning protocol lasted 6 to 9 mo, when sequential blood samples were collected to monitor LFTs. At the same time, the idea of using these sequential samples to detect molecular biomarkers of immune graft injury before a rejection episode could be detected by an alteration in LFTs and by histological changes became more attractive. Theoretically, the detection of molecular biomarkers of rejection by noninvasive tests before increases in transaminases or the appearance of histological damage in the graft in each patient under IS weaning protocol could trigger some clinical strategies to halt the progression of rejection. Some of these measures could be (1) halting the weaning protocol, (2) reverting the doses of IS drugs to the previous higher doses, (3) spacing the time between drug reductions, and (4) excluding the subject from the weaning protocol. Blocking the progression of rejection at the start when only rejection biomarkers are detected and no intense lymphocyte allograft infiltration has occurred could, in theory, spare future nontolerant recipients from the complete immune system reactivation manifested by clinical, biochemical, and histological rejection scenario. This strategy could avoid the "rejection priming effect," which can prevent later attempts of ISW when those patients will be older and more time will have elapsed since the transplant procedure, the 2 most robust clinical factors associated with successful complete ISW.

To achieve this goal, Bonaccorsi-Riani et al analyzed the gene expression of 45 target genes in blood samples collected sequentially from 22 liver recipients that failed the ISW protocol in a multicenter European clinical trial. They found in a univariate analysis 10 genes that were upregulated in the diagnosis of rejection compared with samples

collected at the beginning of weaning, 8 in HCV$^-$ patients and 2 in HCV$^+$ patients. In a stepwise multivariate logistic regression analysis, C-X-C motif chemokine ligand 10 and *FOXP3* were identified as the best signature to discriminate between rejection and baseline samples. The use of this signature in all samples collected sequentially showed that the expression of C-X-C motif chemokine ligand 10 was the most robust predictor of rejection. Its expression increases 1 to 2 mo before the diagnosis of rejection and decreases just after the start of rejection treatment. It is worth noting that the signature was unable to correctly differentiate rejectors from tolerant ones in HCV$^+$ patients.[45] In another study, Shaked et al performed miRNA profiling in serum samples from 69 liver recipients enrolled in 2 American clinical trials. After multiple testing corrections, the expression profile of 31 miRNAs was significantly associated with rejection in the training and tests sets with an FDR adjusted *P* value of <0.001. Then, they submitted the panel of 31 miRNAs to a variable selection model built with GLMNET to define the best rejection predictor, which defined a signature containing 2 miRNAs, hsa-miR-483-3p and hsa-miR-885-5p, as the best diagnostic test to differentiate TCMR from non-TCMR with an AUC of 90% (95% confidence interval, 81%-98%), with 88.9% sensitivity, and 83.3% specificity in the training set ($P=0.0001$). This signature was tested on sequential blood samples collected from 27 patients undergoing ISW, with each patient having at least 2 samples before the biopsy event. They found that miRNA signature separated rejection events from nonrejection events, with 95% confidence intervals in the smoothed plotting at approximately 40 d before the biopsy-proven rejection.[99] In addition, in a multicenter ISW clinical trial in adult liver recipients, the development of de novo DSA was associated with the failure of the weaning.[32] This was confirmed in another lSW study in children, in which the development of de novo DSAs in sequential measurements during the IS weaning was associated with a rejection episode.[33] More studies with the same strategy of analyzing sequential blood samples collected during the weaning protocol but with new technologies as the dd-cfDNA are required to identify new biomarkers associated with the rejection episode in this context.

FUTURE DIRECTIONS

Given the additional information they provide for surveillance of liver allografts, we anticipate that the aforementioned noninvasive assays and alternative analyses of liver allografts will be increasingly incorporated into clinical practice in the future. The immediate utility of these novel approaches is limited by additional cost. However, the continued increase in the utilization of some of these techniques, particularly the DNA/RNA-based assays and multiplex imaging, in medicine outside transplantation (cancer, inflammatory or infectious diseases) will likely help drive down their cost and accelerate their incorporation into clinical transplantation in the near future. As more data become available for each individual, clinicians will face the attendant challenges and opportunities. The emerging field of bioinformatics will likely help with decisions to be made on the basis of the status of allografts. For example, there is an ongoing clinical trial in the United States, Molecular Assessment and Profiling of Liver Transplant Recipients or the MAPLE study, which aims to enroll 1500 liver recipients. In this study, the most advanced molecular and bioinformatics tools, including, dd-cfDNA, blood-based gene expression profiling, microchimerism analysis, machine learning algorithms, metagenomic infections, blood-based disease testing, and molecular analysis of FFPE liver tissue-based gene profiling, will be used to process, analyze, and interpret the protocol planned sequential blood samples and liver tissues. The inclusion of the analysis of liver biopsies in such a study can be used to compare changes eventually found in the blood. It also denotes that we are only starting to decipher the changes in the intragraft molecular pathways during rejection.

Machine Learning and Artificial Intelligence

As more granular and multidimensional data sets reflecting the status of liver allografts become available, predictive modeling of clinical outcomes via artificial intelligence and machine learning algorithms will likely be increasingly explored in transplantation.[6,100] Such strategies, albeit on a smaller scale than what is expected in the near future, have already been investigated through coupling of RNA-based assays, including RNA sequencing, quantification, and microarrays, to create diagnostic and predictive algorithms using these approaches. For example, Shaked et al developed a dual-gene (hsa-miR-483-3p and hsa-miR-885-5p) model to identify TCMR in LT recipients based on a larger profile of 31 miRNAs significantly associated with this outcome. The model showed high specificity and sensitivity in discerning TCMR up to 40 d before clinical evidence of allograft dysfunction.[99] Levitsky et al created a similar 36-probe model using mRNA microarray analysis. Validation using a separate cohort showed that the model successfully distinguished between LT recipients with TCMR from those with healthy allografts.[101] A more recent iteration of this methodology achieved a sensitivity of 0.70 and specificity of 0.81.[9] These models showed an additional capacity for application to IS modulation. Hence, as novel, highly dimensional biomarker data sets are developed, rapid integration of predictive modeling using machine learning and artificial intelligence has the potential to impact the long-term management of LT recipients and may ultimately help prevent complications associated with under-immunosuppression and overimmunosuppression.

CONCLUSION

It is difficult to imagine the complete replacement of liver biopsy with a different biomarker capable of diagnosing and perhaps predicting a TCMR or AMR episode before its clinical manifestation in the early post-LT stage. It is likely that standard histological and immunohistochemical readouts will be important tools for graft immune monitoring and diagnosis during the first year posttransplant in association with tissue transcript profiles or blood biomarkers that will emerge from studies like MAPLE. In contrast, it may be easier to use a biomarker or a composite biomarker in long-term liver recipients for the purpose of monitoring graft immune injury or for IS treatment tailoring instead of liver biopsies. In a recent study, Vionnet et al proposed the use of a composite biomarker including alanine aminotransferase levels, class II DSAs, and liver stiffness to distinguish patients with or without alloimmune injury. These factors were positively associated with TCMR transcript levels in

a population of 190 long-term LT recipients screened to be enrolled in an ISW clinical trial.[58] This study points to a new strategy that should be further investigated for liver graft monitoring in long-term patients.

A large amount of data will likely become available in the coming years. New molecular technologies associated with bioinformatics and machine learning will transform immune monitoring after LT to improve long-term graft and patient outcomes.

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