Contemporary Biomarkers for Renal Transplantation

Subjects: Transplantation

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Renal transplantation (RT) is the preferred treatment for end-stage renal disease. However, clinical challenges persist, i.e., early detection of graft dysfunction, timely identification of rejection episodes, personalization of immunosuppressive therapy, and prediction of long-term graft survival. Biomarkers have emerged as valuable tools to address these challenges and revolutionize RT patient care.

organ/renal/kidney transplantation
precision/personalized medicine
diagnostic/predictive non-invasive biomarkers
immunopathology of acute allograft rejection (AR)
chronic allograft dysfunction (CAD)
glomerular vs. tubular nephron damage
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1. Introduction

Renal transplantation (RT) is currently the optimal treatment option for patients with end-stage renal disease (ESRD), providing survival benefits, improved health-related quality of life, and cost-effectiveness, compared to dialysis [1]. Despite significant improvements in immunosuppressive therapies and surgical techniques, pervasive challenges still remain unaddressed regarding the complex, multi-modal, clinical management of RT patients. These challenges include early detection of graft dysfunction, timely identification of rejection episodes, personalization of immunosuppressive therapy, and prediction of long-term graft survival. Recently, biomarkers have emerged as valuable tools in addressing these challenges, offering the potential to revolutionize the clinical management of RT patients.

Recent advancements in immunosuppressive therapy have reduced acute rejections (ARs) and improved short-term renal allograft half-life [2]. Even so, late allograft loss still constitutes a major clinical issue post RT [3]. Current monitoring of renal allograft function relies upon serum creatinine measurement and needle-core renal biopsy, both of which have limitations. Creatinine levels rise only in later stages of allograft injury and cannot differentiate between specific injury types or predict chronic injury progression. Needle-core renal biopsy, though considered the gold standard, is invasive, cannot be safely performed repeatedly to monitor injury progression, has potential sampling biases, carries a 1–2% risk of significant complications, and its overall predictive power is poor in RT patients [4][5]. In fact, molecular-level tissue sample examination can detect immune response abnormalities before they become histologically evident [6]. The development of non-invasive, reliable, and predictive biomarkers for early diagnosis and monitoring of clinical conditions post-RT is essential for personalized treatment.

Biomarkers represent measurable objective indicators of normal biological processes, pathogenic visceral responses, and/or therapeutic interventions [7], and thus may also provide critical information about the state of the transplanted organ, i.e., the kidney allograft. Assays for proteomic, metabolomic, transcriptomic, and genomic biomarkers, derived from various biological sources, i.e., donor/recipient peripheral blood/serum/lymphocytes or urine, and tissue biopsy specimens have been extensively explored due to their notable clinical potential in RT, namely, to monitor allograft function, detect early rejection, guide immunosuppressive treatments, and predict long-term allograft survival and RT patient outcomes. The inclusion of validated gene transcripts/classifiers in the Banff classification for rejection highlights the growing importance of biomarkers in post-RT pathology [8]. Thus, it is becoming increasingly clear that further integration of these emerging biomarkers into clinical practice could significantly improve patient care and potentially optimize RT outcomes.

Generally speaking, biomarkers could, at least in theory, play a host of essential clinical roles throughout each step of the entire RT process [4], namely: (1) preoperative donor assessment and kidney allograft retrieval—prediction of short-term outcomes/risk of postoperative complications, i.e., delayed graft function (DGF); (2) in the perioperative setting—assessment, identification and characterization of subacute and/or AR processes, thus enabling more timely interventions; (3) postoperatively, for the crucial differential diagnosis between true chronic rejection (CR) vs. chronic allograft dysfunction (CAD)—similar clinically, yet require completely different treatments, with CR being immunologically mediated, whereas CAD is usually the result of various non-immunological pathogenic factors; (4) long-term monitoring of allograft injury occurrence [4]. Furthermore, biomarkers associated with RT patient immune tolerance are also highly coveted and of great importance for clinical management, as they could potentially allow for the progressive tapering or even complete discontinuation of postoperative immunosuppression, thus further reducing the risk of treatment-associated side effects and complications.

Beyond specific clinical context, RT biomarkers can be classified based on their individual capacity to assess immunological vs. non-immunological outcomes. Immunological outcomes are primarily related to rejection and immune tolerance, whereas non-immunological outcomes are mainly related to tissue injury [9]. Conversely, regarding nephron targeted biomarkers, a further classification based on individual histological nephron component specificity, i.e., glomerular vs. tubular, may also prove useful for the better characterization of pathogenesis and a more nuanced understanding of non-specific patient manifestations [10]. However, non-invasive biomarkers are indeed the primary candidates for clinical application in RT management, due to their inherent practicality, ease of assessment and minimal patient discomfort. Promisingly, non-invasive assessments for RT patients currently include: messenger (m)RNA transcripts; lymphocyte phenotype markers; chemokines; micro(mi)RNA; and donor-specific antibodies (DSA), i.e., antibodies that react specifically to antigens from the organ donor [4].

Notwithstanding the potential benefits of RT biomarkers, their clinical application is not without challenges. Overall, the actual utility of RT biomarkers in real-life patient management is largely dependent on their individual evaluation metrics, such as: sensitivity; specificity; positive predictive value; negative predictive value; receiver operating characteristics (ROC) curves. These metrics help determine the biomarker's precision and reliability in identifying a condition or predicting an outcome, which are critical for guiding clinical decisions [11][12]. Moreover, validation of biomarker assay results can be affected by inter-observational variability (differences in results

between evaluators) and inter-laboratory or inter-platform methodological heterogeneity (differences in results due to variations in laboratory methods or testing platforms). These can create discrepancies in biomarker measurements, limiting their predictive power and possibly leading to result misinterpretation [13]. Therefore, before new biomarkers can be confidently integrated into clinical practice, they must undergo thorough validation studies and assay standardization. Validation studies test the biomarker in a large, diverse patient group to ensure its accuracy and reliability across different clinical scenarios. Assay standardization ensures the methods used to detect/measure the biomarker are consistent and reproducible, providing dependable results regardless of where or when the test is conducted.

2. Immunopathology of Nephron Injury and Allograft Rejection

The recent clinical introduction of more potent immunosuppressive drugs has resulted in a decreased incidence of AR. Nonetheless, about 10% of kidney transplant recipients still experience an AR episode within the first year after RT [14]. Although these episodes can generally be treated with intravenous steroids and/or anti-thymocyte globulin, their occurrence can have a negative impact on graft outcome. Routine immunologic laboratory tests are already being used to determine a patient's immunologic sensitization and to assess the risk of adverse graft outcomes. The complement-dependent cytotoxicity test, performed pre-RT, has significantly reduced the incidence of hyper-acute rejection [15]. Similarly, pre-RT human leukocyte antigen (HLA) alloantibody screening aids in optimizing donor selection. Post-RT HLA alloantibody screening assists in identifying the specific type of AR and potential antibody impact on graft function [16].

In fact, AR episodes, which are most prevalent in the first few weeks after transplantation, can be categorized into T-cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR) [17]. Essentially, TCMR involves lymphocyte infiltration and proliferation within the interstitial space of the kidney allograft, which will the subsequently induce cytotoxic effects on renal tubular epithelial cells, causing inflammatory responses, i.e., "tubulitis". Similarly, vascular rejection, a more severe variant of TCMR, involves mononuclear cells invading arteries, leading to arteritis and potentially severe transmural necrosis of allograft vasculature. Conversely, in ABMR, DSA target HLAs or non-HLAs on the donor endothelium, leading to antibody-dependent cellular cytotoxicity and complement activation [16].

DSA refer to the antibodies that a transplant recipient forms against specific HLA antigens found on the donated organ. These antibodies can inflict allograft nephron damage, by inducing multi-lamination of the peritubular capillary basement membranes, or arteriopathy that manifests as intimal fibrosis [18]. The DSA endothelial cell injury can trigger platelet aggregation and leukocyte recruitment, potentially leading to graft failure. Thus, when the allograft is subjected to rapid surges in high-titer DSA, AR occurs, usually either in sensitized recipients, or as de novo responses in non-sensitized patients who do not strictly adhere to their immunosuppressive treatment. Alternatively, CR mediated by DSA occurs in response to a slower emergence of these antibodies, which can be of high or low titer and may be either transient or persistent [18].

While substantial research is being conducted to develop therapeutic strategies aimed at reducing DSA levels [19], the current understanding of how to prevent the initial formation of DSA is still limited. Moreover, risk factors associated with DSA development are not fully defined. Early evidence suggests that specific immunosuppressive treatments could influence DSA formation [20]. Specifically, it appears that treatments based on calcineurin inhibitors are less likely to be associated with DSA formation compared to those based on mTOR inhibitors or lower mycophenolic acid levels [21].

Clinically, microcirculation lesions, C4d deposition in peritubular capillaries, and the presence of DSA in the patient's serum suggest ABMR. However, DSA can be identified in the serum of RT recipients many years before any signs of clinical graft dysfunction appear. Hence, it is crucial to routinely monitor DSA in the follow-up of transplant recipients, even though uniform protocols are not yet in place [22]. Moreover, the onset of de novo DSA (dnDSA) post-RT has been firmly linked to poor graft outcomes in adults as well as children [22][23]. The formation of dnDSA, in general, is associated with lower 10-year graft survival rates, even in pediatric studies [22]. Managing the aftermath of chronic (c)ABMR is typically even more challenging. Given this information, dnDSA are recognized as reliable biomarkers that can predict late acute ABMR, cABMR, transplant glomerulopathy, and graft loss [24]. Even so, their clinical significance is contingent upon certain characteristics of the antibody itself, such as its IgG subclass, which affects its capacity to bind complement cascade components and engage effector cells through Fc receptor binding. For instance, IgG3 subclass dnDSA can bind to complement component (C)1q more efficiently, activate the classical pathway of the complement cascade, and often lead to acute ABMR, whereas IgG4 DSA, which cannot bind Cs, primarily operate through the Fc receptor to magnify alloresponses [25].

Indeed, the transplant recipient's adaptive immune system plays a central role in allograft TCMR. Thus, within this process, alloreactive T-lymphocytes, which represent between 1and 10% of T-lymphocytes overall, interact with mismatched HLAs on donor-derived antigen-presenting cells (APCs) [26]. This interaction, known as direct allorecognition, and the subsequent interaction between recipient APCs and CD4+ T cells, known as indirect allorecognition, promote T cell proliferation and differentiation [27]. Activated CD8+ T cells release perforin and granzyme B, which induce apoptosis of target cells [28], while monocytes and myeloid dendritic cells (DCs) infiltrate the graft and contribute to AR [29][30]. However, innate immunity also plays a role in transplant injury, via intra-allograft complement cascade activation.

Normally, the innate immune system provides a general defense against foreign pathogens by employing the complement system and cellular responses from macrophages and DCs. These cells possess Toll-like receptors (TLRs) that can identify pathogen-related molecular patterns on invading microbes [16]. Importantly, post RT, ischemia reperfusion injury (IRI) is, at least to a certain degree, virtually unavoidable, due to the inherent conceptual and methodological limitations of contemporary surgical strategies [31]. Thus, the process of post-RT allograft TCMR damage is initiated by this pervasive associated mechanism of IRI, which determines tubular cellularity apoptosis, causing the subsequent release of damage-associated molecular patterns (DAMPs). These post tubular injury DAMPs, typically concealed within healthy cells, will then bind to TLRs on DCs, triggering their activation and maturation [32][33]. Furthermore, IRI can also lead to local activation of the complement cascade. The DCs present donor-derived human leukocyte antigen (HLA) target epitopes and co-stimulatory molecules to naïve

T-cells, leading to the differentiation of these cells into interferon (IFN)γ-producing T-helper (Th)1 cells. This, in turn, will further stimulate the maturation of other additional recipient DCs, induce macrophage activation and recruitment, and direct the differentiation of CD8+ T-cells. Concurrently, IRI can also induce a local increase in complement component 3 (C3). When C3 is cleaved by the alternative pathway, C3b is deposited on cellular membranes, instigating the activation of the complement cascade. The breakdown of C3 leads to the release of small fragments, i.e., C3a and C5a, during complement activation, both of which have pro-inflammatory effects. The subsequent formation of the membrane attack complex (MAC) results in lysis of the targeted cell and further release of DAMPs [16].

3. Glomerular vs. Tubular Biomarkers for Allograft Nephron Damage Assessment

Following RT surgery, the kidney allograft may either immediately resume normal functionality or experience a delay of several days or even weeks, i.e., DGF. A lack of normal kidney transplant function can lead to acute kidney injury (AKI) [34][35], nephrotic syndrome (NS) [36][37], and aggravation of pre-existing chronic kidney disease (CKD) [38]. Thus, post RT, it is crucial to monitor specific biomarkers that can detect disease progression and identify which kidney functions are at risk, facilitating the prompt implementation of appropriate treatments [39][40][41]. The administration of immunosuppressants to prevent renal graft rejection can, ironically, lead to progressive renal tissue damage (such as interstitial fibrosis, tubular micro calcifications, and renal tubule atrophy), due to the high toxicity of these drugs. The majority of renal pathological changes affect the glomeruli, proximal and distal tubules, as well as the vascular endothelium.

Renal proximal tubular cells, which have the highest metabolic activity and contain large amounts of mitochondria, lysosomes, and peroxisomes, are typically the first to suffer damage. Other sections of the nephron, such as Henle's loop, distal tubules, and collecting tubules, usually sustain damage later on. There are many biomarkers available to identify injury in different areas of the renal nephron, such as the glomerulus, or the proximal and distal tubules [10].

All in all, scientific advancements in molecular biology, i.e., novel genomic, transcriptomic, proteomic, and metabolomics experimental data, have revealed an array of new, nephron-segment-specific, post-RT biomarkers for allograft damage. There are high hopes for proteins that present nephron specificities or are locally produced at the site of nephron damage. Traditional biomarkers, particularly enzymuria, still hold diagnostic value in assessing renal tubule function. While this abundance of biomarkers, in particular, may in fact reflect that their individual diagnostic value may be limited, the search for a universal integrative biomarker for allograft assessment remains challenging. Instead, identifying putative biomarker proteins useful in diagnosing key allograft disease features is likely to yield better results [10].

Multiple promising biomarkers for kidney damage have been identified, with the most relevant and best-studied being neutrophil gelatinase-associated lipocalin (NGAL), CYC, kidney injury molecule-1 (KIM-1), β2M, and interleukin-18 (IL-18) [42]. Notably, in kidney allograft recipients, urinary KIM-1 expression provides prognostic

information related to the rate of renal function decline, regardless of the underlying kidney pathology [43]. However, validation of these kidney markers in various pathological conditions is still ongoing. High diagnostic value is still held by certain enzymes in diagnosing renal diseases, such as HEX and its isoenzyme HEXB as markers of proximal tubular damage, AAP or GST as markers of the tubular brush border membrane, and cytosolic FBP-1,6 for assessing graft function [10]. A panel of urinary proteins and enzymes may serve as a practical marker for evaluating the nephron function of a transplant kidney and prognosticating the renal allograft's fate. Future biomarker discoveries and research techniques may change the practical approach to treating patients with renal grafts.

4. Biomarkers for Non-Surgical Renal Allograft Complications

Postoperative monitoring of RT patients is a critical aspect of care management [44]. Currently, the standard of care recommended is quarterly measurements of urinary protein excretion, within the first year. Moreover, screening for viral infections, i.e., Polyoma and/or Epstein–Barr virus, using plasma nucleic acid testing, should be done monthly, for at least the first three months post RT, and then every three months, until the end of the first year. A percutaneous renal allograft needle biopsy is necessary if there is an unexplained rise in serum creatinine. The Banff classification system provides standardized criteria for histological diagnosis of AR, scoring inflammation in various renal compartments [8]. However, changes in serum creatinine are not specific to graft injury: variations might indicate an intrinsic renal process like AR or graft infection, or a transient process such as the hemodynamic effects of calcineurin inhibitors or pre-renal volume depletion [44]. AR involves various stages, with clinical signs of graft damage appearing late, following a period of subclinical graft damage [45][46]. Thus, serum creatinine levels may remain unchanged despite significant kidney injury.

Moreover, biopsies can also lead to complications for the transplant recipient [47], and being an in-patient procedure, can be quite costly. Other drawbacks of allograft biopsy include potential sampling errors and/or differences in interpretation among pathologists [48]. Therefore, there is a pressing need for alternative, less invasive, yet more sensitive, post-RT biomarkers for diagnosing acute graft rejection, i.e., subclinical allograft nephron damage. Discovering and validating biomarkers that correlate with and/or can predict AR early on, thus capable of enhancing the objectivity, accuracy and overall efficacy of therapeutic decision making for clinicians, are high priorities among most ongoing RT research initiatives [13]. Through regular sampling, the development of rejection might be predicted before tissue injury actually develops. Biomarker information could also help differentiate high-risk patients from low-risk ones, facilitating individualization of immunosuppressive drug therapy.

5. Immune Tolerance and Therapeutic Drug Monitoring

Drug level monitoring is an important biomarker for assessing the proper use of immunosuppressive drugs in transplant recipients. It is commonly performed for drugs such as tacrolimus, cyclosporine, everolimus, and sirolimus [49]. However, monitoring mycophenolic acid (MPA) using single-sample drug concentrations in the

recipient's blood immediately before the next dose is administered may not accurately reflect the overall drug exposure. To overcome this limitation, MPA area under the curve estimation has been introduced as a more effective clinical tool. However, it requires multiple concentration samplings, which can be less practical, especially in pediatric patients [49][50].

In the case of tacrolimus, intra-patient variability (IPV) refers to fluctuations in blood levels over time in individual patients receiving a fixed dose. High IPV of tacrolimus has been associated with the development of DSA, allograft dysfunction, rejection, transplant glomerulopathy, and late graft loss in adult studies [51]. In pediatric studies, tacrolimus IPV has been correlated with de novo DSA development, but its correlation with rejection, decline in graft function, and graft loss is weaker. This may be due to differences in defining cut-off values, cohort size, and methodological variations [52][53].

Future perspectives in drug monitoring advocate the use of expert systems to estimate drug exposure [54], the development of novel techniques for simultaneous evaluation of multiple drugs, and a shift towards the concept of "time in therapeutic range" [55]. This concept, already employed in other medical fields, can provide more precise predictors of under-suppression and the potential risk of allograft rejection. Advancements in drug monitoring techniques and the use of more comprehensive predictors of drug exposure hold promise for improving individualized immunosuppressive therapy and optimizing transplant outcomes.

Global immunosuppression markers are important for assessing the overall intensity of immunosuppression in transplant recipients. Albeit still subject to scientific scrutiny and clinical exploration, various techniques, including flow cytometry and pathogen-specific T-cell response assays, show promise, but still require further validation and standardization [14]. These biomarkers have the potential to improve individualized immunosuppressive therapy and identify patients who can safely reduce their immunosuppression levels. Simple numeric quantitative measurements of lymphocytes have not proven to be reliable indicators, even for determining the dosage of immunosuppressive agents used for depletion induction. AR can occur even in patients with profound T-cell depletion and without additional immunosuppression [56]. One potential measure of global immunosuppression is the quantification of CD4+ T-cell adenosine triphosphate (ATP) production after polyclonal antibody stimulation in vitro [57]. This assay has only been assessed in a non-controlled trial thus far, and still lacks validation and substantial evidence of its utility, yet it has been marketed commercially as a clinical tool for post-RT monitoring [14].

Indirect assessment of global immunosuppression can be performed by quantifying biomarkers of pre-existing protective immunity. Techniques such as PCR, enzyme-linked immunosorbent spot (ELISPOT), and flow cytometry have been developed to detect pathogen-specific T-cell responses against common viral pathogens like cytomegalovirus (CMV), Epstein–Barr virus, and BK virus [58][59][60]. However, these techniques are labor intensive and lack standardization across transplant centers. The detection of IFNγ production in response to CMV peptides, using currently available, well-validated CMV immune assays, i.e., ELISPOT and/or QuantiFERON, might help standardize monitoring for this viral infection [61][62], but further characterization of the correlations between immunosuppression degree, viremia risk, and allograft rejection risk is needed. Most recently, multiple potentially impactful, novel experimental applications for immune monitoring post RT have been developed, centered around

the most abundant virus of the commensal human virome, the non-pathogenic Torque Teno Virus (TTV), i.e., an anellovirus that does not cause disease directly, but rather replicates based on the immune status of its host [63]. Thus, as TTV viremia has already previously been shown to correlate with the overall level of immunosuppression, while also predicting the occurrence of viral infections, graft rejection, and antibody response after COVID-19 vaccination in lung transplant recipients, it has now been proposed and investigated as a biomarker of functional immunity in RT patients [64]. Apparently, monitoring TTV viremia could be an additional tool for predicting CMV reactivation. However, while these TTV methods have potential in risk prediction, they have not been explicitly tested in drug titration protocols and have not clearly documented a direct drug-infection relationship [63][64].

Flow-cytometry-based assessment of lymphocyte phenotypes has been investigated as a means of gauging immunosuppression intensity. Interestingly, while T-cell phenotypes have not provided significant insights, three studies have observed a B-cell phenotype signature associated with spontaneously immuno-tolerant RT patients [65][66][67]. This unexpected association suggests that transplant recipients may have altered peripheral blood lymphocyte repertoires that warrant further investigation. If validated, an assay based on flow cytometry could be easily adopted in clinical laboratories to prospectively identify tolerant patients, allowing clinicians to reduce immunosuppression and avoid unnecessary adverse drug effects [14].

Even so, clinically stable allograft function, within acceptable parameters, under the long-term absence of immunosuppressive therapy, i.e., operational tolerance (OT), post RT, represents an exceedingly rare phenomenon, with only ~100 cases hitherto reported [68]. However, some studies have identified specific genes that are upregulated in OT patients. In different patient cohorts and using various microarrays, 39 genes were found to be elevated in OT, with 24 of them being B-cell related. CD79b and prepronociceptin were among the most highly expressed OT-related genes [65][66][69]. Furthermore, miR-142-3p was also found to be upregulated in B cells of OT patients [70].

Genomic studies have revealed gene expression changes associated with tolerance. Membrane-spanning 4-domains A1 (MS4A1/CD20), T-cell leukemia/lymphoma 1A (TCL1A), CD79b, tolerance-associated gene 1 (TOAG1), and FOXP3 genes were found to be upregulated in peripheral B cells [71]. A multicenter study reviewed a cohort of kidney transplant recipients to identify an immunosuppression-independent gene signature for predicting tolerance. They identified nine genes, including Ataxin 3 (ATXN3), BCL2-related protein A1 (BCLA1), Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), Gem-associated protein 9 (GEMIN7), Immunoglobulin lambda constant 1 (IGLC1), Membrane-spanning 4-domains A4A (MS4A4A), Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (NFκBIA), RAB40C-member of RAS oncogene family, and TNF, α-induced protein 3 (TNFAIP3) [72]. Additionally, the kidney spontaneous operational tolerance test (kSPOT) program identified 21 genes involved in OT [73]. Among them, Kruppel-Like Factor 6 (KLF6), Basonuclin 2 (BNC2), and Cytochrome P450 Family 1 Subfamily B Member 1 (CYP1B1) were used to develop a three-gene assay with high accuracy for detecting OT [67][73][74].

Overall, the pursuit of a tolerance signature in RT remains challenging due to the small number of OT patients. Biomarker studies are primarily focused on identifying OT in post-RT patients, i.e., screening applications. Various

large-scale approaches, such as kSORT, tCRM, uCRM, and kSPOT, may assist in reclassifying transplant recipients based on immune risk threshold and determining which patients can benefit from immunosuppression withdrawal or minimization [14].

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