

# World Journal of *Transplantation*

*World J Transplant* 2017 December 24; 7(6): 276-363





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*World Journal of Transplantation* (*World J Transplant*, *WJT*, online ISSN 2220-3230, DOI: 10.5500) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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**NAME OF JOURNAL**  
*World Journal of Transplantation*

**ISSN**  
 ISSN 2220-3230 (online)

**LAUNCH DATE**  
 December 24, 2011

**FREQUENCY**  
 Bimonthly

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*World Journal of Transplantation*  
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 E-mail: [editorialoffice@wjnet.com](mailto:editorialoffice@wjnet.com)  
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**PUBLISHER**  
 Baishideng Publishing Group Inc  
 7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA  
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 Help Desk: <http://www.f6publishing.com/helpdesk>  
<http://www.wjnet.com>

**PUBLICATION DATE**  
 December 24, 2017

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## Biomarkers and a tailored approach for immune monitoring in kidney transplantation

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Author contributions: Both Salcido-Ochoa F and Allen JC Jr wrote and revised the paper.

**Conflict-of-interest statement:** There is no conflict of interest between the authors or the participating institutions, and the authors do not have any financial relationships to disclose. The opinions expressed in this editorial are those of the authors and attach to the ideology of the Tregs and HLA Research Force, but might or not reflect their other scientific affiliations. The recommendations and suggestions presented by the authors are mainly aimed to inspire scientific reflections and serve as prototype for future clinical guidelines of biomarker research in kidney transplantation. The authors can be contacted for scholarly discussions, suggestions or corrections.

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Manuscript source: Invited manuscript

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Received: May 17, 2017

Peer-review started: May 23, 2017

First decision: July 20, 2017

Revised: October 16, 2017

Accepted: November 22, 2017

Article in press: November 22, 2017

Published online: December 24, 2017

### Abstract

A literature review on immune monitoring in kidney transplantation produced dozens of research articles and a multitude of promising biomarkers, all in the quest for the much sought after - but perennially elusive - "holy grail" of kidney biomarkers able to unequivocally predict acute transplant rejection *vs* non-rejection. Detection methodologies and study designs were many and varied. Hence the motivation for this editorial, which espouses the notion that in today's kidney transplantation milieu, the judicious use of disease classifiers tailored to specific patient immune risks may be more achievable and productive in the long run and confer a greater advantage for patient treatment than the pursuit of a single "omniscient" biomarker. In addition, we desire to direct attention toward greater scrutiny of biomarker publications and decisions to implement biomarkers in practice, standardization of methods in the development of biomarkers and consideration for adoption of "biomarker-driven" biopsies. We propose "biomarker-driven" biopsies as an adjunctive to and/or alternative to random surveillance (protocol) biopsies or belated indication biopsies. The discovery of a single kidney transplantation biomarker would represent a major breakthrough in kidney transplantation practice, but until that occurs - if ever it does occur, other approaches offer substantial potential for unlocking prognostic, diagnostic and therapeutic options. We conclude our editorial with suggestions and recommendations for productively incorporating current biomarkers into diagnostic algorithms and for testing future biomarkers of acute

rejection in kidney transplantation.

**Key words:** Acute rejection; Banff classification; Biomarker; Human leukocyte antigen matching; Immune monitoring; Immunological risk; Kidney transplantation; Protocol biopsy

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**Core tip:** In kidney transplantation, a multitude of biomarkers have been proposed to predict transplant rejection *vs* non-rejection, but few - if any - have gained acceptance as reliable tools for predicting rejection. However, an approach more likely to be successful would include improved timing of kidney transplant biopsies and judicious use of multiple diagnostic methodologies based on different immune risks and events throughout transplantation. This approach could also aid in improving diagnostic and prognostic kidney transplantation algorithms and in developing more impactful therapeutic options.

Salcido-Ochoa F, Allen JC Jr. Biomarkers and a tailored approach for immune monitoring in kidney transplantation. *World J Transplant* 2017; 7(6): 276-284 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v7/i6/276.htm> DOI: <http://dx.doi.org/10.5500/wjt.v7.i6.276>

## INTRODUCTION

Kidney transplantation provides kidney failure patients the best opportunity to live longer and fuller lives. Indeed, kidney transplantation is recommended as the first option for suitable patients. However, immunosuppressive drugs currently in use for kidney transplantation are not one-hundred per cent effective in preventing acute or subclinical rejection episodes, or premature transplant failure. In addition, immunosuppressive drugs bring a constellation of side effects linked to significant morbidity and mortality. Thus, until the advent of more targeted and effective, less toxic and tolerogenic immunotherapies, the best strategy appears to be tailoring current immunosuppressive ammunition to the specific immune systems affected by kidney transplant patients. However, the tailored immunosuppressive approach stands in contrast to the current protocolised indiscriminate minimisation of immunosuppression, which has proven to be counterproductive in many instances<sup>[1]</sup>. Tailoring immune monitoring strategies to a patient's particular risks of rejection, of transplant loss and of transplant-related complications would certainly be more impactful and cost-effective than the non-judicious use of "in vogue" biomarkers.

Conventional monitoring of kidney transplant patients consists of assessing dynamic changes in serum creatinine levels as well as other laboratory parameters such as proteinuria and immunosuppressive drug levels.

Additionally, some transplant programmes perform surveillance biopsies, and many measure donor-specific alloantibodies (DSA). DSA are clearly markers of an ongoing anti-allograft response and traditionally viewed as late and ominous markers of rejection that are difficult to counteract. DSA are currently under thorough evaluation in the United Kingdom<sup>[2]</sup>. Importantly, most immunosuppressive dose changes in kidney transplantation are guided by drug blood levels and their associated toxicities or as a consequence of infections or rejection episodes. But conventional immunomonitoring strategies are unable to reveal the actual state of the immune system and the body's innate defense system. It would be expected then that accurate information on the detector, effector and regulatory arms of the immune system would aid researchers in their quest for more clinically useful biomarkers with improvements in diagnostic accuracy and outcome prediction. It would be anticipated that biomarkers derived from actual immune processes occurring *in vivo* in kidney transplantation would be more likely to guide physicians in choosing the most suitable immunosuppressive strategy.

A substantial impediment to biomarker discovery and application is that the activated targetable immune pathways vary with the immune risk profile of each donor-recipient pair, as well as with the immunosuppressive regimens selected for the recipient. Making the situation even more complex, activated pathways change dynamically throughout the various transplantation stages in response to immunological and infective events occurring throughout the duration of the kidney transplant, and due to modifications in immunosuppressive drugs. Therefore, it is unlikely that one or even a few universal biomarkers can guide transplant physicians in the best use of immunosuppressive regimens throughout all stages of transplantation. However, a combination of clinical parameters and biomarkers revealing distinct immunological, inflammatory and tolerogenic processes occurring at different stages post-transplantation could provide a more useful guide to clinicians. In striving to provide a more accurate picture of the state of the immune system, and hence of the requirements for specific kidney transplant patients, the ideal strategy would complement the immune biomarker analysis with biomarkers revealing parenchymal tissue injury, repair, fibrosis and senescence. Finally, knowledge of the kinetics and interplay of these processes is essential for a proper interrogation and utilization of the biomarker universe.

## PERFECT BIOMARKER VS TODAY'S REALITY

Biomarker preferred definitions and conceptual framework have been formulated by the Biomarkers Definitions Working Group<sup>[3]</sup>. Our definition of the perfect tailored immunosuppressive biomarker combines the following properties and characteristics: (1) is easily obtained non-invasively from patients to allow

multiple and sequential analyses; (2) is easily detected and detectable prior to clinically observable events; (3) reflects physiopathogenic mechanisms; (4) demonstrates strong immunodiagnostic and theragnostic value to guide selection and changes in immunosuppressive therapies and possess immunopredictive value; (5) correlates with treatment response; (6) anticipates potential clinical outcomes before and after interventions; (7) indicates over-immunosuppression and risk of infection and cancers; (8) inexpensive with rapid turnaround time; and (9) spares the patient from a kidney transplant biopsy. However, given the complexity of the immune system and alloresponses, the perfect biomarker may be just a pipe-dream.

In kidney transplantation, urine is the most attractive sample source for non-invasive biomarker testing and discovery. Urine is also very accessible, and several urine biomarkers have shown great promise. For instance, chemokines CXCL10 and CXCL9, measured by ELISA, were found to be elevated in urine up to 30 d prior to the episode of acute rejection, and importantly, levels decreased with anti-rejection treatment and displayed prognostic value<sup>[4]</sup>. Similarly, higher levels of urinary transcripts for cytotoxic cell products like perforin and granzyme B are found in patients with rejection as opposed to non-rejection states<sup>[5,6]</sup>. Despite the anatomical relationship with the transplant, the kidney does not leak all molecules released by the immune system or injured parenchymal cells into the urine. Many of the leaked molecules are not reliable surrogate markers of rejection, and are even less reliable as markers of tolerance.

On the other hand, whole blood and serum are very accessible, and transcripts for cytotoxic cell products like granzyme B, perforin and granulysin top the list of promising biomarkers to differentiate rejection from non-rejection<sup>[7]</sup>. However, many of the molecules participating in transplant rejection or inflammation are not leaked into the blood compartment or they are diluted. Many cells involved in alloimmune processes and detectable in tissue<sup>[8]</sup> remain or die inside the kidney, or migrate preferentially to draining lymph nodes, which make them inaccessible to the physician's tools. In spite of these limitations, alloreactive memory/effector T cell responses in peripheral blood using an IFN-gamma ELISPOT<sup>[9]</sup>, and the detection of a 17-gene set in peripheral blood using the so-called kidney solid organ response test (kSORT)<sup>[10]</sup> have shown promise to identify kidney transplant rejection at both the subclinical and clinical stages. Thus, as physicians we must learn to take full advantage of available biomarkers by using them in the correct combinations and at optimal sampling times post-transplantation.

It is important to remember that in many cases serum creatinine levels and glomerular filtration rate are of uninformative for detecting kidney transplant dysfunction as a consequence of rejection. Elevation of serum creatinine levels occurs late in the rejection process and indicates overt kidney transplant injury and

nephron loss. At this point, significant alloaggressive mechanisms have commenced, portending the possibility of permanent and irreparable tissue damage and increased risk of refractory rejection. In addition, serum creatinine monitoring precludes the possibility of detecting acute rejection pre-emptively at the state of subclinical rejection. Moreover, small elevations of serum creatinine indicating initiation or progression of the rejection process, may be ignored by patients and physicians with opportunity for early intervention delayed. Serum creatinine is recognized as an imperfect marker for acute kidney dysfunction and a very poor marker for acute rejection; however, its utility might be augmented if taken in combination with other promising non-invasive biomarkers, including certain cytotoxic cell products described above<sup>[4-7]</sup> or others. Taken in combination, immunodiagnostic and immunopredictive properties might be enhanced.

It would be absurd to suggest that urine and blood biomarkers - given the current state of the art - are able to replace kidney transplant biopsy, which is the gold standard for diagnosis of allograft rejection<sup>[11]</sup>. However, the realistic and practical utility of these biomarkers would be to aid physicians in decisions that ultimately expedite a confirmatory transplant biopsy and initiation of anti-rejection therapy thereby minimizing damage to the kidney and enhancing chances of therapeutic success.

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## CURSE OF THE SPECIFIC "MAGICAL" BIOMARKER

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The kidney transplant literature is rife with research in pursuit of a "magical" biomarker capable of identifying onset of kidney transplant rejection with perfect accuracy - with an aim to supplanting the kidney transplant biopsy. But inevitably, pre-study optimism is confuted by post-study outcomes demonstrating that tested markers are not specific for kidney rejection - they cannot distinguish indicators of rejection from those of other disease processes such as BK virus infection, non-rejection sources of inflammation or nonspecific tissue injury. Markers are then labelled as "not-very useful" and dismissed - a possibly premature verdict considering a marker might be still useful for signalling at least that some pathologic events are in progress in the transplant kidney and thereby alerting to the need of a confirmatory biopsy.

For researchers engaged in the perennial search for a biomarker to replace the kidney transplant biopsy, a concomitant enterprise could be mining the depth and breadth of information that remains untapped in a transplant biopsy. It is highly unlikely that urine or blood markers can surpass those extracted from the transplant kidney and the draining lymph nodes as more informative of the condition of the kidney transplant - the invasive nature of biopsy and inaccessibility of lymph nodes notwithstanding. In today's world of kidney transplants, current non-invasive biomarkers will not be perfect predictors as they reveal only partially the complex

interplay of immune and non-immune factors and events occurring inside the kidney transplant. However, we can use them more effectively by understanding their precise biological meaning and clinical value.

On the other hand, tissue biomarkers, classifiers and archetypes obtained from the molecular microscope on kidney transplant biopsies, when combined with the constellation of non-invasive biomarkers, could give physicians the most comprehensive and accurate information upon which to base therapeutic decisions. In this respect, citing the INTERCOMEX Study, the analysis of transcripts in kidney transplant biopsies was able to classify patients with acute kidney transplant dysfunction with high accuracy in those having pure T cell-mediated rejection (TCMR), antibody-mediated rejection (ABMR), mixed rejection and no rejection<sup>[12]</sup>.

The most effective solution - although not the simplest - will involve a finer dissection of the immunopathogenesis of rejection. The purpose would be to achieve greater understanding of the biological meaning and derivation of the presently available biomarkers and potential new biomarkers, to rank them physiopathologically and address their clinical contributions individually and in combination with other biomarkers.

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## **SURVEILLANCE KIDNEY TRANSPLANT BIOPSIES RELOADED**

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Surveillance kidney transplant biopsies play an important role in kidney transplant immune monitoring, especially in patients at high immunological risk for antibody-mediated rejection whose biopsies were performed in the early stages post-transplantation when risk of rejection is higher. The purpose of surveillance biopsies is straightforward: To find remediable problems as early as possible. However, many centres do not perform surveillance biopsies for various reasons, including the following: Biopsies are not part of their academic culture, feasibility issues, historic poor yields and/or poor outcomes - making crucial judicious patient selection - or use of more effective combinations of immunosuppressive drugs. However, surveillance biopsy schedules tend to be somewhat arbitrary and unit specific. They reflect varying physician experience and thresholds among transplant units and are imperfect in consequence of the limited and equivocal signs and symptoms manifested by the alloresponses. Thresholds adjudged warranting a kidney transplant biopsy vary among transplant units and physicians - even thresholds attributable to indication biopsies (also referred to as for-cause or episode biopsies) - when something is obviously going wrong. In addressing arbitrariness in selecting surveillance biopsy time points, current and future biomarkers could be designed not only for diagnosis of rejection - as they might not replace biopsy - but to identify the onset of specific problems or simply to confirm with a transplant biopsy when something wrong (yet to be defined) is occurring at the

subclinical stage. These types of biopsies would not be called surveillance biopsies or indication biopsies, but might be referred to as "biomarker-driven or biomarker-triggered biopsies". Biomarker-driven biopsies would enhance the diagnostic yield of the biopsy procedure as accuracy would likely be higher than a conventional and arbitrarily mandated protocol surveillance biopsy, and they would be more opportune than an indication biopsy. An exciting prospect is the potential to enhance the diagnostic yield and outcome prediction potential of any surveillance, indication or "biomarker-triggered" transplant biopsy by coupling gene expression analysis (the molecular microscope) with the conventional histopathologic grading of the Banff classification like in the INTERCOMEX Study<sup>[12]</sup>.

Inherent in the concept of a "biomarker-triggered" transplant biopsy, is the notion of a more impactful search for biomarkers of subclinical rejection rather than markers of acute rejection. Subclinical rejection biomarkers could trigger an opportune diagnostic kidney transplant biopsy enabling initiation of anti-rejection strategies much earlier. Performance of a marker of acute rejection might not be as good if tested for utility in identifying subclinical rejection. Nevertheless, biomarkers of acute rejection could still have a role in confirming suspicious cases of rejection, as prognosticators of transplant outcomes, or for hypothesis generation in the search for novel biomarkers of subclinical rejection.

Although kidney transplant biopsy is considered the gold standard for diagnosing acute rejection, it is far from ideal. The vision provided of what is occurring reveals patchy, non-uniform rejection throughout the kidney tissue. Consequently, acute rejection can be missed by performing biopsies in randomly selected areas of the kidney transplant. In addition, a biopsy cannot quantify the degree to which the renal parenchyma is inflamed. One possible solution - not yet developed - is an imaging technique that could give a quantifiable assessment of inflammation in the kidney parenchyma. Imaging findings in combination with biopsy results would allow quantification of the extent of rejection and guide better "tailoring" of corrective immunosuppression after rejection episodes.

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## **STATISTICAL ADVANTAGE IN IMMUNE MONITORING**

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From the discussion above and the examples presented, we can also expect that the development of predictors for immune monitoring strategies that incorporate multiple biomarkers, as opposed to just a single biomarker, would have the greatest potential for considerably enhancing prognostic accuracy, especially if incorporated into comprehensive monitoring algorithms that include clinical parameters. One approach to accomplishing this more effectively would be to incorporate tests assessing different biomarkers in prospective studies and clinical trials under more controllable and less heterogeneous

circumstances to investigate potential utility as predictors in kidney transplantation. In clinical trials, biomarkers could be investigated as therapeutic markers to guide the use of interventions or assess response to interventions thereby providing data enabling better kidney transplant outcomes.

## LAYING THE FOUNDATIONAL STONES IN IMMUNE MONITORING

A better understanding of the immunopathogenesis of kidney transplant rejection and the mechanisms of immune adaptation that could potentially lead to transplant tolerance is crucial for the development of more accurate and precise biomarkers in kidney transplantation.

Technological advances now allow us to interrogate the immune system in peripheral blood and other fluids and tissues of kidney transplant patients that give a multidimensional and multifaceted perspective. We are currently able to obtain a very detailed picture of the state of many genes involved in the body's response to kidney transplantation, specifically of their transcriptional and translational products. Nevertheless, a multitude of genetic interactions, their hierarchy and precise clinical translation remain to be deciphered. Sophisticated biomolecular technologies and mass spectrometry-based technologies are robust to identify and discover novel biomarkers, which once validated, will open the way for implementation of other less expensive and more accessible technologies to serve in the clinical detection of those biomarkers. Thus, a multidimensional and multisystem interrogation of different biological systems in kidney transplantation would provide a combinatorial (phenotypic and functional picture) of the actual state of the immune system and its inter-relationships with other bodily systems. Well-equipped and experienced labs will be able to eventually reveal the secret world underlying alloresponses, especially if they commit their full resources and capabilities to achieving the goal.

Until the advent of more robust non-invasive biomarkers able to detect subclinical rejection with greater accuracy, *i.e.*, "biomarker-triggered transplant biopsies", protocolled surveillance biopsies and indication biopsies will continue to play a central role in the discovery of molecular signatures and the evaluation and correlation of novel biomarker candidates.

A comprehensive review article on different types of biomarkers tested and those showing promise in kidney transplantation immunodiagnosis was published recently in this journal<sup>[13]</sup>. However, more critical reviews of the available literature are needed to identify the most promising biomarkers. Admittedly, this is a difficult task given the multitude of biomarker candidates obtained from diverse sources using a range of technologies in typically heterogeneous patient populations. Thus, laboratories aiming to discover and validate biomarkers should consider protocol standardization and judicious selection of testing time points as essential

elements of adequate and well-controlled biomarker-led clinical trials. The creation of advisory and work groups, and opportunities for collaboration and grant applications, should also be promoted with the ultimate aim of advancing the science of biomarker use and immunomonitoring in kidney transplantation.

## RECOMMENDATIONS AND SUGGESTIONS FOR USING BIOMARKERS AND SURVEILLANCE BIOPSIES IN KIDNEY TRANSPLANTATION

It is quite apparent that we are still far from finding biomarkers that can supplant kidney transplant biopsy. Nevertheless, we can proceed methodically and persistently, perhaps not expecting to find the "magical" biomarker but towards a more in-depth and informative interrogation of the patient immune system. With this view in mind, our recommendations and suggestions for utilizing and testing biomarkers in kidney transplantation are summarized in Table 1. These are presented in the context of eight scenarios representing somewhat typically encountered cases. Given the complexity of clinical kidney transplantation, they are by no means all-inclusive or exhaustive. For each scenario, the necessity for customization in addressing different immunological risks should be recognized. Challenges confronting researchers engaged in biomarker development and utilization in kidney transplantation are encountered as well in other branches of nephrology (*e.g.*, biomarkers of acute kidney injury) and other disciplines of Medicine. We believe that the recommendations and suggestions offered have general applicability in other areas of biomarker research. Standard measures for assessing kidney transplant status are omitted from Table 1 as they are standard practice. Therapeutic recommendations or choice of immunosuppressants are not given as they are not within the scope of our biomarker-centred recommendations and suggestions. The interested reader is referred to the references cited<sup>[1,14]</sup>.

## IMMUNOLOGICAL RISK AND HOW IT AFFECTS BIOMARKER RESEARCH

Approaches for objective quantification of immunological risk have been attempted but as yet no reliable risk score has been developed. Immunological risk depends largely on the distinct genetic and antigenic differences between recipients and donors (along with other factors), type and amount of immunosuppression used, the degree of activation of the innate defense system and the set of dynamic alloresponses occurring throughout transplantation. The current or proposed attempts to quantify immunological risk would require an editorial or review article of its own - which will likely come with imperfect approximations - but we would like to bring attention to an important point, which is the



**Table 1 Recommendations and suggestions on the incorporation of biomarkers and surveillance biopsies in kidney transplantation**

Scenario A: Patients with acute kidney transplant dysfunction on whom a kidney transplant biopsy has been performed to exclude rejection

**Recommendations**

- A1 Diagnose rejection if present in kidney transplant biopsies according to the Banff classification (using the most current update; now the 2015 update), and report it in a systematic way
- A2 Quantify BK viremia<sup>a</sup> and BK virus (BKV) nephropathy by specific staining
- A3 Detect anti-HLA antibodies/DSA<sup>d</sup> and define their immunoglobulin class, complement fixing capacities and titres through dilutions

**Suggestions**

- A4 Bank serum, plasma, urine, peripheral blood mononuclear cells (PBMC) and kidney transplant tissue for future biomarker research<sup>c</sup>
- A5 Exclude active infection by cytomegalovirus (CMV) and Epstein-Barr virus (EBV)<sup>a</sup>
- A6 Generate a data base with detailed clinical and immunological variables, ideally, using a standardized data base from a consortium or a large multicentre/multinational collaboration
- A7 Test any experimental biomarker(s) of your choice and correlate it/them with standard clinical variables and a detailed immune profile. The use of validated disease classifiers and archetypes appears to have more diagnostic accuracy than the use of single biomarkers
- A8 Perform a surveillance biopsy if kidney function and other clinical or laboratory parameters do not improve as expected after treatment to exclude persisting rejection or transformation to another type of rejection<sup>b</sup>

Scenario B: Patients with acute kidney transplant dysfunction on whom a kidney transplant biopsy is being considered to exclude rejection

**Recommendations**

- B1 Quantify BK viremia<sup>a</sup>
- B2 Detect anti-HLA antibodies/DSA<sup>d</sup> and define their immunoglobulin class, complement fixing capacities and titres through dilutions; and perform a kidney transplant biopsy if DSA are detected
- B3 Use validated disease classifiers and archetypes (if available) to enhance to pre-test probability for rejection, and perform a kidney transplant biopsy if positive
- B4 If a kidney transplant biopsy is performed, consider the recommendations and suggestions for Scenario A

**Suggestions**

- B4 Bank serum, plasma, urine and PBMC for future biomarker research<sup>c</sup>
- B5 Exclude CMV and EBV infection<sup>a</sup>
- B6 Generate a data base with detailed clinical and immunological variables, ideally, using a standardized data base from a consortium or a large multicentre/multinational collaboration
- B7 Test any experimental biomarker(s) of your choice and correlate it/them with standard clinical variables and a detailed immune profile. The use of validated disease classifiers and archetypes appears to have more diagnostic accuracy than the use of single biomarkers

Scenario C: Patients with: (1) stable kidney function; (2) low immunological risk for ABMR with lack of preformed DSA; and (3) low immunological risk for TCMR or for the synthesis of *de novo* DSA due to no or low degree of HLA mismatch<sup>[16-18]</sup>

**Recommendations**

- C1 Detect anti-HLA antibodies/DSA<sup>d</sup> after a sensitization event (transfusions, pregnancies or other transplants *e.g.*, pancreas after kidney transplantation) and define their immunoglobulin class, complement fixing capacities and titres through dilutions
- C2 Perform a kidney transplant biopsy if DSA are detected, diagnose it according to the Banff classification 2015 update and exclude intra-graft BKV infection by specific staining
- C3 In case of kidney dysfunction, consider the recommendations and suggestions for Scenarios A or B

**Suggestions**

- C4 Test any experimental biomarker(s) of your choice at pre-selected time points and correlate it/them with standard clinical variables and a detailed immune profile. Select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry
- C5 Consider surveillance biopsies that exclude subclinical rejection and banking of kidney transplant tissue for biomarker research<sup>c</sup>. Recommendation to select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry
- C6 Detect anti-HLA antibodies/DSA<sup>d</sup> at your pre-selected time points, to define their immunoglobulin class, complement fixing capacities and titres through dilutions, and correlate them with standard clinical variables and a detailed immune profile. Select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry. There are published consensus guidelines<sup>[19]</sup>, but their recommendations are relatively arbitrary as well
- C7 Bank serum, plasma, urine and PBMC at your pre-selected sampling time points and when kidney biopsies are performed<sup>c</sup>
- C8 Exclude CMV and EBV infection<sup>a</sup>
- C9 Perform a biomarker-driven biopsy if your chosen validated biomarker for rejection (or any other anomaly) turns positive, and bank tissue for further biomarker research

Scenario D: Patients with: (1) stable kidney function; and (2) high immunological risk for ABMR due to preformed DSA (desensitized or not)

**Recommendations**

- D1 Ensure adequate levels of immunosuppression and prevent non-compliance with treatment<sup>e</sup>
- D2 Perform surveillance biopsies to exclude subclinical rejection and banking of kidney transplant tissue for biomarker research<sup>c</sup>. Select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry, but available guidelines<sup>[19]</sup> recommend them within the first 3 (or 6) mo post-transplantation
- D3 Monitor anti-HLA antibodies/DSA<sup>d</sup> and define their immunoglobulin class, complement fixing capacities and titres through dilutions at your pre-selected time points and correlate them with standard clinical variables and a detailed immune profile. Select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry; although there are published consensus guidelines<sup>[19]</sup>
- D4 Detect anti-HLA antibodies/DSA<sup>d</sup> after a sensitization event (transfusions, pregnancies or other transplants, *e.g.*, pancreas after kidney transplantation) and define their immunoglobulin class, complement fixing capacities and titres through dilutions
- D5 Perform a kidney transplant biopsy if DSA are detected, to diagnose it according to the Banff classification 2015 update and exclude intra-graft BKV infection by specific staining

- D6 Perform a biomarker-driven biopsy if your chosen validated biomarker for rejection (or any other anomaly) turns positive, and bank tissue for further biomarker research
- D7 In case of kidney dysfunction, we recommend to perform a kidney transplant biopsy and to consider the recommendations and suggestions for Scenario A

Suggestions

- D8 Test any experimental biomarker(s) of your choice at pre-selected time points and correlate it/them with standard clinical variables and a detailed immune profile. Select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry
- D9 Bank serum, plasma, urine and PBMC at your pre-selected sampling time points and when kidney biopsies are performed<sup>d</sup>
- D10 Exclude CMV and EBV infection<sup>a</sup>

Scenario E: Patients with: (1) stable kidney function; (2) high immunological risk for TCMR and for the synthesis of *de novo* DSA due to high degree HLA mismatch<sup>[16-18]</sup>; and (3) without preformed DSA

Recommendations

- E1 Ensure adequate levels of immunosuppression and prevent non-compliance with treatment<sup>e</sup>
- E2 Detect anti-HLA antibodies/DSA<sup>d</sup>, especially in those with HLA-B and HLA-DRB1 mismatches, thought to be more immunogenic<sup>[16]</sup>, at your pre-selected time points and correlate them with standard clinical variables and a detailed immune profile. Define immunoglobulin class, complement fixing capacities and titres through dilutions. Select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry, although there are published consensus guidelines<sup>[19]</sup>
- E3 Detect anti-HLA antibodies/DSA<sup>d</sup> after a sensitization event (transfusions, pregnancies or other transplants, *e.g.*, pancreas after kidney transplantation) and define their immunoglobulin class, complement fixing capacities and titres through dilutions
- E4 Perform a kidney transplant biopsy if DSA are detected, diagnose according to the Banff classification 2015 update and exclude intra-graft BKV infection by specific staining
- E5 In case of kidney dysfunction, perform a kidney transplant biopsy, especially in those with HLA-B and HLA-DRB1 mismatches, thought to be more immunogenic, and consider the recommendations and suggestions for Scenario A

Suggestions

- E6 Test any experimental biomarker(s) of your choice at pre-selected time points and correlate it/them with standard clinical variables and a detailed immune profile. Select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry
- E7 Suggest surveillance biopsies exclude subclinical rejection and banking of kidney transplant tissue for biomarker research<sup>c</sup>. Select time points based on the modal distribution of rejection in a specific population of patients with similar immunological risk, ideally derived from your own registry
- E8 Bank serum, plasma, urine and PBMC at your pre-selected sampling time points and when kidney biopsies are performed<sup>d</sup>
- E9 Exclude CMV and EBV infection<sup>a</sup>
- E10 Perform a biomarker-driven biopsy if your chosen validated biomarker for rejection (or any other anomaly) turns positive, and bank tissue for further biomarker research

Scenario F: Patients with: (1) stable kidney function; (2) high immunological risk for ABMR due to preformed DSA; and (3) high immunological risk for TCMR and for the synthesis of *de novo* DSA due to high degree HLA mismatch<sup>[16-18]</sup>

Recommendation

- F1 Follow our recommendations and suggestions for Scenarios D and E

Scenario G: Patients with delayed graft function (DGF)

Recommendations

- G1 Perform a kidney transplant biopsy if DGF extends beyond the first week post-transplantation without an obvious explanation, and subsequently every 7-10 d if DGF persists<sup>[14]</sup>
- G2 Detect anti-HLA antibodies/DSA<sup>d</sup> if DGF extends beyond the first week post-transplantation without an obvious explanation, and subsequently every 7-10 d if DGF persists, and define their immunoglobulin class, complement fixing capacities and titres through dilutions
- G3 Perform a kidney transplant biopsy if DSA are detected, to diagnose it according to the Banff classification 2015 update and exclude intra-graft BKV infection by specific staining

Suggestions

- G4 Define lower threshold for performing a kidney transplant biopsy in patients with DGF and pre-formed DSA or with HLA-B and HLA-DRB1 mismatches thought to be more immunogenic<sup>[16]</sup>
- G5 Bank serum, plasma, urine and PBMC at the protocolised sampling time points and when kidney biopsies are performed<sup>d</sup>
- G6 Bank kidney transplant tissue for biomarker research whenever a biopsy is performed<sup>c</sup>
- G7 Test any experimental biomarker(s) of your choice at protocolised time points and correlate it/them with standard clinical variables and a detailed immune profile<sup>c</sup>
- G8 Perform a biomarker-driven biopsy if your chosen validated biomarker for rejection (or any other anomaly) turns positive, and bank tissue for further biomarker research
- G9 Exclude active CMV and EBV infection<sup>a</sup>

Scenario H: Every kidney transplant patient included in a clinical trial

Recommendations

- H1 Bank serum, plasma, urine and PBMC at the protocolised sampling time points and when kidney biopsies are performed<sup>d</sup>
- H2 Bank kidney transplant tissue for biomarker research whenever a biopsy is performed<sup>d</sup>
- H3 Test any experimental biomarker(s) of your choice at the sampling points established by the trial designers and correlate it/them with
- H4 Consider performing surveillance biopsies at important assessment points as per trial protocol (which can help to exclude subclinical rejection and to assess histopathological response to interventions) and banking of kidney transplant tissue for biomarker research<sup>c</sup>

<sup>a</sup>These infections can present with kidney dysfunction, trigger or appear around a rejection episode, but importantly viraemia, especially at high levels, will elicit cytotoxic-type and other immune responses that can interfere with the interpretation of biomarkers. <sup>b</sup>This is another opportunity for biomarker testing, especially if its kinetics post-treatment are known or being tested. <sup>c</sup>When banking samples, we suggest to process them and store them with the vision that they could be analysed using different technologies (*e.g.*, RNA- or proteomics-friendly sample processing), even if those technologies are not available in your lab, as the research world is developing towards more constructive collaborations and cross-validation approaches. In such way,

laboratories will end up with legacy sample banks from highly characterized patients with several follow up times points, in which future technologies (pending improvements or not developed yet) could be easily applied, saving huge time to researchers (no further recruitment and sample acquisition), minimizing the risk of including patients to similar protocols (just because the technology has changed) and maximizing previous patients effort and kindness; at least for pilot, exploratory and cross-validation studies. Seek advice on how to maximise your sample banking from an experienced laboratory. Strict protocols should be devised and followed up and biobanking details of the samples should be recorded (time and date of collection, type of tube, type of anti-coagulant, additives for preservation, if centrifuged the speed of centrifugation in “g”, sample processor – if a person – or a machine, etc.). It is important to consider the easiness of the retrieval process of the data as it is inputted (any free text or absence of drop-down lists from choice answers will result in manual-dependent retrieval, which will be time consuming and expensive. <sup>3</sup>We recommend high resolution tissue typing of HLA-A; -B; -C; -DP; -DQ; and -DRB1,3,4,5 alleles for both donor and recipient. This will ensure more accurate detection of anti-HLA DSA, and the use of algorithms to assess degree HLA mismatching like the HLAMatchmaker<sup>[17,18]</sup>. <sup>6</sup>This recommendation is important for every kidney transplant patient, but seems crucial for patients with augmented immunological risk. <sup>4</sup>For clinical trials, we prefer to recommend rather than just suggest the inclusion of biomarker testing as the incorporation of biomarkers in diagnostic well-designed clinical trials is the best channel to validate biomarkers in a standardized controlled setting and maximize all the benefits from the trial.

differentiation of risk conferred by pre-formed anti-human leukocyte antigen (HLA) alloantibodies from risk conferred by HLA mismatches.

Many centres stratify patients according to the degree of immunological risk based primarily on presence or absence (or titres) of preformed anti-HLA alloantibodies (greater risk if DSA) and cross-match characteristics, and whether or not they have been desensitized. Some centres pay appropriate attention to the degree of HLA mismatches but others do not. Thus, a patient with a negative crossmatch and no anti-HLA antibodies could be deemed in some programmes to have a low immunological risk even with a high degree of HLA mismatch. This type of stratification, based on the presence of preformed alloantibodies, represents risk primarily for immediate or early ABMR due to preformed DSA, particularly in the absence of desensitization or subsequent ABMR episodes (either acute or chronic) and depicts previous sensitization events in the recipient (e.g., pregnancies, transfusions, previous transplants). However, the immunological risk derived from the degree of HLA mismatch between recipients and donors must be considered more explicitly. The antigenic differences provided by the donor genes not present in the recipient are the main drivers of strong *de novo* alloresponses. These can trigger either the development of TCMR or the formation of *de novo* DSA with consequent progression to ABMR<sup>[15]</sup> or both, especially when current immunosuppression is not 100% effective to prevent rejection. In fact, pre-formed alloantibodies are derived from the same principle, *i.e.*, from mismatches in HLA molecules (or other polymorphic antigens) between the fetus and the mother, and the blood or tissue donor and the “pre-transplant” recipient. The degree of HLA mismatch has been traditionally quantified by counting, enumerating or stratifying the number and type of HLA mismatches<sup>[16]</sup>, but more robust algorithms like the HLAMatchmaker<sup>[17,18]</sup> that more specifically assess HLA epitope mismatches can be applied to assess the risk for TCMR (acute or chronic variants) and synthesis of *de novo* DSA. To make things even more complex, kidney transplant patients usually have a combination of immunological factors that put them at risk for both types of rejection. So our recommendations and suggestions have to be tailored to the specific clinical and immunological characteristics of specific patient

populations, and they would need to be implemented in the context of other available useful guidelines<sup>[14,19]</sup>.

## ACKNOWLEDGMENTS

We would like to thank Dr Maria Hernandez-Fuentes, an expert in the field of immune monitoring in solid organ transplantation, for reviewing our manuscript and her useful comments. Similar degree of gratitude goes to the four anonymous reviewers, whose constructive comments increased the quality of this paper. Once more, our deep gratitude to Ms Rachel Liew, our library technician, for helping us finding the least accessible references.

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**P- Reviewer:** Fulop T, Lu K, Sheashaa HA, Taheri S **S- Editor:** Kong JX  
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