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Detection of Antibody-Mediated Rejection in Kidney Transplantation and the Management of Highly Sensitised Kidney Transplant Recipients

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1. Introduction

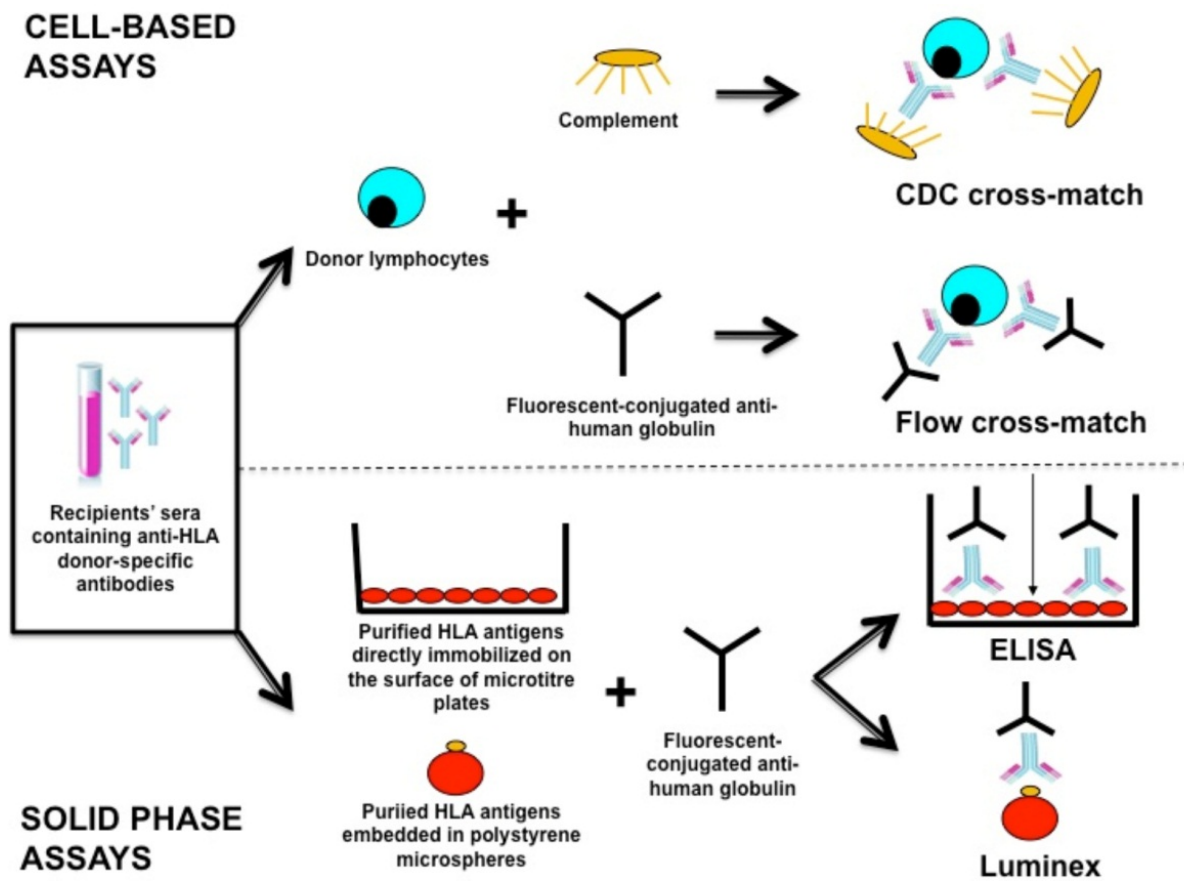
With the evolution in our understanding of the human leukocyte antigen (HLA) system, there have been substantial improvements in the HLA-typing techniques and the ability to detect anti-HLA antibodies, allowing accurate assessment of immunological risk among potential renal transplant candidates. Specifically, flow cytometry and the solid phase assay such as the enzyme-linked immunosorbent assay (ELISA) and Luminex technology have improved the sensitivity of detecting low levels class I and II donor-specific anti-HLA antibodies (DSA). Although there is now established evidence showing the presence of DSA is associated with a greater risk of antibody-mediated rejection (AMR) and early graft loss, the clinical significance of low levels DSA remains unclear. As a result of prior sensitizing events, there has been an expansion in the number of highly sensitized transplant candidates with multiple anti-HLA antibodies. Management of these candidates for the preparation of transplantation continues to be a subject of intense debate. In this chapter, we will discuss the identification of potential clinically relevant DSA detected by the different assays including the 'acceptable' level of clinically significant DSA and the advantage of C1q-positive DSA in further stratifying the immunological risk of transplant candidates. The association between DSA and non-DSA with graft and patient outcomes following kidney transplantation will be discussed in greater detail. Furthermore, we will examine the transplant outcomes of highly sensitized patients undergoing desensitization regimens and to determine the optimal desensitization regimens along with their risks and benefits.

2. Evolution of techniques to detect donor-specific anti-HLA antibodies (Figure 1)

HLA forms part of the major histocompatibility complex (MHC) in humans and MHC antigens are an integral component of the normal functioning of the human immune system. HLA antigens play a crucial role in the recognition of self-antigens and are therefore crucial in the defence of foreign antigens, including donor antigens in solid organ transplantation. HLA antigens are comprised of both class I and II antigens, with class I antigens being expressed on all nucleated cells, whereas class II antigens are being expressed on antigen presenting cells, B cells and endothelial cells [1]. The evolution in our understanding of the HLA system is closely linked to advancements in technology. Traditional serological-based (i.e. antibody-based) low-resolution techniques have been the standard method for HLA typing, enabling efficient and effective anti-HLA antibody detection. However, these techniques are dependent on the availability of specific cell types, cell viability and appropriate anti-sera that are capable of recognising HLA antigens. The emergence of molecular HLA typing techniques over the past two decades has allowed for a more specific and robust method of high resolution HLA typing. In 1982, *Wake et al* described restriction fragment length polymorphism (RFLP), which eventually highlighted the shortcomings of serology-based methods ensuing the establishment of molecular-based HLA-typing for routine detection of anti-HLA antibodies pre-transplantation [2]. Data generated via the genome project and the initiation of polymerase chain reaction (PCR) techniques through the 1980s further refined DNA-based techniques for HLA-typing, which has led to the development of a number of PCR-based techniques still in use to the present day.

Alongside with the advances in the typing of HLA alleles, the techniques used to detect anti-HLA antibodies has evolved from CDC assays to the more sensitive techniques including flow-cytometry and solid-phase assays (e.g. enzyme-linked immunosorbent assay [ELISA] or Luminex), allowing for accurate assessment of pre-transplant immunological risk (e.g. calculated panel reactive antibodies to determine level of sensitization and application of virtual cross-match to determine transplant suitability) [3] (Figure 4).

Since the recognition of the clinical importance of CDC assay in kidney transplantation in the 1960s, CDC cross-match has become the foundation of determining transplant suitability in kidney transplantation [4]. CDC cross-match can detect donor-specific anti-HLA antibodies that may have the potential to induce an anti-HLA antibody-associated hyperacute rejection following transplantation. Donor T and B cells are isolated from peripheral blood mononuclear cells using density gradient separation and incubated in the presence of recipients' sera and complements. If donor-specific anti-HLA antibodies are present, these will bind to specific antigen(s) expressed on donor cells, and with the addition of rabbit serum as a source of exogenous complement, will result in the initiation of the classical complement cascade causing direct damage to the donor cell membrane and therefore making these cells permeable to an important dye. The percentage of cell lysis is quantified and forms the basis of determining transplant candidate's suitability for transplantation with a lysis score of 20% generally considered a contraindication for transplantation. Many laboratories perform CDC assays in



Assays	Complement fixing vs non-complement fixing antibodies	Identify specific HLA antigens	Quantify anti-HLA antibodies	Problems
CDC-XM	Complement fixing antibodies	No	No	No cell targets that expressed only class II antigens
FCXM	Complement and non-complement-fixing antibodies	Yes (class I and II)	Yes	Too sensitive Costly
ELISA	Complement and non-complement-fixing antibodies	Yes (class I and II)	Yes	Too sensitive Costly
Luminex	Complement and non-complement-fixing antibodies	Yes (class I and II)	Yes	Too sensitive Costly

HLA – human leukocyte antigen, CDC-XM – complement-dependent cytotoxicity cross-match, FCXM – flow cytometric cross-match, ELISA – enzyme-linked immunosorbent assay.

Figure 1. Detection of anti-HLA antibodies – differences between cell-based and solid-phase assays.

the presence of anti-human globulin, which augments the sensitivity of this assay by increasing the number of Fc receptors available to bind complements, and/or dithiothreitol (which breaks

down the disulfide bonds in IgM antibodies believed to be of no clinical significance) to reduce the false positivity of these assays [5, 6]. Initial studies evaluating the clinical validity of CDC assays demonstrated that 80% of CDC cross-match–positive kidney transplants and 4% of cross-match–negative kidney transplants were associated with early graft loss, thereby verifying the clinical significance of anti-HLA antibodies in renal transplantation. It is noteworthy that 20% of patients transplanted across a positive cross-match did not lose their grafts [3]. Given that T cells express class I antigens and B cells express both class I and II antigens, the interpretation of T cell together with B cell cross-match will assist in establishing whether class I and/or II anti-HLA antibodies are present. A positive B cell CDC cross-match invariably accompanies a positive T cell CDC cross-match but this may reflect either anti-HLA antibodies to class I antigens and/or multiple antibodies to class I and/or II antigens. However, a positive B cell CDC cross-match may occur in the absence of a positive T cell CDC cross-match and suggest the presence of class II antigens or low levels class I antigens. The presence of a positive T cell CDC cross-match is an absolute contraindication for transplantation whereas a positive B cell cross-match is a relative contraindication because of the uncertainty regarding the clinical significance and the chance of false-positive results [7, 8]. The presence of a positive T cell cross-match is an absolute contraindication for transplantation within the deceased donor kidney allocation algorithm in Australia and New Zealand. \On the contrary, B cell cross-match is not routinely performed and therefore not utilized in the decision-making process for transplantation. With the increasing recognition of the potential importance of a positive CDC B cell cross-match, these results are now often interpreted in the context of solid phase assays. The immunological risk of potential renal transplant candidates are established by regular monitoring and storage of their sera to establish peak and current immune reactivity against a panel of donor cells, termed peak and current panel reactive antibodies. When a potential donor becomes available, donor cells are incubated in the presence of both peak and current sera. The presence of a positive CDC cross-match with peak sera even in the presence of a negative CDC cross-match with current sera poses a contraindication to transplantation, as this suggests suggest immunological memory to donor antigens from prior sensitizing events.

The inability to correlate all graft losses to anti-HLA antibodies detected using CDC assays (i.e. an inability of CDC assays to detect low levels of clinically significant anti-HLA antibodies) has led to the development of the more sensitive cell-based flow cytometric cross-match assays. The fundamental principle that forms the basis of the flow cytometric cross-match assay is similar to that of the CDC assay. Since the description of this assay in the early 1980s, this technique has been widely adopted to determine transplant suitability in many countries [9]. Similar to the CDC assay, flow cytometric cross-match assays require the addition of donor cells to recipients' sera, followed by the addition of a fluorescein-labelled secondary antibody allowing for the detection and quantification of anti-HLA antibodies by flow cytometer expressed as mean channel shifts. Unlike CDC cross-match, flow cross-match identifies both complement-fixing and non-complement-fixing anti-HLA donor-specific antibodies. However, the availability of different subtypes of detection antibodies has allowed clinicians to differentiation between complement-fixing versus non-complement-fixing anti-HLA antibodies [10]. Although an universal mean channel shifts cut-off value corresponding to positive flow cross-match has not been determined, it is generally accepted that the use of a low cut-

off value may disadvantage many transplant candidates as it may detect anti-HLA donor specific antibodies of no clinical significance, especially in the presence of negative CDC cross-match. Nevertheless, several studies have shown that the presence of a positive flow cytometric cross-match with a negative CDC cross-match is associated with a significantly greater risk of AMR and early graft loss with a positive predictive value for predicting AMR of 83% [10, 11].

To avoid problems associated with the availability and viability of donor cells that could affect the accuracy of cell-based assays, solid-phase assays were introduced which have largely circumvented these problems and improved the sensitivity of detection of anti-HLA antibodies [12]. The identification of anti-HLA antibodies using ELISA was first described in 1993 where purified HLA antigens were directly immobilized on the surface of microtitre plates but the basic principle of antibody detection was similar to cell-based assays [13]. The Luminex platform is a solid-phase assay that utilizes polystyrene microspheres (beads), each embedded with fluorochromes of differing intensity attached to one (single-antigen beads) or several HLA molecules (screening beads) to determine anti-HLA antibody specificity. The Luminex assay has been used in many transplant centres to select the appropriate desensitization regimen according to DSA strength and to establish an acceptable DSA cut-off that may allow kidney transplantation to proceed following desensitization [14, 15]. Similar to other assays, the addition of recipients' sera containing anti-HLA antibodies are added to the bead mix, these antibodies will bind to the appropriate beads expressing single or multiple specific antigen(s). A phycoerytherin-labelled secondary anti-human IgG is then added to this mixture and these antibodies will bind to the primary anti-HLA antibody already attached to the beads expressing the antigens. The sample is then passed through lasers, which would independently excite the beads and the phycoerytherin, therefore allowing the laser detector to define antibody specificity [16, 17]. Unlike the CDC assays, Luminex assay detect both complement-fixing and non-complement-fixing anti-HLA antibodies but does not detect IgM autoantibodies or non-HLA antibodies. The concept of virtual cross-match using solid phase assays relies on accurate HLA typing accompanied by evaluation of anti-HLA antibodies. The presence of a negative solid phase virtual cross-match reliably excludes the presence of donor-specific anti-HLA antibodies and is capable of predicting a negative flow cytometric cross-match in >90% of cases and CDC cross-match in 75% of cases. With the continued reliance on using cell-based cross-match assays, especially CDC cross-match assays to determine transplant suitability, a potential disadvantage of virtual cross-match is that transplants may be excluded based on antibody results with unknown clinical relevance [18]. It is generally accepted that solid phase virtual cross-match to identify anti-HLA donor specific antibodies complements the results of cell-based assays to help inform decision-making process with regards to transplant suitability.

3. Association between anti-HLA donor-specific antibodies and transplant outcomes (Table 1)

Despite technological advances in detecting pre-transplant DSA, the incidence of acute and chronic AMR appears to increase over time. However, the true incidence of AMR remains

Study	Cohort	Rejection	Graft survival
<i>Eng H et al</i> [24]	N=471 DD renal transplant recipients 83 T-B+ XM vs 386 T-B- XM IgG DSA in 33% of T-B+ XM patients	Vascular: 19% T-B- XM vs 32% T-B+ XM (p=0.01) DSA+ significantly predict vascular or glomerular rejection	Graft loss: T-B+ 44% vs T-B- 27%
<i>Lefaucheur et al</i> [25]	C N=402 DD renal transplant recipients Peak sera: positive DSA 21% (Luminex) Current sera: positive DSA 19%	PPV for AMR with peak DSA 35% vs current DSA 32% Prevalence of AMR categorized by MFI: MFI <465 – prevalence 1% MFI 466 to 3000 – prevalence 19% MFI 3001 to 6000 – prevalence 36% MFI >6000 – prevalence 51% Peak DSA MFI predicted AMR better than current DSA MFI	5 and 8-year DCGS: Non-sensitized - 89% and 84% Sensitized with no DSA - 92% and 92% DSA-positive - 71% and 61% Relative risk for graft loss if AMR 4.1 (95% CI 2.2 to 7.7) vs no AMR
<i>Lefaucheur et al</i> [26]	C N=237 LD/DD renal transplant recipients All negative T and B-cell CDC-XM 27% class I or II anti-HLA antibody with 52% anti-HLA antibody being DSA	Incidence of AMR: preformed DSA 35% vs no DSA 3% (p < 0.001)	Overall graft survival at 8 years: DSA-positive 68% DSA-negative 77% Graft survival lower in patients with DSA and AMR compared to DSA and no AMR and in non-DSA patients
<i>Mujtaba M et al</i> [34]	N=44 desensitized LD transplant recipients Negative CDC T-cell XM Sensitization = CDC B+ & T+ ± B+ flow XM	Incidence AMR 31% Total MFI and AMR: <9500 7% vs >9500 36% Class II DSA but not class I DSA greater risk of AMR	3-year graft survival was 100% for total MFI <9500 vs 76% for total MFI >9500.
<i>Amico P et al</i> [94]	N=334 LD and DD renal transplant recipients 332 negative T and B cell CDC-XM 67 DSA vs 267 no DSA (Luminex)	Overall incidence of clinical/subclinical rejection including AMR and/or acute T-cell mediated rejection at day 200 post-transplant: DSA-positive 71% vs DSA-negative 35%	5-year DCGS: No DSA 89% vs DSA without AMR 87% vs DSA with AMR 68%
<i>Song EY et al</i> [95]	N=28 LD and DD renal transplant recipients Positive flow XM but negative CDC-T cell XM, 57% positive DSA	BPAR: DSA-positive 56% vs DSA-negative 0% Class II > class I DSA higher incidence of AMR: 100% vs 22% Class II DSA MFI of 4487 predicted AMR with sensitivity of 100% and specificity of 87%.	No difference in graft survival

HLA – human leukocyte antigen, DD – deceased donor, LD – live-donor, CDC-XM – complement dependent cytotoxicity cross-match, DSA – donor-specific antibodies, SAB – single antigen bead, AMR – antibody mediated rejection, DCGS – death-censored graft survival, MFI – mean fluorescent intensity, BPAR – biopsy-proven acute rejection, PPV – positive predictive value.

Table 1. Association between pre-transplant donor-specific antibodies and graft outcomes.

unclear with suggestions that acute AMR may account for up to 7% of all acute rejections (and up to 50% of acute rejection episodes experienced by pre-sensitized patients with positive cross-match); whereas the prevalence of chronic AMR manifesting as transplant glomerulopathy may be as high as 20% at 5 years post-transplant [19, 20]. The growing incidence may be attributed to a number of plausible reasons including: greater acceptance of highly-sensitized candidates for transplantation, the use of non-calcineurin-inhibitor-based immunosuppressive regimen such as mammalian target of rapamycin inhibitors, better detection techniques for DSA, availability of markers of antibody injury such as C4d staining and a greater understanding of AMR, which may have been misinterpreted as chronic allograft nephropathy or undefined rejection in the past [21].

In most countries, a large proportion of renal transplant candidates on the transplant wait-list are sensitized with high PRA levels and have multiple anti-HLA antibodies, which often result in protracted wait-list time [22]. In Australia, 23% of transplant candidates have a peak class I PRA of >20% and these sensitized transplant candidates often have twice as long a waiting time as unsensitized candidates [23]. Pre-transplant DSA is a major immunological hurdle for successful kidney transplantation. The clinical importance of pre-transplant DSA has been clearly established over the past decade and the presence of high levels of pre-transplant class I (HLA-A and B) \pm II (HLA-DR) DSA, typically occurring as a result of prior sensitizing events including previous blood transfusions, HLA-mismatched transplants and/or pregnancy, is associated with inferior graft outcomes, including an increased risk of developing acute and chronic antibody-mediated rejection (AMR), transplant glomerulopathy and late graft loss (Table 1) [24-27]. However, few studies have suggested that the association between pre-transplant DSA and graft survival was restricted to recipients who had developed early AMR or those with high levels of DSA as determined by peak HLA-DSA strength expressed as mean fluorescent intensity (MFI) using Luminex technology and that pre-transplant screening for preformed DSA may not be cost-effective [28, 29]. *Lefaucheur C et al* demonstrated in a large single centre study that renal transplant recipients with a peak pre-transplant DSA >465 MFI determined by Luminex have a significantly higher risk of developing AMR and that recipients with peak DSA >3000 have almost a four-fold increase in the risk of graft loss compared to recipients with peak DSA MFI of <3000 highlighting the importance of using DSA strength to more accurately assess the immunological risk of transplant recipients [29]. There is also increasing evidence demonstrating that the development of *de novo* DSA may occur in over 50% of renal transplant recipients at 2-years post-transplant suggesting that regular monitoring of *de novo* DSA post-transplant may help identify those at risk of developing poorer graft outcome [30]. Several studies have shown that the development of *de novo* DSA (occurring post-transplantation), especially DSA directed against HLA-DQ graft molecules in HLA-class II incompatible graft transplantations, are both associated with acute and subclinical AMR and graft loss in kidney transplant only and/or simultaneous pancreas-kidney transplant recipients and post-transplant monitoring of DSA could potentially help clinicians to individualize the amount of immuno-

suppression to better assess immune reactivity [25, 30-33]. Although there is no current consensus on the level of clinically significant DSA identified by flow cytometric or Luminex assays, most studies have demonstrated that increasing single, peak or total DSA levels were associated with an incremental risk of rejection and/or graft loss [29, 34]. Recent studies have suggested that the detection of C1q-fixing DSA (i.e. the potential to identify DSA that can activate complements by binding C1q) may be more accurate in predicting acute rejection, biopsy C4d-deposition, transplant glomerulopathy and late graft failure following kidney transplantation and the authors suggested that the absence of C1q-positive de novo DSA has a high negative predictive value for transplant glomerulopathy (100%) and graft failure (88%) [35]. However, a recent retrospective study showed that the identification of strong complement-activating DSA (of IgG subclasses 1 and 3) pre-transplant was unlikely to improve AMR risk stratification compared to patients with a combination of both strong and weak/no complement-activating DSA (of IgG subclasses 2 and 4) [36]. The clinical importance of C1q-specific DSA in predicting graft outcome remains controversial and not routinely performed in many transplanting centres [35, 37]. With the greater understanding of HLA antigens and anti-HLA antibodies, innovative techniques have been established to allow transplantation across positive CDC and/or flow cross-match barriers by removing circulating DSA and/or B or plasma cells and the success and outcomes of these initiatives will be discussed later in this chapter.

4. Clinical relevance of non-anti-HLA donor-specific antibodies (Table 2)

Although it is well established that AMR is attributed to the presence of class I and/or II DSA, non-donor HLA-antibodies and other non-HLA antibodies have been implicated in the development of acute and chronic AMR following kidney transplantation. *Opelz G et al* and others have demonstrated that increasing panel reactive antibodies (PRA) in HLA-identical sibling transplants was associated with a greater risk of rejection (defined as functional graft survival) and poorer graft survival (PRA 0% 10-year graft survival 72%, PRA 1-50% 63%, PRA >50% 55%; $p < 0.01$) suggesting that immune response against non-HLA targets may be important in kidney transplantation, especially in the prediction of chronic graft loss [38]. Alloantigenic and tissue-specific autoantigenic targets of non-HLA-DSA and non-HLA antibodies may include various minor histocompatibility antigens, major histocompatibility complex (MHC) class I chain-related gene A (MICA) antigens, endothelial cell, vimentin, collagen V, glutathione-S-transferase T1, agrin, and angiotensin II receptor type I. Table 2 provides an up-to-date summary of the significance of these non-HLA-DSA and non-HLA antibodies in kidney transplantation and discuss the interplay between alloimmunity and autoreactivity in renal allograft rejection [39, 40].

Antibodies	HLA-antigen (Yes/No)	Target antigen	Location	Transplant outcomes
Anti-angiotensin type 1-receptor antibody [96,97]	No	Angiotensin type I receptor (cell-based ELISA)	Endothelial cells	Increased risk of ACR, vascular rejection and AMR ± malignant hypertension
MICA antibody [98]	Yes	Major histocompatibility-complex class I related chain A antigens (Luminex)	Endothelial cells (also fibroblasts, epithelial cells)	Increased risk of rejection and graft failure, remains debatable
Anti-endothelial cell antibody [39,99]	No	Endothelial cell precursors (flow cytometry)	Endothelial cells	Increased risk of acute and chronic rejections
Vimentin antibody [100]	No	Intermediate filament protein (flow cytometry)	Endothelial cells	Increased risk of rejection
Aggrin antibody [101]	No	Highly purified GBM heparan sulphate proteoglycans (ELISA)	GBM	Increased risk of transplant glomerulopathy
Glutathione-S-transferase T1 antibody [40]	No	Glutathione-S-transferase T1 enzyme (ELISA)	Endothelial cells	Increased risk of C4d-negative acute and chronic AMR
Anti-GBM antibody [102]	No	Alpha-3 chain (the Goodpasture antigen) and alpha-5 chain of type IV collagen (ELISA)	GBM	Increased risk of vascular rejection (Alport patients)
Antibodies to MIG (also called CXCL9), ITAC (also called CXCL11), IFN- γ , and glial-derived neurotrophic factor [103]	No	Chemokine or cytokine (ELISA)	Circulating proteins	Association with chronic renal allograft injury
Protein kinase Czeta antibody [104]	No	Protein kinase (microarray)	Kidney and lymphocytes	Increased risk of graft loss
Anti-HLA-Ia antibody [105]	Yes	HLA-Ia alleles	Endothelial cells	Correlate with poorer graft survival, possibly mediated via anti-HLA-E IgG antibody

Abbreviations: MICA – major histocompatibility complex class I chain-related gene A, ACR – acute cellular rejection, AMR – antibody mediated rejection, GBM – glomerular basement membrane, ELISA – enzyme-linked immunosorbent assay, HLA – human leukocyte antigen

Table 2. Association between non-HLA-DSA and non-HLA antibodies and renal transplant outcomes.

5. Complexities in the diagnosis of antibody mediated rejection (Table 3)

The diagnosis of AMR has improved dramatically with the advent of C4d staining and the ability to detect DSA [41]. The diagnosis of acute AMR according to BANFF criteria requires a triad of [1] histological evidence of graft damage including acute-tubular necrosis-like minimal inflammation, capillaritis and/or glomerulitis and/or thromboses and arteritis, [2] immunological evidence of complement activation inferred by C4d positivity in the peritubular capillaries (PTC), and [3] presence of DSA; whereas the diagnostic criteria for chronic AMR requires [1] morphological evidence of chronic damage of the allograft including duplication of glomerular basement membrane, lamination of peritubular capillaries, arterial intimal fibrosis or interstitial fibrosis/tubular atrophy, [2] diffuse C4d deposition in PTC, and [3] presence of DSA [42]. C4d, a complement split product, is formed by the binding and activation of the classical complement pathway by DSA, which then binds covalently to specific target molecules on the endothelium of PTC and is therefore considered a footprint of AMR [43]. The sensitivity and specificity of diffuse PTC C4d staining for the presence of DSA is >95% [44].

Acute antibody-mediated rejection	Chronic antibody-mediated rejection
Peritubular capillary C4d deposition	Peritubular capillary C4d deposition
Circulating anti-HLA donor specific antibody	Circulating anti-HLA donor specific antibody
Morphological evidence of acute tissue injury (e.g. capillaritis, glomerulitis)	Morphological evidence of chronic tissue injury (e.g. transplant glomerulopathy, interstitial fibrosis, tubular atrophy)
Controversies of C4d staining	Useful to detect AMR, diffuse > focal, PTC C4d negative in 60% AMR
Peritubular capillary C4d deposition	Correlates with AMR and graft survival
Glomerular C4d deposition	No association with graft survival or Similar sensitivity and specificity but detecting AMR compared with C4d deposition
Arteriolar C4d deposition	
AMR	
Erythrocyte C4d deposition better PPV in peritubular capillary	
Abbreviation: AMR – antibody mediated rejection, HLA – human leukocyte antigen	

Table 3. Histological criteria for acute and chronic antibody mediated rejection and corresponding table of controversies of relying on peritubular capillary C4d deposition as a marker for antibody mediated rejection.

However, there are concerns regarding whether the presence of C4d within peritubular capillaries is essential for the diagnosis of AMR with reports of C4d-negative AMR being identified. There have been a few studies that have demonstrated an association between glomerular or erythrocyte C4d deposition and the presence of acute and chronic AMR but the clinical significance of these deposits remain debatable.

Problems with C4d staining:

i. Accommodation

The presence of C4d deposition in PTC does not always denote the presence of AMR or tissue injury. In ABO-incompatible renal transplant, the presence of PTC C4d staining often occurs in the absence of tissue injury or AMR, a process known as accommodation and may be observed in >70% of ABO-incompatible transplants; whereas the presence of PTC C4d staining in HLA-incompatible grafts correlates strongly with the presence of AMR [45].

ii. C4d negative AMR

AMR in the absence of PTC C4d staining has been reported more frequently. In an analysis of 173 indication kidney biopsies, *Sis et al* demonstrated that a combination of high expression of endothelial-associated transcripts (ENDAT) detected using microarray on tissue biopsy, suggesting endothelial damage from alloantibody, plus the presence of DSA was strongly associated with morphological evidence of AMR but only 38% of these biopsies had evidence of PTC C4d positivity [46]. Other studies have corroborated this initial finding suggesting that over reliance of C4d positivity to diagnose acute or chronic AMR could miss up to 60% of patients with morphological evidence of AMR and C4d staining should always be interpreted in the context of tissue morphology [47, 48]

iii. Focal versus diffuse C4d staining

It is generally accepted that the detection of C4d in renal allograft biopsies using immunofluorescence staining is more sensitive than immunohistochemical staining [42, 49]. The level of C4d staining appears to have prognostic significance and it is widely accepted that diffuse C4d staining involving >50% of PTC by either technique is considered positive and correlates much more strongly with adverse graft outcome compared to focal C4d staining involving <50% of PTC, but this remains controversial [50]. However, there are other studies suggesting that focal C4d staining is also associated with histological evidence of AMR including glomerulitis and/or peritubular dilatation [51].

iv. Non-PTC C4d staining

Glomerular, arteriolar and/or erythrocyte C4d positivity often occurs in the absence of PTC C4d staining but the clinical significance of these patterns remains unclear. In a retrospective study of 539 indication renal allograft biopsies, *Kikic et al* demonstrated a poor correlation between arteriolar C4d staining and graft survival, whereas linear glomerular C4d staining was strongly associated with graft failure [52]. There has been considerable interest in the detection of erythrocyte C4d deposition (eC4d) by indirect immunofluorescence as a potential surrogate marker of disease activity in patients with systemic lupus erythematosus and may be useful for the monitoring of disease activity and/or response to treatment in these patients [53, 54]. In kidney transplantation, *Haidar et al* showed a greater amount of eC4d in PTC C4d positive samples compared to PTC C4d negative samples. The authors reported that the positive (PPV) and negative predictive value (NPV) of PTC C4d and eC4d for peritubular capillaritis were 28% and 46% for PPV and 93% and 94% for NPV respectively suggesting that monitoring of eC4d may be an useful non-invasive marker of AMR [55].

6. Management of highly sensitized renal transplant candidates with anti-HLA antibodies

The complexity of transplantation has evolved over the years such that many transplanting centres are performing ABO-incompatible transplants and desensitizing highly allo-sensitized transplant candidates to improve their transplant potential. There is an increasing number of transplant candidates who are allo-sensitized to HLA as a result of previous exposure to HLA antigens, typically following blood transfusion, prior transplantation and pregnancy. It is well known that the presence of high levels of pre-transplant DSA is associated with poorer graft outcomes, including the development of acute and chronic AMR resulting in late graft loss [26, 56]. Finding a compatible donor for potential transplant candidates with multiple anti-HLA antibodies is often difficult and these patients may remain on the deceased donor transplant wait-list for a much longer period compared to unsensitized transplant candidates. Paired kidney exchange program is a potential and proven option for highly sensitized patients who have a positive cross-match with their potential live donors to receive a compatible cross-match negative donors [57]. With the greater understanding of HLA antigens and anti-HLA antibodies, innovative techniques have been established to allow transplantation across a 'positive CDC and/or flow cytometric cross-match' barrier resulting from anti-HLA antibodies directed against the donor. Nevertheless, graft outcomes of highly sensitized transplant recipients are poorer compared to compatible transplant recipients, particularly a much greater risk of acute AMR (Table 4).

	Number of patients	AMR incidence (%)	1-year graft survival (%)	2-year graft survival (%)
<i>Lefaucheur et al</i> ^ [26]	43	35	89	89
<i>Thielke et al</i> ^o [70]	51	32	93	81
<i>Magee et al</i> [71]	28	39	92	89
<i>Gloor et al</i> [106]	119	41	89	89
<i>Haririan et al</i> [106]	41	12	90	85
<i>Vo et al</i> [72]	16	30	94	Not reported
<i>Vo et al</i> [#] [62]	76	29	87	84
ANZDATA 2010* [107]				
Primary DD grafts	550	<5%	95	93
Primary LD grafts	296	<5%	96	96

*ANZDATA 2010 – graft failure secondary to AR 2%; #Stratified by donor type – death-censored graft survival at 1 and 2 years for LD 90% and 90%; for DD 82% and 80%. [Note: Of the total 374 recipients, only 51 [13.6%] were DD transplants].

Acute AMR is a strong predictor of inferior graft survival: 1] ^AMR vs no AMR – 1y GS 60% vs 89% (*Lefaucheur et al*); 2] ^oAMR vs no AMR – development of transplant glomerulopathy 44% and 12% (*Gloor et al*).

Abbreviations: ANZDATA – Australia and New Zealand Dialysis and Transplant registry, AMR – antibody mediated rejection, DD – deceased donor, LD – live-donor.

Table 4. Incidence of antibody mediated rejection and graft survival following positive crossmatch kidney transplantation.

	Number	Technique	Outcomes	Complications
<i>Vo A et al</i> [72]	<ul style="list-style-type: none"> • 10/11 LD and 6/9 DD with CDC-XM or FCMX+ (Note: 13/16 had persistently positive XM at time of transplant) 	<ul style="list-style-type: none"> • IVIg 2g/kg day 0 and 30 + rituximab 1g day 7 and 22 (5/16 CDC-XM+) 	<ul style="list-style-type: none"> • Wait-time pre-transplant 144±89m, additional 5±6m (range 2-18) post-desensitisation • 12mGS 94% • 12mPS 100% • 50% AR (30%AMR) 	<ul style="list-style-type: none"> • 44% asymptomatic UTI
<i>Vo A et al</i> [62]	<ul style="list-style-type: none"> • 76 (31 LD & 45 DD) with T cells FCMX+ F/up 18m 	<ul style="list-style-type: none"> • IVIg 2g/kg day 1 and 30 + rituximab 1g day 15 	<ul style="list-style-type: none"> • Wait-time for DD pre-transplant 95±46m, additional 4.2±4.5 post-desensitisation • AMR 29% (11DD/11LD) • 2yGS 84%, PS 95% (LD 90%/100%, DD 80%/91%) • 2yCr - 143µmol/L 	<ul style="list-style-type: none"> • 11% infections, 8% CMV/BKV • 5% mortality (2.5% infections)
<i>Rogers N et al</i> [108]	<ul style="list-style-type: none"> • 10/13 LD with CDCXM+ and DSA+ successful(DSA up to 18,000 MFI) 	<ul style="list-style-type: none"> • Rituximab 375mg/m2 day -14 + 5PP with 0.1g/kg post-4PP + 2g/kg IVIg post-final PP • Induction basiliximab 	<ul style="list-style-type: none"> • 80% Cr <160 • 30% AR (cellular) <3m (Pre-Tx DSA <5000) 	<ul style="list-style-type: none"> • 10% PNF • 10% mortality (sepsis) • 70% transfusions ≥5 units • 30% sepsis • 21% CMV
<i>Haririan A et al</i> [69]	<ul style="list-style-type: none"> • 41 LD with FCMX+ with 27 B/T cell+ (vs historical controls) 	<ul style="list-style-type: none"> • Alternate day PP (mean 4) + post-PP 0.1g/kg IVIg + induction T cell depletion 	<ul style="list-style-type: none"> • 1y GS – 90% vs 98% (historical controls) • 5y GS – 69% vs 81% • Graft half-life 6.8y • 12% AMR 	<ul style="list-style-type: none"> • Infection rates similar
<i>Gloor J et al</i> [106]	<ul style="list-style-type: none"> • 119 LD +CM (52 CDC-XM+) vs 70 controls 	<ul style="list-style-type: none"> • Daily PP with post-PP 0.1g/kg IVIg ± splenectomy or rituximab (d-7) + rATG induction 	<ul style="list-style-type: none"> • 50% AMR and 54\$ TG in CDC-XM+ (vs 1% and 0% controls) • DCGF 46% vs 0% at 2y 	<ul style="list-style-type: none"> • Not reported
<i>Thielke J et al</i> [109]	<ul style="list-style-type: none"> • 49/57 LD FCMX+ successfully desensitised to XM- 	<ul style="list-style-type: none"> • 3-5 PP with post-PP 0.1g/kg IVIg ± rituximab (1-2 doses 375mg/m2) 	<ul style="list-style-type: none"> • 1y DCGS 93% • 1y PS 95% • AR 43% 1y (24% AMR) 	<ul style="list-style-type: none"> • Infection risk with rituximab • 7% CMV
<i>Magee C et al</i> [110]	<ul style="list-style-type: none"> • 29 LD CDC-XM T or B-cell + 	<ul style="list-style-type: none"> • 3x/week PP with 10g IVIg post-PP ± rituximab pre-transplant (375mg/m2) 	<ul style="list-style-type: none"> • 42% ACR and 39% AMR (no difference with rituximab) 	<ul style="list-style-type: none"> • Not significant

	Number	Technique	Outcomes	Complications
<i>Jordan S et al</i> [61]	<ul style="list-style-type: none"> • 98 PRA \geq50% randomised 1:1 to IVIg or placebo (LD and DD) 	<ul style="list-style-type: none"> • IVIg 2g/kg monthly for 4 months or placebo 	<ul style="list-style-type: none"> • Improved DD transplant rate in IVIg group compared to placebo (31% vs 12%, $p=0.01$) • Estimated projected mean time to transplantation is 4.8y for IVIG vs 10.3y for placebo • GS and PS similar 	<ul style="list-style-type: none"> • More headaches in IVIg group
<i>Jordan S et al</i> [111]	<ul style="list-style-type: none"> • N=42 (62% LD) 	<ul style="list-style-type: none"> • LD 1x 2g/kg IVIg • DD monthly 2g/kg IVIg x 4 + pre-Tx 2g/kg IVIg 	<ul style="list-style-type: none"> • 31% AR (<1m), 38% ATG and 23% graft loss from AR • 2y GS 89%, PS 98% 	<ul style="list-style-type: none"> • Not reported

Abbreviations: LD=live-donor, DD=deceased donor, CDC=complement dependent cytotoxicity, FCMX=flow cytometric cross-match, DSA=donor specific antibody, PP=plasmapheresis, AR=acute rejection, rATG=rabbit antithymocyte globulin, GS=graft survival, DCGF=death-censored graft failure, PS=patient survival, AR=acute rejection, AMR=antibody mediated rejection, ACR=acute cellular rejection, TG=transplant glomerulopathy, IVIg=intravenous immunoglobulin, PNF=primary non-function, CMV=cytomegalovirus

Table 5. Relevant studies of desensitization in live and deceased-donor transplantation.

Studies reporting the utilization of desensitisation techniques to allow transplantation in highly sensitized transplant candidates have focussed predominantly on live-donor transplantation, which allows early planning and implementation of treatment at a suitable time (Table 5). A recent paper by *Montgomery R et al* had demonstrated that desensitization of highly sensitized patients for live-donor transplantation was associated with a significant survival benefit compared with waiting for a compatible deceased donor organ. By 8 years, this survival advantage more than doubled suggesting that desensitization protocols to overcome incompatibility barriers in live-donor renal transplantation may be justified [58]. However, the benefit of desensitization of highly sensitized patients on the deceased donor transplant wait-list remains debatable due to the uncertainty of kidney availability [59, 60]. The only randomized study evaluating the benefit of IVIg to improve transplant potential in highly sensitized transplant candidates on the deceased donor transplant wait-list was a double-blind, placebo-controlled, multicentre study whereby 101 patients with PRA >50% who have been waiting for >5 years on the transplant wait-list were randomized to receive IVIg (2g/kg monthly for 4 months) or placebo. The administration of high-dose IVIg was associated with a reduction in PRA levels with 35% of IVIg-treated patients being transplanted compared with 17% of patients receiving placebo suggesting that this regimen was associated with improved transplant potential for highly sensitized patients [61]. This same group modified this initial regimen by adding rituximab and subsequently reported that desensitization of highly sensitized patients with PRA >30% using high dose IVIg (2 doses of 2 g/kg days 1 and 30)

and a 1g dose of rituximab (day 15) reduced the deceased donor transplant wait-list time from 95±46 months to 4.2±4.5 months achieving acceptable rejection rates and graft survival at 24 months [62]. In contrast, a recent prospective cohort study evaluating pre-transplant desensitization with two doses of IVIg (2 g/kg up to a maximum of 120g per dose) plus a single dose of rituximab (375 mg/m²) in highly sensitized kidney transplant candidates with a calculated panel reactive antibody (cPRA) of >90% and had spent >5 years on the deceased donor wait-list did not improve their transplant potential or reduced class I and II cPRA levels. This finding has been corroborated by other studies that have demonstrated that treatment with high dose IVIg in highly sensitized patients (flow cytometric calculated PRA of 100%) on the deceased donor transplant wait-list did not significantly alter their cPRA levels or improved their transplant potential highlighting that the potential benefit of desensitization of highly sensitized transplant candidates on the deceased donor wait-list remain uncertain [63-65].

The optimal desensitization regimen for highly sensitized renal transplant candidates in the context of living related and unrelated donation remains unclear. Most of the current desensitization protocols are modifications of plasmapheresis and intravenous immunoglobulin (IVIg) ± rituximab and have been used successfully to desensitize highly allo-sensitized transplant candidates, therefore allowing transplantation to occur [61, 66-73] (Table 2). However, desensitization of positive CDC or flow cytometric cross-match patients using immunoadsorption with rituximab followed by ongoing immunoadsorption post-transplant appears promising achieving rapid elimination of DSA and excellent short-term graft outcomes [74]. Immunoadsorption appears to be more effective than plasmapheresis in removing circulating DSA and studies have shown that a single pre-transplant immunoadsorption could render a positive cross-match to become negative [75, 76]. Encouraging results have been obtained with the use of bortezomib and/or eculizumab in desensitization protocols to achieve successful transplantation across a positive CDC and/or flow cytometric cross-match barrier but the use of these agents are usually considered adjunctive treatments to standard protocol [77]. Although splenectomy has historically been used in the desensitization protocols for ABO-incompatible transplants and treatment of refractory AMR by removing an essential source of B lymphocytes, this has largely been superseded by B cell depleting agents [78]. These techniques aim to lower the DSA to an 'acceptable' level pre-transplant to allow transplantation to proceed and preventing immediate acute renal allograft injury. Most published studies of desensitization protocols are non-randomized and observational with varying techniques and threshold of detecting pre-transplant DSA, thereby making comparisons between studies difficult. Plasmapheresis with low dose IVIg (0.1g/kg following each plasmapheresis) for 2-3 weeks pre-transplant followed by interleukin-2 receptor antibody or CD3-T cell depletive agent induction is the most common desensitization protocol utilized in many transplanting centres although the duration of treatment pre- and post-transplant would depend on achieving a negative cross-match pre-transplant and on the DSA titres. Studies utilizing this protocol have reported high risk of AMR (between 12-100%) with a reduction in longer-term graft survival (66% at 4 years) despite acceptable short-term graft survival [69, 79, 80]. Although high-dose IVIg (2g/kg) was initially considered for deceased donor kidney transplant candidates, it has been implemented with and without rituximab in positive CDC and/or flow cytometric cross-match live-donor transplant candidates with similar risk of

rejection and graft survival to studies using plasmapheresis and low-dose IVIg [62, 80-82]. A retrospective study by *Stegall et al* showed that CDC T cell cross-match positive renal transplant recipients receiving high dose IVIg alone had a higher rate of AMR [80%] compared to recipients receiving plasmapheresis, low-dose IVIg with rituximab (37%) or plasmapheresis, low-dose IVIg, rituximab and pre-transplant anti-thymocyte globulin (29%) suggesting that high dose IVIg may be inferior to the combination of plasmapheresis and IVIg but it is difficult to draw any firm conclusion from an uncontrolled study [73]. Furthermore, there are suggestions that pre-transplant treatment to lower DSA MFI to <6000 using Luminex is recommended for successful transplantation and is associated with lower risk of AMR but again, this remains debatable [14].

Following successful transplantation, ongoing monitoring of DSA and early recognition of AMR is crucial to avoid early graft loss. On re-exposure to donor antigens against which the recipient is sensitized, memory B lymphocytes in their spleen, bone marrow and lymph nodes undergo an anamnestic reaction leading to the development of antibody-producing cells, which can produce high levels of DSA within days or weeks and therefore, positive cross-match kidney transplantation requires both pre- and post-transplant interventions to continually suppress DSA levels. Although continuing plasmapheresis and/or IVIg post-transplant following successful desensitization of highly sensitized recipients with positive cross-match against the donor is generally accepted, there has been no study addressing the type, amount, duration and cost-effectiveness of such approach [70]. Nevertheless, studies have demonstrated a strong association between the development of *de novo* DSA (especially DQ-DSA or when there is a rise of DSA >500 MFI) and AMR and graft loss suggesting that that long-term monitoring of DSA in highly sensitized patients may be appropriate, especially those receiving class II-incompatible grafts [83-85]. A recent single centre study suggested that post-transplant DSA surveillance followed by pre-emptive initiation of IVIg and plasmapheresis with rising DSA titres have successfully improved long-term graft survival [86].

Intravenous gammaglobulins (IVIg) are effective in the successful management of a number of autoimmune and inflammatory disorders attributed to their immunomodulatory and immunoregulatory properties. IVIg has been suggested in the management of highly sensitized renal transplant patients because it eliminates circulating anti-HLA antibodies, suppresses the production of these antibodies by inducing B cell apoptosis (and also T cells and monocytes *in vitro*) and is a modifier of complement activation and injury [87, 88]. There is now considerable debate among the transplant community regarding the balance between the benefits and harms associated with IVIg desensitising patients with high immunological risks. [89, 90]. One small but significant side effect associated with the use of high dose IVIg is the risk of thrombosis, which may be mitigated by slowing infusion rate (maximum infusion rate of 100mg/kg/hour), aspirin, enoxaparin and intravenous hydration pre- and post-infusion [91]. The important side effects of IVIg along with other agents commonly used in the desensitization protocol are summarized in Table 6. However, it is important to note that many of the side effects associated with desensitization treatment have been reported in non-transplant population but should be recognized and advised to patients receiving these treatments.

Treatment	Actions	Complications	Cost	Comments
Intravenous immunoglobulin [112]	Neutralize circulating anti-HLA antibodies Enhance clearance of anti-HLA antibodies Inhibit complement activation Induce B cell apoptosis Inhibitory effects on other immune cells such as macrophages and natural killer cells by binding to their Fcγ receptors	Thrombotic events Acute renal failure Haemolytic anaemia Aseptic meningitis Anaphylactoid reactions	US\$8700 for 120g	Infusion related adverse events related to osmolality, minimized by slowing infusion rate Reduce thrombotic events by using aspirin, heparin/enoxaparin, intravenous fluids Newer preparation, iso-osmolar products have higher titres of antiA±B, resulting in higher rates of haemolysis
Plasmapheresis [113]	Removal of circulating anti-HLA antibodies	Hypotension Bleeding diathesis Potential blood-borne pathogen transmission if replacement with fresh frozen plasma is required (rare)	US\$2000 per session	Non-selective removal of antibodies
Immuno-adsorption [74, 114]	Removal of circulating anti-HLA antibodies	Similar complications as plasmapheresis	US\$1600 per session	Higher plasma volume exchange resulting in higher antibody removal rate may be achieved over plasmapheresis. More selective IgG removal compared to plasmapheresis
Rituximab [115]	Chimeric murine/human monoclonal antibody that binds to CD20 on pre-B and mature B lymphocytes	Infection (fungal and other opportunistic) Progressive multifocal leukoencephalopathy	US\$3900 for 700mg	Similar effectiveness using smaller dose
Bortezomib [116, 117]	Proteasomal inhibitor causing apoptosis of plasma cells	Fatigue, weakness Gastrointestinal disturbances (common, mild) Anaemia, thrombocytopenia (mild, transient) Peripheral neuropathy (mild, transient)	US\$1322 for 3.5mg	Role in desensitization unclear
Eculizumab [118, 119]	Humanized monoclonal antibody against C5 preventing the formation of membrane attack complex (C5b-9)	Meningococcal infections (rare, severe) Other infections especially with encapsulated bacteria Anaemia (rarely serious), leukopenia Hypertension, headache, gastrointestinal upset (common, mild)	US\$5990 per 300mg	Role in desensitization unclear Requires meningococcal vaccination at least 2 weeks prior to transplant

Abbreviation: HLA – human leukocyte antigens.

Table 6. Complications and cost of desensitization treatment.

In the absence of large randomized controlled trials, the optimal desensitization protocol is unclear. Observational data have reported desensitization protocols comprising of high or low-dose IVIg and plasmapheresis with or without rituximab and other newer agents such as bortezomib and eculizumab may be beneficial in selected patients, the rate of AMR remains extremely high (up to 50% in pre-sensitized positive cross-match patients undergoing desensitization) and may not be justified in circumstances such as in patients with very strong pre-transplant DSA levels [19]. The lack of treatment effectiveness among highly sensitised individuals is not unexpected, because most recommended treatment options such as plasmapheresis, IVIg and rituximab have minimal effects on plasma cells, the critical element of anti-HLA antibodies production, and AMR. Clinicians should discuss with their patients about the complexities and the potential side effects associated with any desensitisation protocols, taking into considerations the underlying immunological risks of the potential transplant candidates, the potential benefits against the short and longer-term harms such as infection and cancer risks. Specifically transplant candidates with prior sensitizing events and have DSA (even at low levels) against potential donor (e.g. husband to wife transplant) are at significant risk of AMR after transplantation despite r adequate desensitization. If desensitization is undertaken, this should be initiated 2-3 weeks post-transplant to ensure adequate removal of anti-HLA DSA pre-transplant with at least a negative CDC cross-match (or reduction in flow cytometric cross-match results) and persistent reduction in DSA MFI below 2000-5000. Transplantation should be abandoned if there is rebound of high titres DSA and/or the crossmatches remained unchanged/positive following desensitisation protocols. Although the benefit or cost-effectiveness of post-transplant DSA monitoring \pm protocol biopsies in improving post-transplant graft outcomes remains unclear, it is well established that *de novo* DSA and rising pre-transplant DSA are associated with a greater risk of rejection and poorer graft survival [32, 92, 93]. However, there is no data suggesting that early interventions in renal transplant recipients who develop *de novo* DSA or rising DSA would result in an improvement in graft outcomes. Nevertheless, prospective monitoring of pre-existing DSA or for *de novo* DSA \pm protocol biopsies should be considered and appropriate treatment instituted in those who develop histological evidence of rejection. Several proposed desensitization and post-transplant follow-up algorithms for positive cross-match highly sensitized recipients are available but the cost-effectiveness and outcomes of these programs remains unknown [80].

7. Conclusion

Despite the availability of more potent immunosuppression, the incidence of AMR continues to be an important cause of graft loss. Nevertheless, with the evolution of more sensitive molecular-based HLA-typing and the ability to detect DSA, clinicians have the necessary facts to critically appraise the immunological risk of each transplant candidate. However, there continues to be debate on several major issues including the role of non-DSA in transplantation, the appropriate DSA threshold, complexity in the diagnosis of acute and chronic AMR and the optimal desensitization protocol for highly sensitized patients. As there continues to be an increase in the number of highly sensitized renal transplant candidates on the transplant wait-

list as a result of prior sensitizing events, future studies addressing all these unanswered issues are critical.

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