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REVIEW

Technical challenges and clinical relevance of single antigen bead C1q/C3d testing and IgG subclass analysis of human leukocyte antigen antibodies

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SUMMARY

Luminex single antigen bead assays revolutionized human leukocyte antigen (HLA) antibody detection owing to their superior sensitivity compared to conventional methods. Nevertheless, the advent of higher sensitivity came at the expense of difficulty in clinical decision-making, since not all luminex detectable antibodies are clinically relevant. Therefore, new tools such as C1q/C3d assays and IgG subclass analysis emerged with the aim to discriminate the inert antibodies from the deleterious ones. Here, we provide an overview on the technical challenges related to these different HLA antibody detection systems and briefly refer to the recent literature regarding the clinical relevance of these assays, mainly in the field of kidney transplantation.

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Introduction

Pre-existence or *de novo* development of donor-specific antibodies (DSA) directed to mismatched human leukocyte antigen (HLA) is known to be an important risk factor for developing antibody-mediated rejection (ABMR), and a major cause of inferior graft survival [1–3]. While currently available immunosuppressive agents are effective in controlling T cell-mediated rejection and improving short-term graft survival, they are inadequate in preventing or reversing ABMR, making this the leading cause of late graft loss [4,5]. The landmark study by Patel and Terasaki in 1969, in which they introduced the complement-dependent cytotoxicity (CDC) assay, has clearly proven that complement activation by pre-existing DSA is a strong predictor of hyperacute rejection [6]. The CDC assay, while allowing for detection of complement-fixing antibodies, is limited in sensitivity and specificity. The introduction of a flow cytometric crossmatch, detecting both complement-fixing and noncomplement-fixing DSA increased the sensitivity but did not solve the specificity problem [7]. A considerable breakthrough came with the development of luminex single antigen bead (SAB) assays enabling characterization of HLA antibodies with unprecedented sensitivity and specificity. These SAB assays were originally designed to detect all IgG isotypes of HLA antibodies, irrespective of their capacity to fix complement.

Variations on SAB assays to determine DSA that can bind complement have been introduced since, as well as efforts to determine IgG subclasses, all aiming at a better stratification of DSA into high and low risk.

In this review, we provide an overview of the recent literature on HLA antibodies as defined by luminex SAB, C1q, C3d assays and IgG subclass analysis by

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focusing on the technical challenges associated with the above-mentioned methods and briefly touch on the clinical relevance of the information obtained from these assays.

Role of DSA in development of ABMR

Antibody-mediated rejection, acute or chronic, can occur as a result of both complement-dependent and independent pathways upon binding of antibodies to mismatched donor antigens on graft endothelium [8–10]. Complement activation occurs through highly regulated cascades of enzymatic reactions via three distinct pathways (classical, lectin, and alternative). The antibody-dependent classical pathway of complement activation is triggered upon binding of C1q, the recognition unit of C1 complex (C1qrs) to Fc portions of antigen-bound IgM or IgG [11]. While at least two IgG molecules in close proximity are required for C1q to bind to the Fc portions of the IgG molecules, optimum complement activation with high affinity C1q binding occurs when IgG molecules on the cell surface form hexamers [12]. Docking of C1q to the CH2 domain in Fc regions of multiple antigen-bound IgG results in activation of C1r and C1s serine proteases of C1 complex leading to cleavage of C4 and C2, which generate the common effector C3/C5 convertases responsible for amplifying the complement response in all three pathways. While complement split products at initial phases such as C3a and C5a can serve as proinflammatory anaphylatoxins, ultimate formation of C5b-C9 membrane attack complex leads to cell lysis.

When circulating antibodies bind to HLA expressed on graft endothelium and initiate the classical pathway of complement activation, local inflammation can lead to graft thrombosis, tissue ischemia and eventually graft failure. Staining of biopsy material for the deposition of C4d, a C4 cleavage end product on peritubular capillaries, has widely been accepted as a diagnostic marker for ABMR, although C4d-negative antibody-mediated rejections exist as well [13–15]. Besides complement activation, HLA antibodies can initiate endothelial cell activation through crosslinking of HLA antigens expressed on the surface of endothelial cells. Activated endothelial cells trigger downstream intracellular signaling events leading to increased proliferation, cytokine expression and recruitment of leukocytes. Eventually, crosslinking of HLA on endothelial cells by DSA may lead to acute or chronic ABMR independent of complement activation, depending on the concentration of DSA [9,16]. Indeed, in a recent study on the use of complement component C5 inhibitor eculizumab for desensitization, a lower incidence of ABMR at 3 months was found [17], but despite these encouraging initial findings, the incidence of chronic ABMR at 1- to 2-years post-transplant was not significantly reduced [18]. Similarly, Eskandry *et al.* [19] described no effects on late ABMR by complement inhibition using an anti-C1s antibody, even though profound classical pathway inhibition and clear reduction of C4d positivity in renal biopsies were observed.

Why luminex SAB data should be interpreted with caution

In the standard IgG SAB assay, serum samples are incubated with fluorescent polystyrene microbeads, each coated with a single specificity of recombinant HLA molecules. Binding of HLA antibodies to specific beads is detected by using a phycoerythrin (PE)-conjugated anti-human IgG secondary antibody yielding a mean fluorescence intensity (MFI) value. Importantly, luminex SAB assays are semiquantitative at best, since MFI values do not reflect antibody titers [20]. Furthermore, false-positive reactions are observed because of antibody reactivity to denatured HLA molecules on the beads [21–24] while false-negative results are because of the so-called prozone effect or interference of serum substances other than IgG [25–30]. Although not part of the commercial protocol provided by the manufacturers of the IgG SAB assays, serial dilutions, heat inactivation or hypotonic dialysis of the serum samples as well as pretreatment with dithiothreitol (DTT) or ethylenediaminetetraacetic acid (EDTA) are means to circumvent the prozone effect or interference of serum components such as IgM and complement in the detection of circulating IgG antibodies [20,25,26,31]. Luminex SAB assay kits are manufactured by two companies. Because of the differences between the two kits in the antigen composition, antigen density and integrity, sample dilution and manufacturing processes, MFI values obtained from kits of these two vendors display a moderate to weak correlation and therefore should not be compared quantitatively. While the majority of discrepancies for assigning antibody positivity or negativity accounts for HLA antibodies with relatively low MFI values (<1000–1500), significant overlap between two manufacturers can be observed for HLA antibodies at high MFI values [32–34].

Many studies using SAB technology revealed pre-existence or *de novo* DSA particularly directed at HLA class II to be much more prevalent than was previously

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found by using less sensitive methods such as CDC or enzyme-linked immunosorbent assay (ELISA) [2,35,36]. Nevertheless, not all patients with DSA as defined by these highly sensitive SAB assays experience ABMR or poor graft survival [3], questioning the clinical relevance of HLA antibodies only detected in SAB assays. This differential pathogenicity of HLA antibodies could be attributed to differences in IgG subclass composition and complement-fixing capacity, not detected in standard SAB assays. Therefore, modified SAB assays measuring C1q binding to HLA antibodies or C3d deposition on beads have been introduced in recent years [37,38]. In addition, several research groups have modified SAB assays in order to be able to analyze the IgG subclass distribution of HLA antibodies [39–41].

The C1q and C3d assays: principle and limitations

The C1q SAB assay (One Lambda, Canoga, USA) aims at defining HLA antibodies that have the capacity to bind C1q. Serum samples to be tested with the C1q assay are first heat-inactivated to eliminate interference of endogenous complement components. Sera are incubated together with recombinant C1q, after which binding of both antibody and C1q is detected by a PE-labeled anti-C1q antibody (Fig. 1). Since there is no selection for IgM or IgG, C1q-binding HLA antibodies detected can be either IgM or IgG isotype. Modified versions of the assay such as anti-human globulin enhancement or two-step detection of C1q-binding HLA antibodies have been utilized by several groups in order to increase sensitivity [42–44].

Whereas C1q binding to antigen-bound antibody is the first step in classical pathway complement activation, binding of recombinant C1q to HLA antibodies does not necessarily imply that all downstream events in the complement cascade will take place, as has been shown for human monoclonal HLA antibodies [45]. In this regard, the subsequently introduced C3d assay (Immucor Transplant Diagnostics, Stamford, USA) might be a more accurate tool to predict *in vivo* complement activation. Furthermore, the C3d assay may have a higher sensitivity in comparison to the C1q assay mainly because C1q binding to IgG in turn triggers packed accumulation of split products such as C3d on beads [46]. As schematized in Fig. 1, the C3d assay resembles the C1q assay in methodology but does not utilize a recombinant complement product. Following an initial incubation of serum samples with SABs, standardized human serum is added as source of complement, followed by an anti-human C3d detection antibody.

Soon after the introduction of the C1q assay, it was questioned whether C1q test results could be predicted by MFI values. Initial studies did not show a correlation of C1q binding with high MFI in standard SAB assays but these studies did not apply any pretreatment of the serum samples to eliminate a possible prozone effect in the standard assay [8,37]. Other studies in which standard SAB samples were pretreated showed that the majority of C1q-positive HLA antibodies manifested with high MFI values in the standard assay [42,47–51]. Furthermore, although only shown for HLA class I, HLA antibody specificities with high IgG MFI but low C1q MFI were exclusively found for SABs on which more than 30% denatured HLA was detected, implying the requirement of native antigen on the beads for efficient complement activation by HLA antibody binding [52].

The observed correlation between IgG MFI values and C1q results is not surprising because C1q requires a certain density of antigen-bound IgG molecules in close proximity for high affinity binding and activation [12]. Yell *et al.* provided direct evidence that C1q positivity is a reflection of antibody concentration rather than the biological property of the IgG molecules. They achieved conversion from C1q− DSA to C1q+ DSA by concentrating the sera, leading to a higher MFI [47]. Furthermore, Wiebe *et al.* [48] showed that an IgG MFI of 10 126 in EDTA-treated serum samples could predict C1q positivity with 100% sensitivity and 99% specificity with 99% of C1q-positive beads having an EDTA MFI titer of 1:1024. Although the C3d assay is a relatively newer product and thus tested less often in comparison to the C1q assay, similar concerns regarding the association of antibody strength/MFI with C3d status apply [38]. In a small study of Claisse *et al.* [53], C1q and C3d status were defined in serum samples before and after plasmapheresis. Both C1q (33%) and C3d (35%) positivity before plasmapheresis decreased to 13% and 16% upon plasmapheresis with 91% of concordance between two assays. Those that remained C1q+ or C3d+ after plasmapheresis had higher MFI in comparison to the MFI of C1q− and C3d− beads, confirming the strong association of high MFI with C3d and C1q status. Similar to the study of Wiebe *et al.*, a high predictive capacity of 10240 IgG MFI for C1q positivity, and IgG MFI of 7629 for C3d positivity with 84% sensitivity and 97% specificity was shown [48,53]. A recent study by Courant *et al.* [54] confirmed that both C1q and C3d positivity could be predicted with an IgG

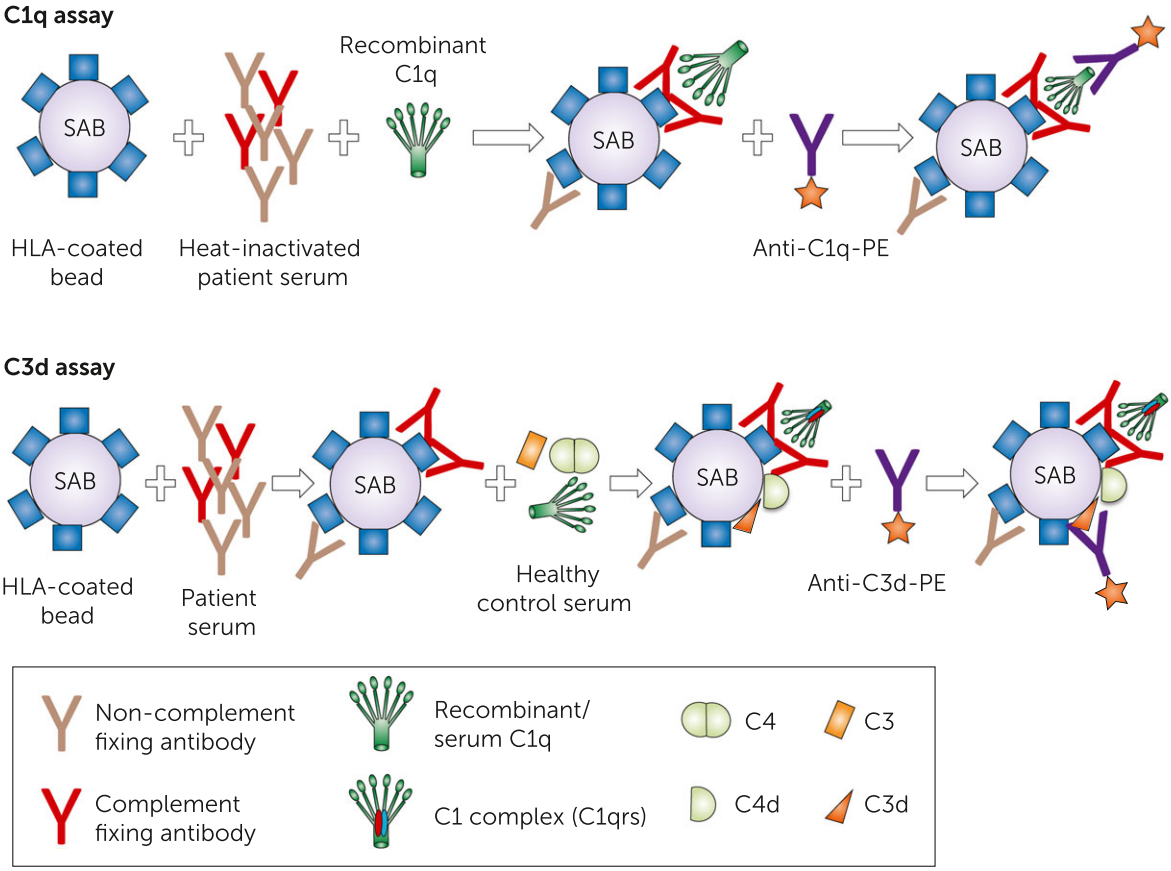

Figure 1 Principles of C1q and C3d assays. (C1q assay) Heat-inactivated patient serum is incubated with single antigen beads (SABs) and recombinant C1q. Following a wash step, phycoerythrin (PE)-conjugated anti-C1q antibody is added to detect C1q-binding HLA antibodies. (C3d assay) Patient serum is first incubated with SABs. Following binding of HLA antibodies to the beads, a healthy control serum as the complement source is added for further incubation. Following a wash step, PE conjugated anti-C3d antibody is added to detect C3d deposition on the beads.

MFI > 3800 in EDTA-treated serum samples. In conclusion, while the MFI threshold is inconsistent, multiple studies could predict complement-fixing luminex results by the MFI level in the regular luminex assay.

IgG subclass analysis

There are four subclasses of IgG (IgG1, IgG2, IgG3, and IgG4) in humans eliciting different effector functions through their constant domains that can be bound by complement or Fc receptors on leukocytes. Diversity in effector functions of antibodies is regulated via antibody class switching during germinal center reactions in secondary lymphoid organs through follicular T-helper cells in response to protein antigens such as allogeneic HLA.

IgG1 and IgG3 antibodies are known to strongly activate complement. While IgG1 is the most abundant subclass in serum and hence the most efficient in complement-mediated lysis, IgG3 is known to have the highest affinity for complement possibly because of its long hinge region enabling it to bind more C1q [55]. Regardless of IgG2 being a poor activator of complement in many circumstances, it can efficiently bind C1q and activate complement in case of high antigen density. IgG4 does not have any complement-activating capacity; however, can theoretically modify or block classical complement activation mediated by other IgG subclasses [56].

With a lack of standardized tests, several groups modified the luminex IgG SAB assay in order to gain insight into the IgG subclass distribution of serum HLA antibodies simply by replacing the polyclonal pan-IgG detection antibody with IgG subclass detection antibodies [39–42,57–59]. While the end concentrations of the detection antibodies in different studies vary from 0.25 to 20 μg/ml, lack of appropriate controls to determine the specificity of these detection antibodies might have

resulted in overestimated results because of the crossreactivity between different subclasses [39,40,59]. Furthermore, when testing for IgG subclasses, serum samples should be pretreated by similar means as for pan-IgG SAB assay to avoid complement interference (by complement components or IgM) or competition of IgM with IgG for the same epitope on the bead [26,29,31].

A commonality in all subclass studies is the decreased sensitivity in comparison to pan-IgG SAB assays because of the use of monoclonal secondary antibodies to replace the polyclonal pan-IgG antibody. Accordingly, around 7–17% of HLA antibodies detected using pan-IgG assay with MFI values ranging from 781 to 3584 were found to be negative for IgG1–4 subclasses [39,40,59]. As a consequence of this low sensitivity of the subclass assay, investigators generated various ways of calculating cutoff values for each subclass, making it difficult to compare the findings between different studies [39,40].

Not surprisingly, IgG1 is the most predominant subclass detected (isolated or in combination with other subclasses). In a study on patients with pretransplant DSA, it was shown that 39% of DSA was IgG1 and/or IgG3, 7% was IgG2 and/or IgG4, and 54% of the DSA was a mixture of both complement-fixing or weak/non-complement-fixing subclasses [40]. Although IgG2 and IgG4 are weak/noncomplement-activating subclasses, their relative concentrations in the presence of IgG1 and IgG3 could be critical when they compete for the same epitopes. Hönger *et al.* [60] used IgG1–4 subclass variants of a chimeric mouse/human monoclonal HLA class II antibody to show that IgG2/IgG4 antibodies profoundly inhibited C1q binding when present at onefold to fourfold higher levels than IgG1 and IgG3, and completely hampered C1q binding when present at a level of 10-fold excess.

Clinical relevance of modified Luminex SAB assays

Pretransplant prognostic value

The main purpose of pretransplant HLA antibody testing is to define unacceptable antigens to which strong, complement-fixing antibodies are present in the recipient. In this regard, a positive CDC crossmatch (CDC-XM) is considered a contraindication for transplantation. While higher sensitivity of flow cytometry crossmatch (FC-XM) test enables detection of low titer HLA antibodies of all IgG subclasses, DSA detected by solid phase assays alone or in the presence of a positive FC-XM result were shown to increase the risk of ABMR and graft failure albeit not accounting for high risk in every individual patient [3,61–63]. Therefore, the presence of DSA which are not considered a contraindication for transplantation can be used for risk stratification and adaptation of immunosuppressive protocols. In theory, definition of unacceptable antigens by C1q or C3d luminex assays could replace the need for prospective CDC-XM assays. This notion was supported by a study in which CDC-XM positivity correlated with the presence of pretransplant C1q+ DSA provided that the CDC-XM was positive because of HLA-specific antibodies [64]. However, several studies reported the presence of pretransplant C1q+ DSA despite negative CDC-XM results [50,51,65]. While in some studies these C1q+ DSA were predictive of ABMR or inferior graft survival, either alone [66,67] or in conjunction with IgG3 status [68], many other studies have shown no effect whatsoever of these parameters [8,50,51]. Therefore, if C1q-defined DSA would be used for virtual crossmatching, a proportion of patients are likely to be wrongfully denied a transplant. On the other hand, if complement-binding characteristics of pretransplant DSA such as C1q and IgG3 have the ability to stratify patients into high and low risk for allograft loss as shown by Viglietti *et al.* [68], administration of pretransplant complement-targeting therapies could help transplantation of particularly highly sensitized patients having these kinds of DSA.

Post-transplant prognostic value

It is well known that post-transplant pre-existing or *de novo* DSA will lead to inferior graft survival in only a subset of patients. Loupy *et al.* showed that patients with IgG DSA (pre-existing and/or *de novo*) determined within the first-year post-transplant had a five-year graft survival of 83% versus 94% for patients without DSA. When focusing on C1q positivity, they showed that patients with C1q+ DSA had a five-year graft survival of only 54%, indicating that C1q positivity increases the chance of inferior graft survival [8]. Several studies confirmed these findings by showing a strong association of post-transplant persisting or *de novo* appearing C1q+ DSA with acute ABMR, transplant glomerulopathy and allograft loss in both adult and pediatric kidney transplant recipients [65,69–76].

However, Wiebe *et al.* [48] showed that *de novo* DSA C1q status was not associated with graft loss after adjusting for nonadherence and clinical phenotype in a multivariate model, suggesting that compliance is an

important factor to take into consideration in such studies.

Clinical relevance of the C3d assay was first demonstrated by Sicard *et al.* [38]. At time of ABMR, 85% of the patients with C3d+ DSA had C4d deposition in the graft. Interestingly, whereas C4d deposition did not correlate with higher risk of graft loss, presence of C3d+ DSA was found to have strong association with increased risk of graft loss, even in patients with low MFI IgG DSA (<3500). Recently, Pelletier *et al.* [77] showed that patients with C3d+ *de novo* DSA had a higher incidence of acute rejection, ABMR and graft loss compared to those with C3d− DSA.

Several studies showed that the presence of C3d+ DSA was superior to C1q+ DSA in stratifying patients at risk for kidney graft failure either at time of first DSA detection or at time of ABMR diagnosis [38,78,79]. On the contrary, Eskandary *et al.* [80], while showing a tendency toward a superior predictive value for C1q+ DSA over C3d+ DSA, reported that complement SAB assays did not provide additional information in comparison to standard SAB assays on predicting the ongoing ABMR. Similarly, a recent study by Courant *et al.* [54], in a mix cohort of patients with pre-existing and *de novo* DSA at the time of an indication biopsy, confirmed that neither C1q nor C3d SAB assay was able to independently predict ABMR and graft loss.

Nevertheless, IgG subclass properties of DSA can also be responsible for allograft injury occurring via complement-dependent or independent pathways. Similar to the pretransplant setting, both the presence of IgG3 and C1q+ DSA after transplantation were superior in predicting risk for allograft loss compared with the mere presence of DSA [68]. A recent study by Lefaucheur *et al.* [59] reported that presence of IgG3 and/or IgG4 DSA in the first-year post-transplant correlated well with acute ABMR and subclinical ABMR, respectively. The authors also showed that patients with IgG4 DSA had more chronic lesions such as transplant glomerulopathy and interstitial fibrosis/tubular atrophy (IF/TA) in biopsies, again suggesting that chronic ABMR may be resulting from complement-independent pathways. The fact that IgG4 responses develop after persisting or repeated antigen exposure may as well explain why IgG4 is accumulated more in the later stages of the allograft injury and may indicate a prolonged humoral alloimmune response [81]. In line with these findings, Guidicelli *et al.* [43] showed that long-term exposure to noncomplement-binding *de novo* DSA detected at 2 and 5 years after transplantation resulted in lower 10-year graft survival similar to the effects of complement-binding *de novo* DSA on graft survival.

Because there is no standardized treatment for ABMR, clinical application of ABMR treatment greatly varies between centers while effectiveness of currently available treatment options is limited [82]. With some novel treatments directly affecting the complement system, the complement-fixing ability of DSA may guide the choice of treatment as well as how to monitor for treatment success.

In a study on acute ABMR treatment by plasmapheresis and IVIG, Ramon *et al.* [83] found that post-treatment conversion of DSA from C1q+ to C1q− showed a better specificity and positive predictive value to predict graft survival as well as response to therapy than a reduction of ≥50% in IgG DSA MFI. Recently, Viglietti *et al.* [84], showed that C1q+ DSA after ABMR treatment with plasma exchange, IVIG, and rituximab was capable of identifying potential patients who will experience future allograft loss. A recent interim report by Lefaucheur *et al.* [85] showed that in patients with C1q+ DSA, eculizumab treatment was found to abrogate the ABMR phenotype with a lower incidence of rejection at 3 months in comparison to those patients receiving plasma exchange and IVIG. In their further analyses, the authors showed that patients with C1q+ DSA benefited eculizumab treatment more than those with C1q− DSA, suggesting a prognostic value for C1q assay in predicting response to treatment, although the response observed was limited to patients with pre-existing DSA. These observations need to be confirmed once the studies are completed, but already warrant further studies in cohorts without pre-existing DSA.

Conclusion

Until now, several assays utilizing luminex SAB technology have been developed to determine the complement-binding capacity of HLA antibodies in addition to IgG subclass analysis. Among these assays, the noncommercially available IgG subclass variants suffer most from lack of appropriate control samples, control beads and therefore remain to be a very expensive assay with low sensitivity requiring a robust standardization. Nevertheless, if the high predictive capacity of IgG3 DSA with increased risk of allograft loss can be confirmed using standardized assays in independent groups, this parameter may be a better tool for risk stratification than the standard IgG luminex SAB assay.

At the other hand, results on utilization of C1q assays in the pretransplant period are not in consensus and

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these assays do not seem to have clear prognostic value when used in the pretransplant period in conjunction with CDC-XM.

The majority of studies found a correlation between post-transplant C1q/C3d status and ABMR development and/or graft loss, indicating that these assays may be used for post-transplant monitoring. Importantly, one should realize that the majority of complement-fixing antibodies detected in these assays will be detected in standard SAB assays with high MFI.

While the quest for assays that can distinguish non-deleterious DSA from the harmful ones is ongoing, the data up till now do not unequivocally show additional benefit of using C1q, C3d, or IgG subclass SAB variants for clinical purposes.

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Conflicts of interest

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