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Risk Stratification Before Living Donor Kidney Transplantation in Patients With Preformed Donor-specific Antibodies by Different Crossmatch Methods

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Background. Preformed donor-specific HLA antibodies (DSA) are a well-known risk factor in kidney transplantation. There is still considerable debate, however, about the optimal risk stratification among patients with preformed DSA. Additionally, data on the prognostic value of different crossmatch assays in DSA-positive patients are scarce. **Methods.** DSA-positive living kidney transplant recipients were selected from a multicenter study examining 4233 consecutive renal transplants. An additional 7 patients from 2 further centers were included. Flow cytometric crossmatches (FXM), Luminex-based crossmatches, and virtual crossmatches based on C1q- and C3d-binding antibodies (C1qXM and C3dXM) were performed retrospectively using pretransplant sera and lymphocytes isolated from fresh samples. These samples were obtained from 44 donor and recipient pairs from 12 centers. Clinical outcome data and the control group without DSA were compiled from the previous study and were supplemented by data on 10-y death-censored graft survival (10yGS). **Results.** Between 19% (C3dXM) and 46% (FXM) of crossmatches were positive. Crossmatch-positive patients showed high incidences of antibody-mediated rejection (AMR) within 6 mo (up to 60% in B-cell FXM+ patients). The incidence of AMR in crossmatch-negative patients ranged between 5% (FXM–) and 13% (C1qXM–). 10yGS was significantly impaired in patients with positive T-cell FXM and total FXM compared with both patients without DSA and those with DSA with negative FXM. **Conclusions.** Especially FXM are useful for risk stratification, as the outcome of DSA-positive, FXM-negative patients is similar to that of DSA-negative patients, whereas FXM-positive patients have both more AMR and decreased 10yGS. Because of their lower sensitivity, the significance of Luminex-based crossmatches, C1qXM, and C3dXM would have to be examined in patients with stronger DSA.

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The risk for antibody-mediated rejections (AMR) and graft loss is significantly increased for patients, in whom antibodies against any of the donor's HLAs are detected before kidney transplantation.[1,2] There is still considerable debate, however, on the optimal risk stratification among patients with preformed donor-specific HLA antibodies (DSA).

Although many DSA characteristics can be determined in vitro (such as IgG subtype, ability to bind complement, and mean fluorescence intensity in single-antigen bead [SAB] assays), there are conflicting data on the prognostic value of these characteristics for the clinical course of the patient. Crossmatches with donor cells are less sensitive than solid-phase methods for antibody testing but are unique in their ability to consider not only the cumulative effect of multiple DSA directed against different HLAs but also the expression level of HLAs on the donor cells.[3] This is especially important as there are huge differences in the number of HLAs per cell not only depending on the HLA locus but also between different individuals with identical HLA typing.[4]

Most studies examining the prognostic value of cell-based crossmatches were performed many years ago.[5-8] While the majority of those studies showed an impaired clinical outcome in crossmatch-positive patients, they were unable to differentiate between DSA-positive/crossmatch-positive and DSA-positive/crossmatch-negative patients because sensitive single-antigen testing was unavailable in those days. Therefore, it remains unclear whether the increased risk in crossmatch-positive patients was only because of an increased prevalence of DSA in this group. Nowadays, sensitive determination of DSA by single-antigen testing before kidney transplant is standard of care in most countries, thus there is less interest in additional cell-based crossmatches to identify patients with DSA. There is, however, an overwhelming request for sophisticated risk stratification within the group of DSA-positive patients. The data for this application of cell-based crossmatches are very limited: Couzi et al[9] showed that 21 DSA-positive patients with a positive flow cytometric crossmatch (FXM) had more rejections than 11 DSA-positive but FXM-negative patients. Kwon et al,[10] however, did not detect any difference in AMR ratio or graft survival between DSA-positive, FXM-positive and DSA-positive, FXM-negative patients.

During recent years, several possible alternatives to cell-based crossmatches have been proposed (eg, a Luminex-based crossmatch[11] or virtual crossmatches with in vitro complement-binding antibodies[12]), but for those methods also, the prognostic value is unclear.

Therefore, we aimed to examine the usefulness of cell-based crossmatches for risk stratification among patients with preformed DSA by performing FXM and comparing the risk for AMR and graft loss between crossmatch-positive and crossmatch-negative patients. Additionally, alternative crossmatch methods, such as a Luminex-based crossmatch and virtual crossmatches with in vitro C1q- or C3d-binding antibodies, were evaluated. Most study patients of the present cohort originate from a previous study examining 4233 consecutive German kidney transplants in 18 transplant centers.[1] The crossmatches were performed with stored pretransplant sera and donor cells isolated from fresh blood samples. Therefore, only patients with preformed DSA before living donation could be included.

MATERIALS AND METHODS

Patients and Specimen

Inclusion criteria were living kidney donation with known pretransplant DSA during the years 2012 until 2015. Most patients were recruited from a previous study examining the prognostic value of preformed DSA in patients from 18 German transplant centers.[1] This study comprised a total of 4233 consecutive renal transplant patients, of whom 4132 had complete follow-up data. Of these patients, 1324 had received a living donor transplant and 106 had received a kidney from a living donor despite known preformed DSA. Of these 106 patients, stored pretransplant sera were available in 84 patients. An additional 7 patients from 2 other German transplant centers were included, resulting in 91 patients with available pretransplant sera.

Clinical outcome data were obtained from the previous study as well as the control group of 1218 living donor kidney

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F.M.H. and M.V. have shared authorship.

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transplant patients without preformed DSA.[1] In 22 control patients without DSA and AMR within 6 mo, where the exact date of the first AMR was missing, the date of the first AMR was assumed to equal the median date of the first AMR of the other control patients (8 d posttransplant). To assess long-term graft and patient survival, current data for the included patients were provided by the transplant centers in 2023.

New EDTA and heparin blood samples were obtained from 44 kidney donor and recipient pairs from 12 transplant centers (Augsburg, Berlin, Dresden, Essen, Hamburg, Kiel, Lübeck, Munich Ludwig-Maximilians-University, Munich Technical University, Regensburg, Stuttgart, Tübingen). All these patients had negative complement-dependent cytotoxicity crossmatches with unseparated cells or T lymphocytes before transplantation, and only 2 patients had positive complement-dependent cytotoxicity crossmatches with B lymphocytes after treatment with dithiothreitol. Lymphocytes isolated from fresh donor and patient samples were used to perform FXM and Luminex crossmatches with stored pretransplant recipient sera, as well as for additional HLA typing if needed for the virtual crossmatches.

The study was approved by the ethical committee of the University of Lübeck (protocol No. 15-132) and adheres to the Declaration of Helsinki.

Flow Cytometric Crossmatches

All T- and B-cell FXM were performed at the Institute for Transfusion Medicine in Essen as described previously.[8,13] Of note, the T-cell FXM was performed using a biotin–streptavidin amplifier complex. Thereby, the signal is enhanced 3-fold and the assay is highly sensitive. The B-cell FXM was performed after pretreatment with 2 mg/mL pronase for 30 min at 37 °C. IgG in patient sera bound to either $CD3^+$ T cells or $CD19^+$ B cells was acquired using FACSCalibur (BD Biosciences, Franklin Lakes, NJ). For the T-cell FXM, the cutoff for positive reactions was defined as mean fluorescence intensity (MFI) value exceeding negative control plus 22; for the B-cell FXM, it was defined as MFI value exceeding negative control plus 25.5.

The total result of the FXM was calculated as follows:

- Patients with DSA against HLA class I only:
  - FXM with T cells negative: total FXM result negative.
  - FXM with T cells positive: total FXM result positive.
  - FXM with T cells undetermined (because of positive autologous crossmatch): total FXM result equals FXM with B lymphocytes.
- Patients with DSA against HLA class II only:
  - Total FXM result equals FXM with B lymphocytes.
- Patients with DSA against HLA class I and class II:
  - FXM with B cells negative: total FXM result negative.
  - FXM with B cells positive: total FXM result positive.
  - FXM with B cells undetermined and FXM with T cells positive: total FXM result positive.
  - FXM with B cells undetermined and FXM with T cells negative or undetermined: total FXM result undetermined.

Luminex Crossmatches

The LIFECODES Donor Specific Antibody Assay (Immucor, Norcross, GA) was performed at the Institute for Transfusion Medicine in Hannover according to the manufacturer's protocol. After isolation of donor lymphocytes, they were treated with a lymphocyte lysis buffer. The donor lysate was incubated with microspheres coated with monoclonal antibodies specific for HLA class I or class II to allow binding of HLA molecules. After the incubation with patient serum/controls, phycoerythrin-conjugated antihuman IgG is added as a secondary antibody. Data acquisition was done using the Luminex 200 Flow Analyzer (Luminex, Austin, TX) and data analysis was performed using the MATCH IT! Antibody Analysis Software (Immucor, Norcross, GA). Each control bead has an equation for calculating the cutoff value for HLA class I and class II capture beads; the cutoff value is calculated and subtracted from the MFI value of the capture bead, resulting in an adjusted MFI value. There is a total of 3 control beads, resulting in 3 adjusted values. A sample is considered positive if 2 or more adjusted MFI values are positive. Only Luminex crossmatch results corresponding to the DSA class were considered (eg, only Luminex crossmatch class II for patients with only class II DSA).

Virtual Crossmatches With In Vitro Complement-binding Antibodies

C1q-binding antibodies were tested at the Institute for Transfusion Medicine in Lübeck according to the manufacturer's instructions using SAB kits (C1q Screen assay [One Lambda/Thermo Fisher Inc, Canoga Park, CA] in combination with conventional SAB for HLA class I and class II). For the C1q assay, patient sera were additionally heat-inactivated at 56 °C for 30 min. C3d-binding antibodies were analyzed at the Institute for Transfusion Medicine in Hannover by the LIFECODES C3d Detection Assay (Immucor, Norcross, GA) in combination with conventional SAB for HLA class I and class II. The antibody specificities were compared with the donors' HLA typing for HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3/DRB4/DRB5, HLA-DQA1, HLA-DQB1, HLA-DPA1, and HLA-DPB1. If C1q- or C3d-binding antibodies were present against any of the donor's HLAs, the virtual crossmatch was considered positive. If the donor typing was insufficient to determine whether the recipient's HLA antibodies were donor-specific (eg, missing typing for DP), additional donor typing by next-generation sequencing was performed at the Institute for Transfusion Medicine in Hannover.

Two donors without material for additional typing had to be excluded in the evaluation of the virtual crossmatches with C3d- or C1q-binding antibodies because a partially missing HLA typing did not allow to categorize the virtual crossmatch as positive or negative.

Statistics

For descriptive statistics, differences between groups were described using the Pearson chi-square test and Mood median test, where appropriate.

Death-censored graft survival was evaluated with Kaplan-Meier curves and significant differences were determined using log-rank analyses. A P value of <0.05 was considered significant. Calculations were performed by the program IBM SPSS Statistics (IBM Corporation, Armonk, NY).

RESULTS

Patient characteristics are displayed in Table 1. All patients received triple immunosuppression (95% with tacrolimus, mycophenolate mofetil, and steroids) and most patients

TABLE 1.

Characteristics of included patients (N = 44)

Recipient age, y	46.5 (40–54)
Recipient sex	
Female	24 (55%)
Male	20 (45%)
Previous transplantation	13 (30%)
Preemptive kidney transplantation	8 (18%)
Dialysis before transplant, mo	17 (3.5–28.5)
Current PRAs, %	0 (0–31.5)
Highest PRA, %	19.5 (0–73.5)
Mismatches HLA-A, HLA-B, HLA-DR	4 (3–5)
DSAs against	
Only HLA class I	15 (34%)
Only HLA class II	20 (45%)
Both HLA class I and class II	9 (20%)
Maximum DSA strength, MFI	3000 (1013–10 539)
Cumulative DSA strength, MFI	3250 (1113–11 496)
Donor age, y	53 (45–59)
Donor sex	
Female	23 (52%)
Male	21 (48%)
Cold ischemic period, min	139 (113–169)
Induction therapy	
None	3 (7%)
ATG	8 (18%)
IL-2 receptor antagonist	17 (39%)
Anti-CD20 antibody	4 (9%)
IL-2 receptor antagonist and anti-CD20 antibody	7 (16%)
ATG and anti-CD20 antibody	5 (11%)
Initial immunosuppression	
Cyclosporin A/mycophenolate mofetil/steroids	2 (5%)
Tacrolimus/mycophenolate mofetil/steroids	42 (95%)
Desensitization therapy	
None	28 (64%)
ABO-incompatible transplantation	8 (18%)
Desensitization due to DSA	8 (18%)

Categorical values are given as n (%) and numerical values as median (interquartile range).
ATG, antithymocyte globulin; DSA, donor-specific antibody; IL, interleukin; MFI, mean fluorescence intensity; PRA, panel-reactive antibody.

additionally received induction therapy (mainly an interleukin 2 receptor antagonist). Desensitization by immunoadsorption or plasmapheresis was performed in 18% of patients because of ABO-incompatible transplantation, and another 18% because of DSA. In case of ABO-incompatible transplantations, desensitization was performed by treatment with anti-CD20 antibodies, intravenous immunoglobulins, and serial immunoadsorptions or plasmapheresis sessions until the isoagglutinin titer was 4 or lower. Similar procedures were used for desensitization caused by DSA but without uniform goals for reduction of DSA strength. Two of the patients with desensitization caused by DSA did not receive anti-CD20 antibodies.

The proportion of patients with positive crossmatches ranged from 19% for the virtual crossmatch with C3d-binding antibodies to 46% for the total result of the FXM (Figure 1). Five FXM with T lymphocytes, 15 FXM with B lymphocytes, 7 total results for the FXM, and 3 Luminex crossmatches had to be excluded from this evaluation because of positive reactions of both autologous and donor cells. FXM for 2 patients and Luminex crossmatch for 2 other patients were invalid and could not be repeated because of lack of available samples.

For all different crossmatch techniques, AMR within 6 mo posttransplant occurred more frequently in crossmatch-positive than in crossmatch-negative patients (Figure 2). This difference was significant for all methods except for the virtual crossmatch with C1q-binding antibodies. The risk for AMR was highest in patients with a positive B-cell FXM (60%), and similar to DSA-negative patients in patients with a negative FXM with B cells, a negative total result of the FXM or a negative Luminex crossmatch.

Death-censored graft survival seemed to be impaired in all patients with positive crossmatches already early after transplantation. This difference was significant for T cell and total FXM results, as well as for the virtual crossmatch with C1q-binding antibodies, but only showed a trend for the B-cell FXM (Figure 3). Neither the Luminex crossmatch nor the virtual crossmatch with C3d-binding antibodies showed a significant association with graft survival.

In addition to the aforementioned results for 44 patients with available fresh donor blood samples, virtual crossmatches were also calculated for all 91 DSA-positive patients with available pretransplant sera (see **Table S1, SDC** [http://links.lww.com/TXD/A679] for patient characteristics). Despite the doubled number of patients, the difference in graft survival between C1q-crossmatch-positive and C1q-crossmatch-negative DSA-positive patients lost significance. The difference in AMR between C1q-crossmatch-positive and C1q-crossmatch-negative patients was still not significant, even if reactions with beads known to be susceptible to false-positive reactions (HLA-C-alleles and B*44:02) were considered negative (**Figures S1 and S2, SDC**, http://links.lww.com/TXD/A679). The incidence of AMR in C1q- or C3d-crossmatch-negative patients, however, was significantly higher than in patients without DSA.

There was neither a significant difference in AMR ratio or graft survival between desensitized and non-desensitized

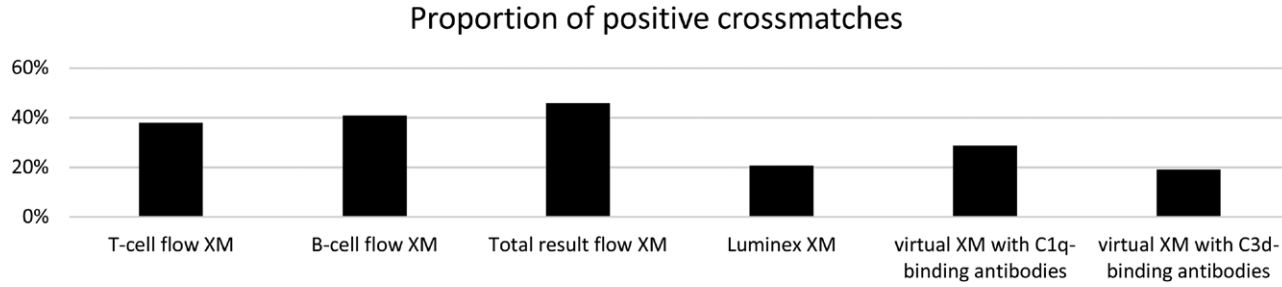

FIGURE 1. Proportion of positive pretransplant crossmatch (XM) in 44 living donor kidney transplant recipients with preformed donor-specific HLA antibodies. Patients with a positive autologous XM or insufficient donor typing for the virtual XM were excluded from this evaluation.

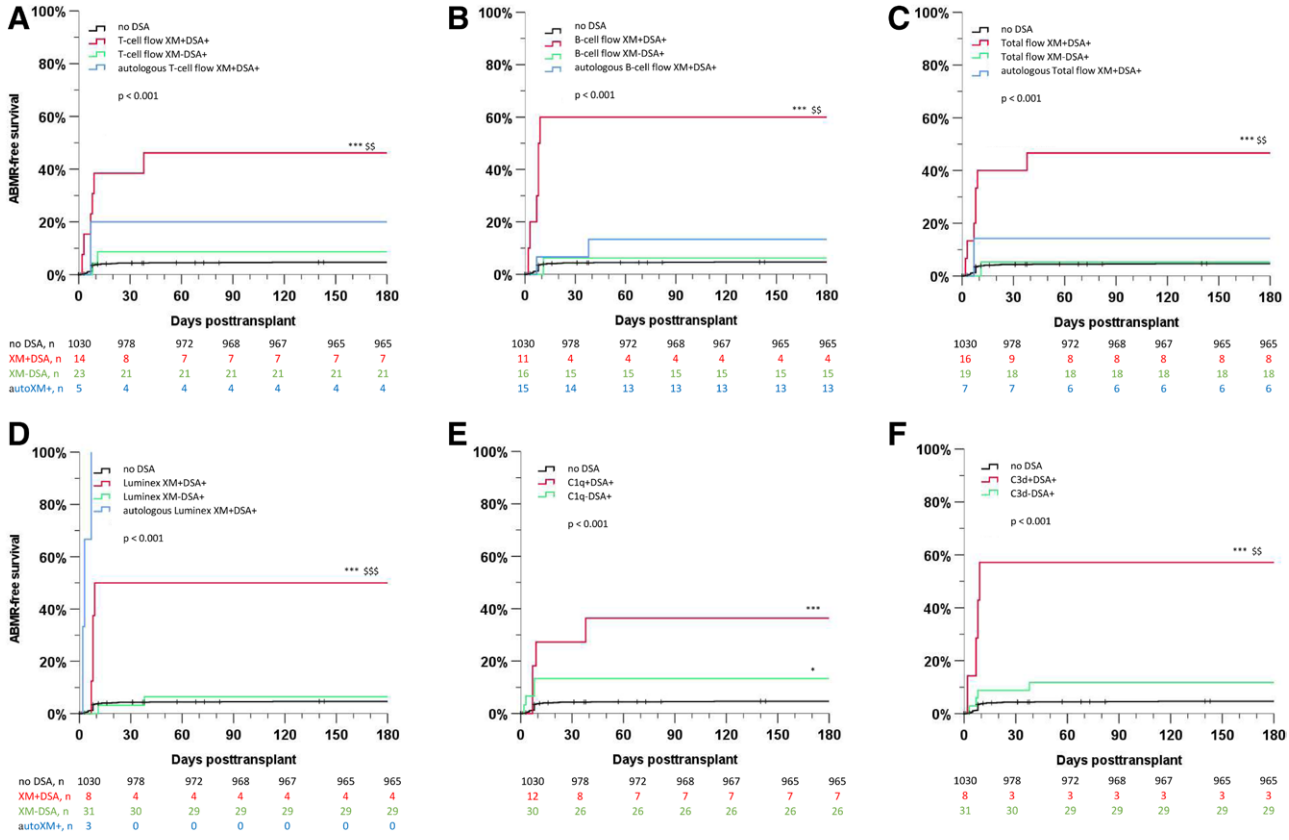

FIGURE 2. AMR-free survival during the first 6 mo posttransplant according to flow (A–C), Luminex (D), and virtual XM results with in vitro complement-binding antibodies (E and F). Data for the control group of DSA-negative patients were extracted from the original study.[1] $^{*}P < 0.05$ vs patients without DSA, $^{***}P < 0.001$ vs patients without DSA, $^{\$\$}P < 0.01$ vs patients with negative XM, $^{\$\$\$}P < 0.001$ vs patients with negative XM. AMR, antibody-mediated rejection, DSA, donor-specific antibody; XM, crossmatch.

patients nor an association between the DSA class (only class I, only class II, or both) and transplant outcomes.

DISCUSSION

This study is unique in comparing different alternative crossmatch techniques in the same study population of living donor kidney recipients with preformed DSA. All crossmatch techniques were able to stratify the risk of AMR within 6 mo posttransplant, but only positive results in the T-cell FXM, the total FXM, and the virtual crossmatch with C1q-binding antibodies were associated with significantly reduced graft survival.

Cell-based crossmatches with donor cells are influenced not only by the cumulative effect of multiple DSA directed against different HLAs but also by the various expression levels of HLAs on the donor cells.[3] Furthermore, the unaltered presentation of HLAs on the cells allows differentiation between true DSA against donor HLA and false-positive reactions of the SAB assay due to denatured HLA on the beads (so-called natural antibodies).[14-16]

Therefore, positive FXM are assumed to identify at-risk patients. However, before our study, there were very little data to support this assumption, as most of the studies were performed before the implementation of SAB assays. Therefore, no comparison between FXM-positive and FXM-negative but DSA-positive patients could be performed in those studies. Thus, a worse clinical outcome in FXM-positive patients could just have been the effect of most crossmatch-positive patients being DSA-positive, whereas most crossmatch-negative patients were DSA-negative. Our data clearly show that FXM are able to identify patients with poor prognosis among the group of DSA-positive patients.

In patients with a negative B cell or total FXM, not only graft survival but also incidence of early AMR was comparable to DSA-negative patients. Irrespective of the exact type of FXM (T cell, B cell, or total result), crossmatch-positive patients had increased incidences of AMR and decreased graft survival. Only the decreased graft survival for B-cell FXM-positive patients missed significance, presumably, because too many patients had to be excluded because of a positive autologous crossmatch. Generally, B-cell FXM are sensitive to false-positive results. In the current study, pronase treatment was used routinely to reduce this problem. Calculating a total FXM result from the measured results of the T-cell and B-cell FXM in consideration of the DSA class allowed both to get valid FXM results for most patients and to consider B-cell information whenever possible.

Our findings confirm the results of Couzi et al,[9] who detected an increased risk for acute rejections in 21 DSA-positive patients with a positive FXM compared with 11 DSA-positive, but FXM-negative patients. There was no difference in graft survival in that study, which might be because of a relatively short follow-up period of crossmatch-positive transplants of about 2 y only.

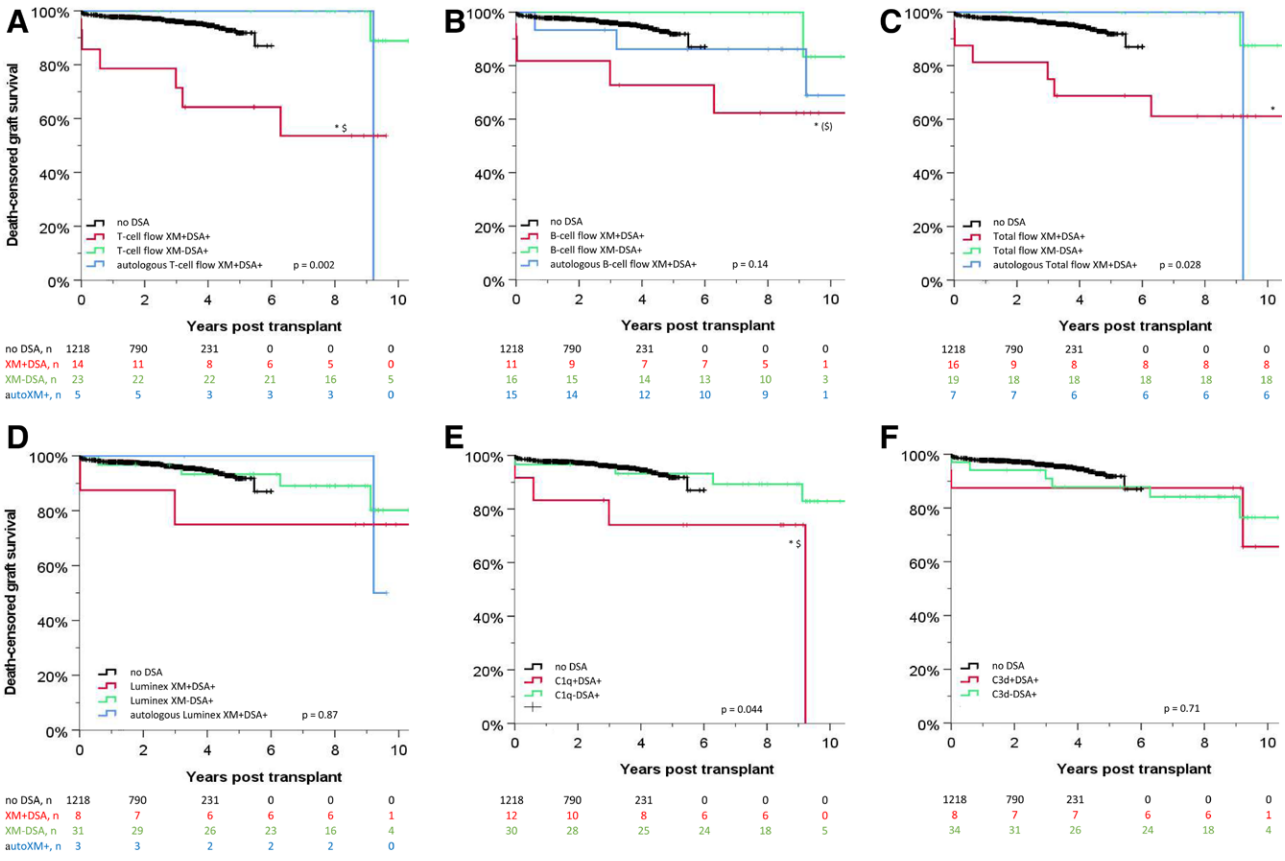

FIGURE 3. Graft survival according to flow (A–C), Luminex (D), and virtual XM results with in vitro complement-binding antibodies (E and F). The follow-up period for the control group of DSA-negative patients is shorter, as these data were extracted from the original study.[1] *$P < 0.05$ vs patients without DSA, $^{\$}P < 0.05$ vs XM-negative patients, $^{(\$)}P = 0.06$ vs patients with negative XM. DSA, donor-specific antibody; XM, crossmatch.

In contrast to our results, Kwon et al[10] did not find any difference in AMR ratio or graft survival between DSA-positive, FXM-positive and DSA-positive, FXM-negative patients. However, these groups were not treated equal as all FXM-positive patients, but not DSA-positive and FXM-negative patients received a desensitization therapy with anti-CD20 monoclonal antibodies and serial plasmapheresis until the FXM was converted to negative. Therefore, an increased risk in FXM-positive patients might have been ameliorated by desensitization.

There are some limitations of this study. As we evaluated real-world data from 12 transplant centers, there were no uniform protocols for DSA or AMR management. Most centers did not perform protocol biopsies, so subclinical rejections might have been missed. We aimed to get as accurate data as possible by summarizing not only biopsy-proven AMR but also presumed AMR in the absence of biopsies. Despite the multicenter nature, the sample size is too low to differentiate the results according to DSA class or specific pretreatment protocols. Nevertheless, the presented data are still one of the largest cohorts of DSA-positive patients in whom the prognostic value of crossmatch tests has been evaluated.

The Luminex crossmatch and the virtual crossmatches with in vitro complement-binding antibodies were less sensitive than the FXM. Therefore, the number of positive crossmatches was lower, preventing clear information about the significance of positive crossmatch results in these tests. Additionally, some presumably false-positive reactions for C1q-binding antibodies impaired the prognostic value of this assay. This effect is among others caused by antibodies binding on denatured HLAs on the SABs.[14] Further studies including patients with strong DSA would be needed to determine the prognostic value of these tests.

In conclusion, this study is the second report about an increased incidence of AMR in FXM-positive, DSA-positive patients compared with FXM-negative, DSA-positive patients. It is the first to demonstrate a significantly reduced graft survival in FXM-positive, DSA-positive patients compared with FXM-negative, DSA-positive patients. Patients with negative total or B-cell FXM, however, had no inferior outcome compared with DSA-negative patients.

Therefore, the presented data substantiate that FXM are still useful in the era of comprehensive DSA testing as they enable risk stratification in DSA-positive patients before kidney transplantation.

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