Research Article

C1Q Assay Results in Complement-Dependent Cytotoxicity Crossmatch Negative Renal Transplant Candidates with Donor-Specific Antibodies: High Specificity but Low Sensitivity When Predicting Flow Crossmatch

José M. Arreola-Guerra, Natalia Castelán, Adrián de Santiago, Adriana Arvizu, Norma González-Tableros, Mayra López, Isaac Salcedo, Mario Vilatobá, Julio Granados, Luis E. Morales-Buenrostro, and Josefina Alberú

1Department of Transplantation, National Institute of Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga 15, Colonia Belisario Domínguez, Sección XVI, Tlalpan, 14080 Mexico City, Mexico
2Histocompatibility Laboratory, National Institute of Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga 15, Colonia Belisario Domínguez, Sección XVI, Tlalpan, 14080 Mexico City, Mexico
3Department of Nephrology-Mineral Metabolism, National Institute of Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga 15, Colonia Belisario Domínguez, Sección XVI, Tlalpan, 14080 Mexico City, Mexico

Correspondence should be addressed to Luis E. Morales-Buenrostro; luis.buenrostro@yahoo.com and Josefina Alberu; josefinaalberu@hotmail.com

Received 29 March 2016; Revised 22 June 2016; Accepted 13 July 2016

Academic Editor: Andreas Zuckermann

Copyright © 2016 José M. Arreola-Guerra et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The aim of the present study was to describe the association of positive flow cross match (FXM) and C1q-SAB. Methods. In this observational, cross-sectional, and comparative study, patients included had negative AHG-CDC-XM and donor specific antibodies (DSA) and were tested with FXM. All pretransplant sera were tested with C1q-SAB assay. Results. A total of 50 donor/recipient evaluations were conducted; half of them had at least one C1q+Ab (n = 26, 52%). Ten patients (20.0%) had DSA C1q+Ab. Twenty-five (50%) FXMs were positive. Factors associated with a positive FXM were the presence of C1q+ Ab (DSA C1q+ Ab: OR 27, 2.80–259.56, \( P = 0.004 \), and no DSA C1q+ Ab: OR 5, 1.27–19.68, \( P = 0.021 \)). The cutoff point of immunodominant LABScreen SAB DSA-MFI with the greatest sensitivity and specificity to predict FXM was 2,300 (sensitivity: 72% and specificity: 75%). For FXM prediction, DSA C1q+ Ab was the most specific (95.8%, 85–100) and the combination of DSA-MFI > 2,300 and C1q+ Ab was the most sensitive (92.0%, 92.6–100). Conclusions. C1q+ Ab and LABScreen SAB DSA-MFI were significantly associated with FXM. DSA C1q+ Ab was highly specific but with low sensitivity.

1. Introduction

An almost 50-year-long history has been written on the search for a clinically relevant assay that could predict antibody-mediated graft injury. The story dates back to 1969, when Patel and Terasaki demonstrated for the first time the highly significant correlation between a positive complement-dependent cytotoxicity (CDC) crossmatch (XM) assay and hyperacute or accelerated acute graft rejection in kidney transplant recipients [1]. Ever since, it has been generally accepted that a positive CDC-XM is a contraindication to transplantation. The assay, however, is insensitive for the detection of low HLA donor-specific alloantibody (DSA) levels.

The newer solid phase assay, Luminex Single Antigen Beads (SAB) assay, is highly sensitive and specific for the target antigen when compared with the CDC assay but less predictive of transplant outcome since it detects both complement-binding (pathogenic antibodies) and less clinically relevant, non-complement fixing anti-HLA antibodies.
FXM result. 

ficity, MFI, and capacity to bind C1q, among others) and the capacity of some antibodies' characteristics (antigenic speci-

CDC-XM. It also evaluated the association and the predictive power of DSA by Luminex-SAB (LABScreen SAB) and showed a negative AHG-CDC-XM but DSA against their potential living donors (as detected by Luminex-SAB); then according to institutional protocol all of them were tested with FXM. For this study, the frozen sera samples were tested with the C1q-SAB.

Forty-one renal transplant candidates were evaluated with their respective potential living donors; five of them had 2 potential donors and 2 of them had 3 potential donors, so a total of 50 events were analyzed.

The Histocompatibility Laboratory at our institution is a reference center for pretransplant testing of patients from different transplant centers in Mexico, and most of the patients included in this report were referrals from other national centers.

HLA Typing. HLA typing is obtained for A, B, C, DR, and DQ antigens by molecular biology techniques (Micro SSP™ HLA DNA Typing Trays, One Lambda, Canoga Park, CA).

Crossmatch. In our setting, XM is performed by direct CDC-XM on total and separated T and B lymphocytes. T and B cells are isolated with magnetic beads (FluoroBeads®, One Lambda, Canoga Park, CA, USA). CDC is performed on untreated serum and in serum with added dithiothreitol and enhanced with goat IgG anti-human kappa (One Lambda, Canoga Park, CA, USA). All patients are transplanted once a negative CDC T and B cell crossmatch is established, unless they harbor DSA in which FXM is performed. FXM technique used in our laboratory is the one described by ASHI (ASHI Laboratory Manual, Fourth Edition, Volume II, 2000). In this scenario, a T cell positive FXM (>40 channel shifts) contraindicates the transplant, while a B cell positive FXM (>100 channel shifts) is approved only for the first transplant.

Antibody Assessment. DSA determinations were performed with Luminex LABScreen Single Antigen Beads (LABScreen SAB), class I and class II (One Lambda, Canoga Park, CA, USA), and were systematically obtained as part of the patient's immunological risk profile. Before DSA determination sera were treated with dithiothreitol at a final concentration of 0.005 M, at 37 °C during 30 minutes followed by centrifugation at 3000 x g for 1 minute; after that, the antibody detection described for Luminex using antigen coated beads is followed (ASHI Laboratory Manual, Fourth Edition, Volume II, 2000). For this analysis, DSA ≥ 400 MFI was considered positive.

HLA antigen typing and DSA results were obtained in every patient in the Histocompatibility Laboratory database.

C1q-SAB. Class I and class II C1q-SAB assays (One Lambda, Canoga Park, CA, USA) were performed in all 41 previously cryopreserved (−70°C) serum samples and according to the manufacturer’s directions. Briefly, heat inactivated serum (56°C for 30 minutes) was spiked with 150 mg/mL purified human C1q in HEPES buffer (One Lambda) to ensure equal
functional amounts of C1q per sample. Single Antigen Beads were added to the mixture and incubated for 20 minutes at room temperature, followed by addition of phycoerythrin conjugated anti-human C1q. Beads were washed twice and analyzed on a LABScan200 flow analyzer. C1q antibodies MFI threshold for positivity was 400.

Sera sample from the same date the FXM was performed was frozen until tested for C1q assay in October 2013, using the same reagent lot.

2.1. Statistical Analysis. Descriptive statistics were used according to the type of analyzed variable. Categorical variables were reported as relative and absolute frequencies. The distribution of continuous variables was evaluated with the Kolmogorov-Smirnov test. Variables with a normal distribution are presented as means and standard deviations, while those with an abnormal distribution are expressed as medians and their interquartile range (IQR).

Between-group comparisons of categorical or ordinal variables were performed with Chi^2 or Fisher’s exact test (accordingly). Continuous variables were analyzed with Student’s t-test or Mann-Whitney’s U test. Logistic regression analysis was used to predict FXM. A P value < 0.05 was considered significant.

We explored the optimum cutoff point to predict a positive FXM by MFI of the immunodominant DSA according to ROC curve. Sensitivity, specificity, and predictive values with 95% CI were calculated for a positive FXM prediction.

The STATA version 11.1 statistical package and Excel 2013 were used.

3. Results

The 41 renal transplant candidates included in the study were evaluated between January 2012 and December 2013; five had 2 potential donors, 2 had 3 potential donors, and all were included in the analysis. A total of 50 evaluations of donor/recipient pairs were conducted. The average recipient age was 35 years (±12.72, min–max 8–68), and that of donors was 42.2 years (±11.9, min–max 22–72). Of the 41 studied potential recipients, 23 (56%) were male while 29 (58%) of the potential donors were female.

3.1. HLA-Ab and DSA by LABScreen SAB in 41 Renal Transplant Candidates. The 41 analyzed renal transplant candidates had a median number of HLA-Ab of 12 (interquartile range (IQR) 6–28, min–max 2–74) and a median MFI of the dominant HLA-Ab of 10,128 (IQR 3,813–15,407, min–max 696–24,470). The antigenic specificity of these Abs was against HLA-B (n = 14, 34%) and HLA-DQ (n = 13, 31%).

The median number of DSA was 1 (IQR 1–2, min–max 1–6). The DSA-MFI was 2,138 (IQR 1,160–7,147; min–max 493–23,715). The antigen specificity of these Abs was almost against HLA-B (n = 14, 34%) and HLA-DQ (n = 13, 31.7%).

The median of number of DSA was 1 (IQR 1–2, min–max 1–6). The DSA-MFI was 2,138 (IQR 1,160–7,147; min–max 493–23,715). The antigen specificity of these Abs was against class II antigens in 62.0% of cases, homogeneously distributed between HLA-DQ and DR antigens (n = 16 and 15, resp.).

3.2. Complement Fixing HLA Antibodies (C1q+Ab). Over half of the included population had at least one CFAb or C1q+ Ab (n = 26, 63.4%). The dominant C1q+ Ab had a C1q-SAB MFI of 14,177 (IQR 8,169–16,359, min–max 614–30161). The MFI of coincidental LABScreen SAB was 13,209 (IQR 10,308–18,562, min–max 832–24470). Most dominant CFAbs (greater MFI in C1q-SAB) were class II (n = 18 of 26). Antibodies against HLA-DQ antigens were the most prevalent (n = 12 of 26).

Upon evaluation of the donor/recipient pairs (n = 50), ten patients (20.0%) had donor-specific C1q+ Ab, and one patient had two antibodies; therefore, 11 DSA C1q+ Abs were detected, with a median C1q MFI of 9,542 (IQR 8,169–14,546, min–max 1,202–30161). The MFI of coincidental LABScreen SAB was 14,045 (IQR 12,829–18,562, min–max 956–23,715). Correlation between fluorescence values was poor (r^2 = 0.19), as shown in Figure 1. Most of these antibodies were against HLA-DQ antigens (n = 9), one was against HLA-A antigen, and one was against HLA-DR antigen.

3.3. FXM Results. Twenty-five (50%) of a total of 50 FXMs were positive. Sixteen patients were positive against B lymphocytes, 3 were positive against T lymphocytes, and 6 were positive against both cell types. The median FXM channels against B cells were 232 (IQR 157–297, min–max 130–560) and against T cells were 227 channels (IQR 76–259, min–max 44–390). Among the 22 patients with a positive FXM against B lymphocytes, 5 had anti-class I DSA, 10 had anti-class II DSA, and 7 had DSA against both classes. Among the 9 patients with a positive FXM against T lymphocytes, 4 had anti-class I DSA, 1 had anti-class II DSA, and 4 had DSA against both classes.

3.4. Factors Associated with a Positive FXM. Table 1 shows the analysis of factors associated with a positive FXM. A greater proportion (nonsignificant) of patients with FXM had a history of previous renal transplantation (50.0% versus 26.1%, P = 0.092). It is evident that the presence of DSA C1q+ Ab and the DSA LABScreen SAB MFI, particularly DSA
Table 1: Factors associated with a positive FXM. DSA determinations are expressed according to the LABScreen SAB results. MM = mismatches.

|                                | All samples (n = 50) | Positive FXM (n = 25) | Negative FXM (n = 25) | P value |
|--------------------------------|----------------------|-----------------------|-----------------------|---------|
| Recipient age, m (SD)          | 35.0 ± 12.7          | 33.8 ± 11.2           | 36.2 ± 14.1           | 0.510   |
| Recipient male gender, n (%)   | 23 (46.0)            | 9 (36.0)              | 14 (56.0)             | 0.156   |
| Donor age, m (SD)              | 42.2 ± 11.9          | 43.7 ± 10.6           | 40.7 ± 13.1           | 0.385   |
| Donor male gender, n (%)       | 21 (42.0)            | 12 (48.0)             | 9 (36.0)              | 0.390   |
| Previous transplant, n (%)     | 18 (36.8)            | 12 (50.0)             | 6 (26.1)              | 0.092   |
| HLA MM, med (IQR)              | 4 (4–6)              | 4 (3–6)               | 4 (4–6)               | 0.899   |
| % PRA class I, med (min–max)   | 15 (0–90)            | 14.0 (2–90)           | 9 (0–100)             | 0.466   |
| Class I DSA, n (%)             | 29 (58.0)            | 14 (56.0)             | 15 (60.0)             | 0.774   |
| Class II DSA, n (%)            | 35 (70.0)            | 18 (72.0)             | 17 (68.0)             | 0.758   |

**HLA antibodies analyzed by LABScreen SAB**

|                                | All DSA, m (min–max) | MFI DSA, med (IQR)     | n of all HLA-Ab, med (IQR) | MFI all HLA-Ab, med (IQR) | HLA-A DSA, n (%) | MFI HLA-A DSA, med (IQR) | HLA-B DSA, n (%) | MFI HLA-B DSA, med (IQR) | HLA-C DSA, n (%) | MFI HLA-C DSA, med (IQR) | HLA-DR DSA, n (%) | MFI HLA-DR DSA, med (IQR) | HLA-DQ DSA, n (%) | MFI HLA-DQ DSA, med (IQR) | HLA-DRC1q+Ab, n (%) |
|--------------------------------|----------------------|-----------------------|-----------------------------|---------------------------|------------------|-------------------------|------------------|-------------------------|------------------|-------------------------|------------------|-------------------------|------------------|-------------------------|------------------|
| n of DSA, m (min–max)          | 1.72 (1–6)           | 1.84 (1–6)            | 1.6 (1–4)                   | 0.769                     |
| MFI DSA, med (IQR)             | 2,138 (1,160–7,147)  | 5,166 (1,457–12,829)  | 1,457 (1,113–2,293)         | 0.001                     |
| n of all HLA-Ab, med (IQR)     | 12 (6–28)            | 12 (10–26)            | 8 (4–28)                    | 0.164                     |
| MFI all HLA-Ab, med (IQR)      | 10,128 (3,813–15,407)| 13,312 (5,681–18,666) | 3,937 (2,192–13,312)        | 0.006                     |
| HLA-A DSA, n (%)               | 16 (32.0)            | 7 (28.0)              | 9 (36.0)                    | 0.544                     |
| MFI HLA-A DSA, med (IQR)       | 1,294.5 (763–4,155.5)| 4,467 (3,141–11,526)  | 828 (664–1,113)             | 0.003                     |
| HLA-B DSA, n (%)               | 9 (18.0)             | 5 (20.0)              | 4 (16.0)                    | 1.00                      |
| MFI HLA-B DSA, med (IQR)       | 1,427 (759–2,669)    | 2,669 (1,427–5,670)   | 838 (706.5–1,190)           | 0.141                     |
| HLA-C DSA, n (%)               | 12 (24.0)            | 5 (20.0)              | 7 (28.0)                    | 0.508                     |
| MFI HLA-C DSA, med (IQR)       | 1,866 (843.5–2,739.5)| 2,362 (2,303–2,505)   | 1,247 (612–2,974)           | 0.329                     |
| MFI HLA-DR DSA, med (IQR)      | 20 (40.0)            | 9 (36.0)              | 11 (44.0)                   | 0.564                     |
| MFI HLA-DQ DSA, med (IQR)      | 1,752.5 (1,177.5–3,986)| 5,145 (1,457–8,651)  | 1,515 (1,127–2,216)         | 0.048                     |
| MFI HLA-DQA1q+Ab, n (%)        | 21 (42.0)            | 13 (52.0)             | 7 (32.0)                    | 0.152                     |
| MFI HLA-DQA1q+Ab, med (IQR)    | 7,147 (1,247–14,710)| 12,829 (1,578–17,976) | 1,615 (908.5–5,710)         | 0.042                     |

**HLA antibodies by C1q assay**

|                                | All C1q+ Ab, n (%)   | DSA C1q+ Ab, n (%) | Non-DSA C1q+ Ab, n (%) | HLA-A C1q+ Ab, n (%) | HLA-B C1q+ Ab, n (%) | HLA-C C1q+ Ab, n (%) | HLA-DP C1q+ Ab, n (%) | HLA-DQA1q+Ab, n (%) |
|--------------------------------|----------------------|-------------------|------------------------|----------------------|----------------------|----------------------|----------------------|---------------------|
| All C1q+ Ab, n (%)             | 26 (53.0)            | 19 (76.0)         | 7 (28.0)               | 0.001                |
| DSA C1q+ Ab, n (%)             | 10 (20.41)           | 5 (20.0)          | 7 (28.0)               | 0.508                |
| Non-DSA C1q+ Ab, n (%)         | 16 (32.65)           | 10 (40)           | 6 (24.0)               | 0.023                |
| HLA-A C1q+ Ab, n (%)           | 6 (12.0)             | 4 (16.0)          | 2 (8.0)                | 0.667                |
| HLA-B C1q+ Ab, n (%)           | 8 (16.0)             | 6 (24.0)          | 2 (8.0)                | 0.247                |
| HLA-C C1q+ Ab, n (%)           | 3 (6.0)              | 1 (4.0)           | 2 (8.0)                | 1.0                  |
| HLA-DP C1q+ Ab, n (%)          | 6 (12.0)             | 3 (12.0)          | 0                      | 0.235                |
| HLA-DQA1q+Ab, n (%)            | 19 (38.0)            | 16 (64.0)         | 3 (12.0)               | <0.001               |
| HLA-DRA1q+Ab, n (%)            | 8 (16.0)             | 7 (28.0)          | 1 (4.0)                | 0.049                |

against HLA-A, HLA-DR, and HLA-DQ antigen specificities, are the most significant factors associated with a positive FXM.

By logistic regression analysis, the LABScreen SAB MFI of dominant DSA was also significantly associated with a positive FXM (for each 1,000 MFI: OR 1.26, 95% CI 1.06–1.49, and P = 0.007). This is also evident when the variable is divided at different cutoff points (Figure 2).

Also, a positive C1q-SAB assay was associated with a positive FXM (OR 8.14, 2.29–28.9, and P = 0.001). When this was analyzed by DSA and non-DSA, both remained significant (Figure 3).

Since the most strongly associated variables to the positive FXM were DSA LABScreen SAB MFI and a positive C1q-SAB assay, we conducted a multivariate logistic regression analysis that revealed an interaction phenomenon. When the patients were grouped according to HLA-Ab LABScreen SAB MFI greater than or under 5000, we detected that the most important association of C1q+ Ab with a positive FXM occurred in the group with DSA LABScreen SAB MFI values <5,000 (OR 5.04, 95% CI 1.1–22.9, and P = 0.037) and there was no association in the group with MFI >5000 (OR 1.0, 95% CI 1.0–0.99, and P = 0.485).

After analysis of the median positive channels for T and/or B cells among the 25 positive FXMs in terms of positive or negative C1q-SAB assay, no relation was established. This information is shown in Figure 5, which also specifies the channel shift values of all positive FXMs, those cases with DSA C1q+ Ab among the positive and negative FXM, the MFI for the dominant DSA by LABScreen SAB, and the total
Table 2: Sensitivity, specificity, PPV, and NPV of DSA C1q+ Ab; C1q+ Ab (DSA and non-DSA); DSA LABScreen SAB MFI > 2,300; and DSA > 2,300 plus C1q+ Ab (DSA and non-DSA).

|                          | DSA C1q+ Ab | C1q+ Ab (DSA & non-DSA) | DSA-MFI > 2300 | DSA > 2300 plus C1q+ Ab (DSA & non-DSA) |
|--------------------------|-------------|-------------------------|----------------|----------------------------------------|
| Sensitivity              | 36% (15.1–56.8) | 76.0 (57.2–94.7)      | 72.0 (52.4–91.6) | 92.0 (79.3–100)                        |
| Specificity              | 95.8% (85.7–100) | 70.8 (50.5–91.1)      | 75.0 (55.5–94.4) | 54.1 (32.1–76.1)                      |
| PPV                      | 90 (66.4–100)     | 73.08 (54.1–92.05)    | 73.47 (55.5–94.4) | 67.65 (50.45–84.84)                    |
| NPV                      | 58.9 (42.2–75.6)  | 73.91 (53.7–94.03)    | 72.0 (52.4–91.6) | 86.67 (66.13–100)                     |

The cutoff point with the greatest sensitivity and specificity was 2,300 (Figure 4).

Table 2 shows the diagnostic performance of the C1q+ Ab (DSA and non-DSA) and LABScreen SAB DSA-MFI > 2,300 and their effect together (C1q+ Ab and MFI > 2300). This last construct yielded the best sensitivity and negative predictive value, while the DSA C1q+ Ab had the best specificity.

4. Discussion

The detection of relevant DSA has been limited since the most sensitive and specific method currently used (Luminex-SAB) does not discriminate between clinically relevant antibodies and those that are inert. This is of particular importance in the pretransplant scenario, where antibody generation precedes the donor’s antigenic stimulus; in the posttransplant period, the stimulus for the generation of de novo antibodies is unequivocally originated by renal graft.

Flow cytometry has significantly contributed to improved patient selection and it is the current standard when selecting transplant candidates. After an appropriate HLA typing and antibody determination by Luminex-SAB, patients with DSA+ and/or FXM+ have been found to be at risk for acute rejection. Some groups have reported that this risk is associated with the presence of DSA even if the FXM is negative [18] while others have found that the presence of DSA detected only by SAB in the context of a negative crossmatch is not associated with an increased risk of acute rejection [19, 20]. However, neither scenario conditions an...
## Figure 5: Characteristics of DSA and FXM results.

The first two lines show B cell and T cell channel shift results for each positive FXM test; the next line depicts complement fixing HLA-Ab (C1q+ in filled cells) where a dashed square mark indicates DSA C1q+. The next three lines show the mean DSA-SAB MFI, number of DSA, and dominant DSA-SAB MFI. MFI = mean fluorescence intensity, DSA = donor-specific antibodies, \( N \) = number, C1q+ HLA-Ab = C1q positive HLA antibodies, and FXM = flow crossmatch.

| FXM B | \( \text{C1q}^+ \text{Ab} \) | Mean DSA-MFI | \( N \) DSA | MFI Dom DSA |
|-------|----------------|----------------|-------------|-------------|
|       |                |                |             |             |
| Positive FXM |                |                |             |             |
| FXM T |                |                |             |             |
| Clq+ Ab |                |                |             |             |

| FXM B | \( \text{C1q}^+ \text{Ab} \) | Mean DSA-MFI | \( N \) DSA | MFI Dom DSA |
|-------|----------------|----------------|-------------|-------------|
|       |                |                |             |             |
| Negative FXM |                |                |             |             |
| FXM T |                |                |             |             |
| Clq+ Ab |                |                |             |             |

### Absolute Risk

One of the most representative studies included 21 patients with FXM+ and DSA+ and revealed a greater risk of acute antibody mediated rejection (AMR) when compared to negative FXM patients [21]. Among these patients, 6 (28.5%) developed AMR, while the remaining 15 (66.66%) did not present any antibody mediated events [21]. Due to the broadly documented evidence on the mechanisms of acute rejection in the early posttransplant period and their direct relation with complement activation as a pivotal physiopathological mechanism, the Clq-SAB assay could potentially contribute to the elucidation of the association between complement activation, a positive FXM, and the transplant’s outcome.

Our study included the samples of sensitized patients in whom a high prevalence of HLA-Ab capable of binding complement (C1q+ Ab) was documented (\( n = 26, 53.06% \); in 10 cases (20%), these HLA-Ab targeted a potential donor’s antigenic specificity (DSA Clq+ Ab). In these 10 cases, most were against HLA-DQ (9/11 Clq+ Ab), which is in stark contrast with the original Clq-SAB assay report that detected a greater proportion of anti-class I antibodies capable of binding complement [16]. All 10 cases had a previous renal transplant, which could explain the predominance of HLA-DQ antibodies [22]. Subsequent reports proved that HLA-DQ antigenicity and that of class II antigens in general could generate \textit{de novo} antibodies capable of activating complement, leading to AMR and graft loss [23]. It is important to mention that DP specificity was no typing, which constitutes a limitation of the present analysis, although the relevance of HLA-Ab versus DP specificities has not been clearly recognized as deleterious at this moment [24]. In clinical practice in our particular setting, patients with DSA against a potential living donor and a negative AHG-CDC-XM are not infrequent. Following current guidelines, these patients should be evaluated by FXM to determine whether the transplant may proceed without the need for any desensitization maneuvers [6].

We observed that 25 (50%) of our FXMs were positive. Of these, only 9 (36%) had DSA Clq+ Ab. This number reflected the high specificity in predicting FXM+ (95.8%). Ten more patients with a positive FXM were non-DSA Clq+ Ab. This same situation occurred in 6 of 25 patients with a negative FXM. Compared to patients with no CFAbs, both the non-DSA Clq+ Ab group and the DSA Clq+ Ab group were at
significant risk for a positive FXM (OR 5, 95% CI 1.27–19.68, and \( P = 0.021 \), and OR 27, 95% CI 2.80–259.56, and \( P = 0.004 \), resp.). Cases with DSA C1q+ Ab and negative DSA by IgG-SAB have been attributed to IgM antibodies or a prozone phenomenon; in this series, however, we did not detect any cases with these characteristics. All patients included in this study had DSA that can explain the positive FXM. To attribute this positivity to a non-DSA C1q+ Ab cannot be clearly biologically sustained. Complement activating of antibodies against donor-specific epitopes may be a possibility. Several studies have emphasized the importance of antibody specificity against epitopes that may be shared by more than one HLA antigen and this would certainly denote a limitation to serologic determination by Luminex. Moreover, it is also possible that we are detecting a more immunologically robust group of patients with the ability to generate more functional antibodies. In either case, this study is not designed to affirm or reject these proposals.

Based on these findings and being well aware of the study's methodology limitations, particularly in terms of its pretransplant and cross-sectional design, we must emphasize the clinical usefulness of our results. Above all, only in 9 of 25 patients with a positive FXM could an explanation hinge on complement activation. Flow cytometry is known to detect donor-specific antibodies that bind to the cell membrane regardless of their functionality. However, the clinical significance of FXM+ in the absence of complement fixing DSA remains to be determined. We must emphasize that two-thirds of FXM+ and DSA+ patients will never develop early AMR phenomena [21]. However, complement-independent mechanisms of endothelial cell damage by anti-HLA antibodies have been proposed. HLA class I antibody binding to endothelial cell activated the mTOR pathway via Src/FAK/paxillin complex, leading to Akt, ERK, and S6RP phosphorylation in a manner dependent on the association of the HLA class I protein with integrin [25, 26]. Recently, an experimental model of HLA-DR antibody stimulation of microvascular endothelial cells (ECs) and allograft damage showed activation of ECs with F(Ab')2 fragment of HLA-DR Ab which led to phosphorylation of Akt, ERK, and MEK and increased in IL-6 production by ECs cocultured with allogenic PBMCs in an Akt-dependent manner. Furthermore, preactivation of ECs with anti-HLA-DR antibody redirected endothelial cell allogenicity toward a proinflammatory response by decreasing amplification of functional Treg and by further increasing IL-6-dependent Th17 expansion [27]. Notably, both the presence of DSA C1q+ Ab and the LABScreen SAB fluorescence values were significantly related to FXM+. Moreover, 9 of the 10 patients with DSA C1q+ Ab had dominant DSA fluorescence values above 10,000 and only one case had MFI of 956. On the other hand, two patients had dominant DSA > 10,000 but were negative for C1q-SAB.

Definitely, the C1q+ SAB value as a pretransplant screening tool will have to be further evaluated, initially, in pretransplant serum samples of transplanted patients with DSA, determining their FXM status and their need for desensitization strategies if it was required. In case an adequate association with the short-term transplant outcome is detected, a randomized clinical trial based on C1q-SAB results should be posited. In this regard, circulating DSA detected on bead arrays was recently evaluated and whether detection of complement fixation using C1q, C4d, or C3d assays in parallel to IgG MFI allowed for improved prediction of AMR was investigated and it was found that IgG MFI add predictive accuracy for silent AMR in DSA+ long-term recipients, and detecting complement fixation did not add independent diagnostic advantage [28].

To our knowledge, this is the first study attempting to associate FXM with the C1q-SAB assay, and it could well contribute to the elucidation of potential interacting mechanisms.

In conclusion C1q+ Ab and LABScreen SAB DSA-MFI were significantly associated with FXM. DSA C1q+ Ab was highly specific but with low sensitivity. The clinical significance of FXM+ in the absence of complement fixing DSA remains to be determined.

### Competing Interests

The authors declare that they have no competing interests.

### Acknowledgments

The authors sincerely thank Chemist Mario Abad from ABALAT, S.A. de C.V., for his kind donation of the reactants used in this study's C1q-SAB assay.

### References

[1] R. Patel and P. I. Terasaki, “Significance of the positive crossmatch test in kidney transplantation,” *The New England Journal of Medicine*, vol. 280, no. 14, pp. 735–739, 1969.

[2] R. Pei, J.-H. Lee, T. Chen, S. Rojo, and P. I. Terasaki, “Flow cytometric detection of HLA antibodies using a spectrum of microparticles,” *Human Immunology*, vol. 60, no. 12, pp. 1293–1302, 1999.

[3] E. M. Gibney, L. R. Cagle, B. Freed, S. E. Warnell, L. Chan, and A. C. Wiseman, “Detection of donor-specific antibodies using HLA-coated microparticles: another tool for kidney transplant risk stratification,” *Nephrology Dialysis Transplantation*, vol. 21, no. 9, pp. 2625–2629, 2006.

[4] R. A. Bray and H. M. Gebel, “Strategies for human leukocyte antigen antibody detection,” *Current Opinion in Organ Transplantation*, vol. 14, no. 4, pp. 392–397, 2009.

[5] A. A. Zachary, J. T. Sholander, J. A. Houpt, and M. S. Leffell, “Using real data for a virtual crossmatch,” *Human Immunology*, vol. 70, no. 8, pp. 574–579, 2001.

[6] H. E. Feucht, H. Schneeberger, G. Hillebrand et al., “Capillary deposition of C4d complement fragment and early renal graft loss,” *Kidney International*, vol. 43, no. 6, pp. 1333–1338, 1993.

[7] M. Crespo, M. Pascual, N. Tolkoff-Rubin et al., “Acute humoral rejection in renal allograft recipients: I. Incidence, serology and clinical characteristics,” *Transplantation*, vol. 71, no. 5, pp. 652–658, 2001.
[9] M. Wahrmann, M. Exner, H. Regele et al., “Flow cytometry based detection of HLA alloantibody mediated classical complement activation,” Journal of Immunological Methods, vol. 275, no. 1-2, pp. 149–160, 2003.

[10] M. Wahrmann, M. Exner, B. Haidbauer et al., “[C4d] FlowPRA screening—a specific assay for selective detection of complement-activating anti-HLA alloantibodies,” Human Immunology, vol. 66, no. 5, pp. 526–534, 2005.

[11] G. Bartel, M. Wahrmann, M. Exner et al., “In vitro detection of C4d-fixing HLA alloantibodies: associations with capillary C4d deposition in kidney allografts,” American Journal of Transplantation, vol. 22, no. 10, pp. 1471–1475, 2010.

[12] G. Chen, F. Sequeira, and D. B. Tyan, “A new combo-flow crossmatch (CFMX) for simultaneous detection of IgG and complement fixing antibodies (CFABS),” Abstracts/Human Immunology, vol. 74, pp. 1–49, 2013, Abstract 7-OR.

[13] A. M. Patel, C. Pancoska, S. Mulgaonkar, and F. L. Weng, “Renal transplantation in patients with pre-transplant donor-specific antibodies and negative flow cytometry crossmatches,” American Journal of Transplantation, vol. 7, no. 10, pp. 1471–1477, 2007.

[14] D. Redondo-Pachón, J. Pascual, M. J. Pérez-Sáez et al., “Impact of preformed and de novo anti-HLA DP antibodies in renal allograft survival,” Transplant Immunology, vol. 34, pp. 1–7, 2016.

[15] E. J. Lepin and E. F. Reed, “Complement-independent mechanisms of anti-HLA antibodies in transplant arteriosclerosis and accommodation,” Current Opinion in Organ Transplantation, vol. 16, no. 1, pp. 10–15, 2004.

[16] P. T. Jindra, Y.-P. Jin, E. Rozenburg, and E. F. Reed, “HLA class I antibody-mediated endothelial cell proliferation via the mTOR pathway,” Journal of Immunology, vol. 180, no. 4, pp. 2357–2366, 2008.

[17] J. Lion, C. Taffin, A. R. Cross et al., “HLA class II antibody activation of endothelial cells promotes Th17 and disrupts regulatory T lymphocyte expansion,” American Journal of Transplantation, vol. 16, no. 5, pp. 1408–1420, 2016.

[18] F. Eskandary, G. Bond, N. Kozakowski et al., “Diagnostic contribution of donor-specific antibody characteristics to uncover late silent antibody-mediated rejection-results of a cross-sectional screening study,” Transplantation, 2016.