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Key Words: Antibody Mediated Rejection, Anti-HLA detection, kidney allograft survival

Running Title: Detection of anti-HLA antibodies, AMR and kidney allograft survival

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Abstract

Pre-sensitizing alloantibodies may represent a grave danger in organ transplantation, increasing the risk of antibody mediated rejection (AMR) and graft loss. However, not all antibodies are harmful to the graft. In our study of a cohort of 325 deceased donor renal allograft recipients, the patients were determined eligible to receive an allograft based on a negative complement dependent cytotoxicity (CDC) crossmatch (XM). Yet at the time of transplantation, many candidates displayed donor specific antibodies (DSA) by more sensitive methods, such as solid phase assays (SPA, Luminex) or flow cytometry crossmatch (FCXM). The majority of the patients who were DSA positive by either SPA (67%) or FCXM (66%) presented an AMR-free clinical course post-transplantation. Among the patients who developed AMR (N=29), 76% proved clinically manageable and did not lose the graft. Analysis of the DSA mean fluorescence intensities (MFI) of Luminex showed no statistically significant difference between patients who experienced AMR episodes and those who did not. Importantly, many of the patients with AMR, did not test positive for DSA by SPA (20/29) or FCXM (14/29). Despite false-positive and false-negative results, the detection of DSA by SPA or FCXM was positively associated with AMR, but not with actuarial graft survival. The field of organ transplantation has always struggled to reconcile two opposing goals: improving transplantation outcome while increasing access to transplantation. SPA and FCXM appear to be over-sensitive and defining patients as “sensitized” according to these methods would block access to transplantation for many candidates who would otherwise benefit greatly from receiving the allograft. Nevertheless, SPA and FCXM are invaluable tools, assisting clinicians to gauge AMR risk and tailor immunosuppression the post-transplantation immunological monitoring accordingly.
Introduction

In the past few decades solid organ transplantation has surged forward in leaps and bounds to a point where it has become almost routine. The United Network for Organ Sharing (UNOS) database indicates that nearly 80,000 people are listed as candidates for kidney transplantation, with tens of thousands more added each year [http://www.unos.org/data]. Advances such as calcineurin inhibitors, molecular typing of HLA, T cell- and B cell-targeted immunosuppression, modern induction therapies and desensitization protocols have greatly improved graft and patient survival. Nevertheless, some old issues (1,2) prominently among which are the deleterious effects of donor specific antibodies (DSA) on transplantation outcome remain a challenge (3, 4).

The newest methods for detection of DSA in the circulation of transplant candidates are highly standardized, fast and cost effective as far as labor is concerned. Yet a definite pronouncement on their effectiveness in predicting transplantation end-points such as graft loss and acute antibody mediated rejection (AMR) is still under intense scrutiny and hotly debated (5-8). Solid phase assays (SPA), such as the Luminex platform, allow the determination of DSA specificity by use of single HLA antigen coated beads gives a relative indication of the antibody strength and level in the circulation by returning results to the user in the form of mean fluorescence intensity (MFI). Since the specificity of the detected antibodies is known, treatment protocols which include Rituximab or Anti-Thymoglobulin (ATG) do not affect solid phase assay results, whereas they do interfere with cell based antibody (Ab) detection methods. SPAs also eliminate the need to work with live donor cells, which require highly skilled technicians for the traditional test of crossmatching by complement dependent cytotoxicity (CDC). Modern donor-specific-antibody detection techniques now allow unparalleled sensitivity in the detection of antibodies.
Therein also lies the greatest shortcoming of solid phase assays, which eliminates the most direct way of evaluating the interaction of patient’s antibodies with a potential donor’s cells. Detection of anti-HLA antibodies in the circulation of a transplant candidate could automatically characterize the patient as “sensitized”, reducing his/her transplantation options. If the goal is minimizing transplantation risk, the improved sensitivity of SPAs would appear as a boon and would dictate the exclusion of any donor against which the recipient has DSAs, since the antibodies may represent a potential risk. However, the complete picture is much more complex. Patients listed for kidney transplantation have generally already undergone renal failure and require frequent dialysis, a procedure that is very expensive and greatly affects the patient’s quality of life and lifespan (9). Successful kidney transplantation returns the patient to a relatively normal life. A University of Maryland study points out that the costs of transplantation and first year care are quickly offset by the much lower costs of post transplant maintenance compared to that of continued dialysis (10). UNOS statistics show that 88% of living-related, 78% of deceased-donor allografts remain functional at the 3 year time point. This statistic holds true even for highly sensitized patients (i.e. 75% 3-year graft survival among patients with >80% panel reactive antibodies (PRA), http://www.unos.org/data). In the light of this information, the decision NOT to transplant is neither ethical nor economical, if allosensitization proves clinically manageable.

Since the sensitivity of antibody detection is greatly improved, a few pertinent questions arise. Should the definition of a sensitized patient expand or depend on the threshold of the detected antibody? And if so, should this occur despite evidence that the antibody is not cytotoxic? Are low titer DSAs deleterious to a graft? If not, what is the threshold beyond which transplantation becomes unadvisable?
To answer these questions, we analyzed the predictive value of the SPA data from a cohort of recipients of deceased donor kidneys (N=325). We also evaluated the consistency of results obtained by SPA, flow cytometry crossmatch (FCXM), and more traditional techniques based on complement dependent cytotoxicity (CDC) for crossmatching (XM) and determining the frequency of Panel Reactive Antibodies (PRA). Finally, DSA detected by SPA (DSA/SPA) and FCXM were analyzed together to evaluate their usefulness for prediction of AMR.

**Materials and Methods**

**HLA typing**

Recipients and donors were HLA-A, B, C, DR and DQ typed using reagents from One Lambda (Canoga Park, CA) for low resolution PCR-SSP.

**Screening of anti-HLA antibodies**

Sera were obtained from each transplant candidate at monthly intervals and screened for lymphocytotoxic antibodies against HLA-A, B, C, DR, and DQ antigens on a reference panel of magnetically sorted T and B lymphocytes from 70 unrelated donors. The frequency of panel reactive antibodies (PRA) and their HLA class I and/or class II specificity was determined by tail-analysis (11-14).

To discriminate between anti-HLA and non-HLA IgG, quarterly collected sera were tested by LABScreen mixed microbeads coated with purified HLA-A, B, C, DR and DQ antigens. All sera with anti-HLA antibodies were further tested on beads coated with single HLA class I (LABScreen single antigen class I LS1 A04) and class II (LS2 A01) antigens (One Lambda)
which identify SPA-S I and II respectively. SPA were performed using a Luminex 100 LS fluoroanalyzer (Luminex Inc., Austin, TX). Based on validation results obtained for each lot of reagents by testing sera from 20 healthy males the MFI and standard deviation (SD) of the cutoff between negative and positive were set at 1000 ± 500 (14).

**Donor cross-matching techniques**

Two samples of sera with high (>10%) PRA, which had been obtained within 6 months prior to transplantation and one “current serum” (CS) collected at the time of transplantation were cross-matched in serial doubling dilutions with magnetically sorted T and B lymphocytes from the donor. The sera were also tested for reactivity with donor and autologous T and B lymphocytes by FCXM, as described by Karpinski et al (15). The samples were tested within 48 h by SPA-MIX and SPA-S I and SPA-S II. All procedures and the interpretation of results were conducted as described before (14).

**Diagnosis of AMR**

Renal allograft biopsies were performed and processed according to established protocols (14). According to the Banff classification, a diagnosis of AMR requires three features: 1. morphologic evidence of acute tissue injury, (e.g. acute tubular injury, neutrophils and/or mononuclear infiltrates in peritubular capillaries and/or glomeruli, and/or capillary thrombosis; intimal arteritis/fibrinoid necrosis/intramural or transmural inflammation in arteries); 2. immunopathologic staining of C4d in peritubular capillaries; and 3. documentation of DSA. Because the aim of this study was to determine the diagnostic specificity of different DSA techniques, DSA was not an /a priori/ criterion for diagnosis of AMR. However, all cases had
morphologic tissue injury and C4d staining in peritubular capillaries (i.e. were "suspicious for AMR" by Banff criteria) (16).

**Immunosuppressive therapy**

Patients received induction therapy with 6 mg/kg anti-thymocyte globulin (Thymoglobulin, Genzyme Transplant, Cambridge, MA), tacrolimus (0.1 mg/kg/day), mycophenolate mofetil (2 g/day) and corticosteroids (methylprednisolone) at a dose of 500 mg intraoperatively tapered to steroid withdrawal on postoperative day number 5. For treatment of AMR, plasmapheresis (one volume exchange reconstituted with albumin) was performed on an every-other-day basis either until CDC detectable DSA were eliminated or until clinical improvement was obtained, as determined by return to baseline serum creatinine level or urine output increase. Standard IVIG or Cytogam (100 mg/kg) (CMV Hyperimmune-IV Ig; MedImmune Inc., Gaithersburg, MD) was administered after each plasmapheresis treatment. Additionally, methylprednisolone boli (500 mg QD × 3) and subsequent taper were also administered upon the diagnosis of AMR. Finally, all patients received tacrolimus and either mycophenolate mofetil or mycophenolic acid immunosuppression. Tacrolimus levels were maintained at a target range of 15–20 ng/ml during the treatment of AMR. Transplants were re-biopsied after treatment as necessary.

**Statistical analysis**

Actuarial survival was estimated using Kaplan-Meier method, with P values calculated by log-rank statistics. Multivariate analysis was performed using Cox proportional hazards Model to identify independent predictors of acute antibody-mediated rejection (17). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were
calculated. Sensitivity was defined as the number of true positives divided by the sum (true positives + false negatives). Specificity was defined as the number of true negatives divided by the sum of true negatives and false positives. PPV was calculated as true positives / (true positives + false positives), and NPV was calculated as true negatives / (true negatives + false negatives). Accuracy was calculated as (true positives + true negatives) / total number of the patients studied. True positive were DSA+ with AMR, true negatives were DSA- with no AMR, false positives were DSA+ with no AMR, and false negatives were DSA- with AMR (14, 18). Student’s t-test for unpaired data was used to compare the strongest MFI of DSA in patients with and without AMR. Categorical variables were compared with Fisher exact test or the chi-square test. All statistical analyses were performed with SPSS software, version 14.0. (SPSS, inc., Chicago, IL).

Results

Demographic characteristics

A cohort of 325 patients, who have undergone kidney transplantation at Columbia Presbyterian Medical Center after 2004, was recruited for this study (Table 1A). The majority (N = 260) received a primary renal graft, while the rest received secondary or tertiary grafts (N = 65). All patients were adults and received kidney transplants from deceased donors. Multivariate analysis of this cohort using the Cox proportional hazards model reveals PRA detected by CDC, DSA detected by SPA or FCXM to be significant AMR prognostic factors. Female gender, and the number of transplants (but not age or number of HLA mismatches) also appear to be predictors of AMR. (Table 1B).
Predictive value of complement fixing PRA, DSA/SPA and FCXM

All patients had a CDC negative crossmatch with sorted T and B cells. Twenty nine of 325 renal allograft recipients (8.9%) had one episode of AMR or more. Of these AMR positive patients 5 lost the graft within the first year post-transplantation and 7 developed an irreversible episode before the end of the 4th year of observation. The actuarial 4 year graft survival of patients who developed AMR was 76% (N=29), and without AMR 90% (N=296) (Fig. 1A).

The PRA frequency was higher or equal to 10% prior to transplantation in 83% of the patients with AMR (24/29). By comparison, 35% (105/296) of the patients who did not develop AMR displayed antibodies within the same range of PRA (Table 2A). Hence, although the development of cytotoxic allo-antibodies increases the risk of AMR significantly, it does not preclude transplantation in patients without cytotoxic DSA.

Within the AMR+ group, the frequency of patients with DSA/SPA against HLA-class I only, class II only or both class I and II was 21% (6/29), 7% (2/29) and 3% (1/29), respectively. Twenty patients without DSA/SPA (69%) developed AMR. However, 63% (10/16), 75% (6/8) and 67% (2/3) patients within the same DSA positive groups remained AMR-free (Table 2B).

Within the AMR+ group, the frequency of patients with a positive FCXM on donor T cells, B cells or both T and B cells was 17% (5/29), 10% (3/29) and 24% (7/29) respectively, while 48% (14/29) were FCXM negative (Table 2C). Among patients who showed FCXM positive results with donor T cells, B cells or both 54% (6/11), 83% (15/18), 61% (11/18) respectively, did not develop AMR.
We compiled the SPA and FCXM testing data to determine whether, if taken together, the two methods have a better AMR-predictive value than either method alone. DSA detected by both SPA and FCXM (SPA+FCXM+), SPA only (SPA+FCXM-), or FCXM only (SPA-FCXM+) were present in 21% (6/29), 10% (3/29), 31% (9/29) of the patients with AMR. There were 57% (8/14), 77% (10/13), 73% (24/33) patients who tested positive by both SPA and FCXM, SPA(only), or FCXM(only), yet did not develop AMR. Eighty-six percent of the patients without detectable DSA by either method (254/296) remained AMR free.

Statistical analysis showed that the corresponding specificities were 0.97, 0.97 and 0.92, and accuracy values were 0.95, 0.92 and 0.86. The positive predictive values (PPV) of combined testing were 0.43, 0.23 and 0.27 and negative predictive values (NPV) were 0.93, 0.92 and 0.93, respectively. In 38% of the patients with AMR (11/29) anti-donor antibodies were not detected by either SPA or FCXM.

Taken together these results suggest that prediction of AMR by use of SPA and/or FCXM still falls short of the time-tested CDC methods, since a maximum of 63% (18/29) of patients with DSA detected by SPA, FCXM or both SPA and FCXM developed AMR, compared to the 83% (24/29) incidence of AMR in patients with PRA >10%. Most importantly, 70% (42/60) of the patients (who were determined to be DSA positive by SPA and/or FCXM yet remained AMR-free) would have been denied a transplant, if eliminated on the basis of one or both of these methods. Considering the cost of dialysis, morbidity and mortality associated with long-term waiting time such an overcautious approach raises serious ethical concerns.

On the positive side, however, awareness of SPA or FCXM-detectable-DSA increases the likelihood that these patients will be followed up closely for early diagnosis and treatment of
AMR. This information is crucial to centers which are equipped to treat AMR by appropriate desensitization protocols.

The arbitrariness of MFI thresholds

The ever increasing effort to improve the sensitivity and reliability of histocompatibility testing has resulted in overregulation of laboratory standards, vehement discussions among experts and suggestion “to use the same antibody testing method and apply uniform value for defining strong and weak antibodies” (19). Such efforts would be well justified if based on statistics rather than case reports.

To illustrate the difficulty of applying rigorous standards to the interpretation of test results, we present in Table 3 our analysis of MFI values for DSA detected in patients with and without AMR. The statistical difference between the mean MFI’s of the two groups did not reach significance, and the standard deviation of these values indicates an overlap which precludes the use of arbitrary “laboratory thresholds”. When the minimal DSA (Mean minus SD) in patients with AMR is compared to the maximal DSA (Mean plus SD) in patients without AMR, a reasonable decision about what should be considered positive or negative cannot be made. A more conservative approach, in which any value above that observed in known negative control sera, seems safer and more objective.

Alloantibody detection, AMR and allograft survival

The rate of renal allograft survival in presensitized patients has long been known to be lower than in nonsensitized recipients. Patients from our cohort which displayed low PRA (<10%, N=196) had better graft survival rates in the short term (97%, 95% and 94% at 1, 3, 6 months
post-transplantation) compared to their high PRA counterparts (93% graft survival throughout the 1-6 month period, N=129). However, in the absence of AMR episodes, 4-year survival was 90% in recipients with PRA ≥10% (N=105) and 88% in those with PRA <10% (N=191) (Fig. 1A, P=N.S.). Since all patients had a negative CDCXM with the donor, these results indicate the reliability of CDCXM for identifying recipients who do not display complement fixing DSA (Fig. 1A). However, in patients with high PRA who developed AMR (N=24) graft survival decreased to 81% at 4 years and in those with low PRA and AMR (N=5) it reached only 40% after 2.5 years. This suggests that the development of AMR has a negative effect on the length of graft function and that it can be caused not only by cytotoxic, but also by noncytotoxic antibodies which are not detectable by CDCXM (P=0.0005).

To determine whether transplantation of patients with such noncytotoxic antibodies should have been precluded by excluding recipients with SPA/DSA, we analyzed the actuarial survival in patients with and without SPA-detected DSA and AMR (Fig. 1B). The survival was highest (90%) in DSA/SPA-negative, AMR-negative recipients (N=278), and reached only 76% at 3 years in DSA/SPA-positive patients (N=5) consistent with the possibility that SPA detects DSA which cause AMR and relatively early failure of the graft. Graft survival was also 76%, however, in DSA/SPA-positive, AMR-negative recipients (N=18) indicating that the presence of SPA/DSA does not necessarily result in humoral rejection and graft loss. Furthermore, the lowest graft survival rate (73%) was seen in DSA/SPA-negative, AMR-positive recipients (N=20), indicating that factors other than anti-HLA DSA may cause AMR and graft failure (P=0.02).
Analysis of graft survival in patients with and without positive FCXM and AMR showed no significant differences, between patients who were FCXM-positive and AMR-positive (N=15), FCXM-positive, AMR-negative (N=32), FCXM-negative AMR-positive (N=14) and FCXM-negative, AMR-negative since at 4 years 76%, 83%, 68% and 90% of the grafts were functioning in these groups (Fig. 1C, P=0.07).

As stated before, all patients in this cohort had a negative CDXM with the donor. Most patients did not display DSA prior to transplantation as determined by either FCXM or SPA (SPA–FCXM–, N=265), while 14 patients were DSA+ by both tests (SPA+FCXM+, N=14). Another 46 patients showed antibodies only by one detection method (SPA+FCXM–, N=13 and SPA–FCXM+, N=33). Log-rank statistical analysis shows that graft survival rates in the four groups were not significantly different (Fig. 1D, P=0.21). Although the DSA/SPA–FCXM– (double negative) group appeared to have a better graft survival rate at 4 years (89%) than the DSA/SPA+FCXM+ (double positive) group (74%), there was no statistically significant difference between these groups (P=0.18).

**Discussion**

HLA are the most polymorphic genes within the human genome, and the antigens which they encode account for the generation of the majority of antibody responses against an organ allograft. However, they are by no means the only polymorphic human genes, and responses against other antigens can and do occur in allo-transplantation (20). Albeit rarely, anti-MICA and anti-endothelial antibodies (21, 22) have been implicated in AMR. While the Luminex SPA method is excellent for identifying anti-HLA antibodies, non-HLA antibodies represent a “blind-spot” for this type of testing. Essentially the SPA will always return a false-negative result, if the
target is not an HLA antigen. Cell based methods are less susceptible to this type of problem, since the donor cells used for testing emulate the comprehensive antigenic makeup of the prospective graft with much greater fidelity.

Another caveat is the presence in the sera of IgM DSA, which are generally considered harmless. However, the B cells that produce them may activate and undergo isotype switching to IgG after encountering cognate antigens provided by the allograft (23, 24). In spite of these considerations, most laboratories which use CDC for the direct cross-match do not test and/or disregard the presence of IgM alloantibodies. Also, both screening and crossmatching of sera on sorted B cells, as originally proposed some 30 years ago (2, 25), has entered clinical practice only in recent years. We found B cells to be a more reliable target for direct cross-matching by CDC because they express both HLA class I and class II antigens. The higher density of HLA class I antigens on professional APC (B cells, monocytes and dendritic cells) is an added advantage not only for the induction of T cell (CD8+) responses to MHC class I/peptide complexes but also for more humble and trivial reasons such as their increased susceptibility to cytotoxic anti-HLA class I alloantibodies.

In spite of our traditional bias in favor of testing alloantibodies on sorted T and B cells, we are bound to recognize the significantly lower impact of anti-B cell and/or anti-HLA class II antibodies on allograft survival, compared to the effect of anti-T cell or anti-HLA class I antibodies. Again, the immunologic reason is self-evident because non-professional APCs, such as endothelial cells lining the graft’s vasculature express HLA class II antigens at a very low density unless exposed to inflammatory cytokines. Thus, anti-HLA class II antibodies may play a role at the time when inflammation caused by trauma or by ongoing acute cellular or humoral
rejections induce the upregulation of class II antigens on target endothelial cells. It implies that while important for post-transplant monitoring of recipients’ immune response, anti-HLA class II antibodies may have little impact on early allograft function.

This brings into question the endless discussions on detection, specificity and quantitation of detected antibodies. Although legitimate from the transplant physicians’ point of view, this question should not be asked by histocompatibility workers before techniques for quantitating the antigen/antibody ratio for individual donor HLA antigens have been developed. While antibody titration using donor lymphocytes as targets is justifiable, titration by SPA - where the amount of antigens on the beads and on patients’ cells may differ - is not.

Continuing along the same line of reasoning, what is the rationale of proposing high resolution typing of HLA class I and II antigens in solid organ transplantation? Is there a sole statistical study demonstrating humoral rejection in patients mismatched for a single allelic subtype rather than for an HLA antigen (as defined by low resolution typing)? Is there any clinical evidence that anti-DP antibodies affect allograft survival in a well studied population or are such claims based on rather anecdotal case reports? The finding of a particular antibody in a patient with AMR may be coincidental rather than representing the real cause of that event. To document the importance of allele specific antibodies or of anti-DP antibodies one should look at donor-recipient pairs matched for all antigens other than the one(s) in question.

Before increasing the cost of histocompatibility testing by adding more standards and regulations should we not consider it our responsibility to provide periodically statistical reports on the effect of antibodies on allograft survival in the patient population which we serve?
New UNOS regulations require the amendment of PRA >80% with the analysis of antibody specificity, so as to improve the efficacy of kidney allocation and appropriately give patients additional points.

If, however, such regulations were expanded to patients with lower PRA, an unacceptable volume of useless additional work would be imposed on the laboratories unless, we would be willing to return to the traditional HLA matching rules still in effect in Europe. Are European populations less heterogenous than Americans? Recent statistics on the rate of immigration in various Western European countries raises some doubt about the homogeneity of their populations.

We do not advocate a return to the HLA matching across the board given the excellent results which we now see, not only in non-sensitized recipients but even in patients with non-cytotoxic anti-HLA DSAs. In view of such results we urge the transplant community to remember the results obtained by Oscar Salvatierra and his followers in the era of donor-specific transfusions (DST). There was an impressive increase in renal allograft survival regardless of the use of cyclosporine (26). They found that more than 70% of the patients did not produce cytotoxic DSA and that survival in these patients was equal to that obtained in recipients of HLA identical grafts. Most likely these patients would have been positive if tested by SPA. In the light of their experience, we feel that understanding the nature of “good and bad” antibodies should be our priority. The possibilities that DST induce the development of suppressor cells, generation of anti-idiotypic antibodies, and eliminate reactive clones of cells were discussed 20 years ago, and should still remain the object of our research (27).
Also, the discovery that weak anti-HLA antibodies are protective rather than deleterious to the graft implies the need of re-evaluating our current and past experience (28).

**Figure Legends**

**Figure 1.** Actuarial graft survival in recipients with or without AMR, and (A) with CDC-detected PRA at high (≥10%) or low (<10%) frequency (P=0.0005), (B) with or without DSA/SPA (P=0.02), and (C) with or without DSA detected by FCXM (P=0.07). (D) Actuarial survival in patients with or without DSA detected by SPA and/or FCXM (P=0.21). Statistical significance among groups was analyzed by log-rank tests.
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can induce endothelial cell survival or proliferation depending on their concentration.
Fig. 1

A. Graft Survival (%)

B. Graft Survival (%)

C. Graft Survival (%)

D. Graft Survival (%)
Table 1

A. Demographics and characteristics of the patient population (N=325)

<table>
<thead>
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<th>Transplant type</th>
<th>N</th>
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<tr>
<td>Primary allograft</td>
<td>260</td>
<td>(80)</td>
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<td>Re-transplant</td>
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<td>(20)</td>
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<table>
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<tr>
<th>Gender</th>
<th>N</th>
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<td>Male</td>
<td>201</td>
<td>(62)</td>
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<tr>
<td>Female</td>
<td>124</td>
<td>(38)</td>
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<table>
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<tr>
<th>Age (Mean ± S.D.)</th>
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<tr>
<td></td>
<td>32 ±13</td>
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<tr>
<td>(Range)</td>
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<table>
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<tr>
<th>HLA-A, B, DR mismatch</th>
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<tr>
<td>0</td>
<td>6</td>
<td>(2)</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>(2)</td>
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<tr>
<td>6</td>
<td>70</td>
<td>(22)</td>
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B. Multi-variate analysis of AMR prognostic factors using the Cox proportional hazards model

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<th>Factor</th>
<th>Coefficient</th>
<th>Standard error</th>
<th>P value</th>
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<td>Age</td>
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<td>N.S.*</td>
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<td>Gender</td>
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<td>0.0335</td>
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<td>Number of HLA mismatch</td>
<td>0.2329</td>
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<td>N.S.*</td>
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<td>Primary Transplant versus Re-graft</td>
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<td>CDC PRA&gt;10%</td>
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<tr>
<td>DSA / SPA</td>
<td>0.8304</td>
<td>0.4472</td>
<td>0.0634</td>
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<tr>
<td>FCXM</td>
<td>1.3998</td>
<td>0.4118</td>
<td>0.0007</td>
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</table>

*N*Not Significant (N.S., *P*>0.05)
### Table 2

**A. Incidence of AMR in patients with high and low PRA**

<table>
<thead>
<tr>
<th>PRA &gt;10%</th>
<th>AMR +</th>
<th>AMR -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 (83%)</td>
<td>105 (35%)</td>
<td>129</td>
</tr>
<tr>
<td>PRA &lt;10%</td>
<td>5 (17%)</td>
<td>191 (65%)</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>296</td>
<td>325</td>
</tr>
</tbody>
</table>

\[ P<0.0001 \]

**B. Incidence of AMR in patients with or without DSA/SPA**

<table>
<thead>
<tr>
<th>DSA by SPA</th>
<th>AMR +</th>
<th>AMR -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti HLA-class I only</td>
<td>6 (21%)</td>
<td>10 (3%)</td>
<td>16</td>
</tr>
<tr>
<td>Anti HLA-class II only</td>
<td>2 (7%)</td>
<td>6 (2%)</td>
<td>8</td>
</tr>
<tr>
<td>Anti HLA class I and II</td>
<td>1 (3%)</td>
<td>2 (1%)</td>
<td>3</td>
</tr>
<tr>
<td>None</td>
<td>20 (69%)</td>
<td>278 (94%)</td>
<td>298</td>
</tr>
</tbody>
</table>

\[ P<0.0001 \]

**C. Incidence of AMR in patients with or without FCXM detected DSA**

<table>
<thead>
<tr>
<th>FCXM</th>
<th>AMR +</th>
<th>AMR -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell XM + (only)</td>
<td>5 (17%)</td>
<td>6 (2%)</td>
<td>11</td>
</tr>
<tr>
<td>B cell XM + (only)</td>
<td>3 (10%)</td>
<td>15 (5%)</td>
<td>18</td>
</tr>
<tr>
<td>Both T and B XM+</td>
<td>7 (24%)</td>
<td>11 (4%)</td>
<td>18</td>
</tr>
<tr>
<td>FCXM -</td>
<td>14 (48%)</td>
<td>264 (92%)</td>
<td>278</td>
</tr>
</tbody>
</table>

\[ P<0.0001 \]

**D. Incidence of AMR and combined SPA and FCXM results**

<table>
<thead>
<tr>
<th>SPA and FCXM</th>
<th>AMR +</th>
<th>AMR -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPA+ FCXM +</td>
<td>6 (21%)</td>
<td>8 (3%)</td>
<td>14</td>
</tr>
<tr>
<td>SPA+ FCXM -</td>
<td>3 (10%)</td>
<td>10 (3%)</td>
<td>13</td>
</tr>
<tr>
<td>SPA- FCXM +</td>
<td>9 (31%)</td>
<td>24 (8%)</td>
<td>33</td>
</tr>
<tr>
<td>SPA- FCXM -</td>
<td>11 (38%)</td>
<td>254 (86%)</td>
<td>265</td>
</tr>
</tbody>
</table>

\[ P<0.0001 \]
Table 3. Relationship between DSA MFI determinations by SPA and AMR development

<table>
<thead>
<tr>
<th></th>
<th>AMR+</th>
<th>AMR-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=6</td>
<td>N=10</td>
</tr>
<tr>
<td>MFI (HLA Class I)</td>
<td>4842 +/-3438</td>
<td>7157 +/-4618</td>
</tr>
<tr>
<td></td>
<td>N=2</td>
<td>N=6</td>
</tr>
<tr>
<td>MFI (HLA-Class II)</td>
<td>7187 +/-4716</td>
<td>6421 +/-1212</td>
</tr>
<tr>
<td></td>
<td>N=1</td>
<td>N=2</td>
</tr>
<tr>
<td>MFI (HLA CLASS I+II)</td>
<td>5000</td>
<td>4104 +/-430</td>
</tr>
</tbody>
</table>