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Original article

History of a previous renal transplant as the most important event for C3d positive HLA antibodies and IgG subclass identification

La historia de un trasplante renal previo es el evento más importante para identificar subclases de IgG de los anticuerpos anti-HLA y positividad del ensayo C3d

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ABSTRACT

Introduction: Antibodies against HLA antigens are the main immunologic barrier for kidney transplantation. These antibodies either preformed, or developed de novo after transplant, precede antibody-mediated rejection, which is the most important cause of graft failure. The capability to further analyze these antibodies is allowing the creation of immunologic risk profiles that are associated with higher rejection rates and graft loss. Some of the most studied risk characteristics of the antibodies are the Mean Fluorescence Intensity (MFI), HLA antigenic specificity, IgG subclasses and the antibodies' ability to fix complement. The objective of this study is to describe the characteristics of pre-transplant HLA antibodies and the sensitizing events related to them (previous kidney transplant, pregnancies and transfusions). **Material and methods:** This is an observational, transversal and descriptive study. Sera samples from previously sensitized

RESUMEN

Introducción: *Los anticuerpos anti-HLA son la principal barrera inmunológica para el trasplante renal. Ya sea preformados y/o desarrollados de novo postrasplante, son los precursores del rechazo humoral, el cual es la principal causa de pérdida del injerto. La caracterización de dichos anticuerpos (Acs) ha delineado un perfil de riesgo asociado con rechazo y pérdida del injerto, siendo la intensidad media de fluorescencia (MFI, por sus siglas en inglés), las especificidades antigénicas HLA, las subclases de IgG y la capacidad de fijación del complemento algunas de las más exploradas. El objetivo del presente estudio es la caracterización de los Acs anti-HLA en la etapa pretrasplante y los factores sensibilizantes asociados (trasplante renal previo, embarazo y transfusiones).* ***Material y métodos:*** *Estudio transversal, observacional y descriptivo de suero de pacientes sensibilizados, potenciales receptores de trasplante. Los sueros fueron evaluados con: determinación de Acs anti-*

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potential kidney transplant recipients were evaluated; anti-HLA antibodies were determined using single antigen beads (SAB) (Luminex), as well as IgG subclasses of these antibodies and its ability for complement activation using the C3d assay. **Results:** A total of 89 patients' sera samples were analyzed, with a mean age 33.3 years (± 13.8) and 60.2% males (n = 54). History of previous kidney transplant was found in 20.4% (n = 18) of the patients, history of at least one previous pregnancy 17.2% (n = 15) of the female patients and previous transfusions in 60.2% (n = 54). A total of 1,771 anti-HLA antibodies were detected, 367 (20.1%) with at least one IgG subclass and 216 (12.1%) complement activation capacity (C3d+). The factors found to be related with a higher detection rate of IgG subclasses were: SAB MFI > 4,000 (OR 15.9, 95% CI 11.2-22.4, p = < 0.01), positive C3d assay (OR 3.4, 95% CI 2.4-4.9, p = < 0.01) and HLA-B specific antibodies (OR 2.3, 95% CI 1.7-3.2, p = < 0.01). The factors found to be associated with a positive C3d assay were: SAB MFI > 4,000 (OR 1.15, 95% CI 1.10-1.21, p = < 0.01), HLA-DQ specific antibodies (OR 4.37, 95% CI 2.21-8.66, p < 0.01), HLA-DR specific antibodies (OR 10.24, 95% CI 5.07-20.65, p < 0.01), HLA-B specific antibodies (OR 0.35, 95% CI 0.18-0.67, p = < 0.01) and the detection of at least one IgG subclass (OR 15.8, 95% CI 7.79-32.2, p = < 0.01). Patients with a history of a previous kidney transplant were found to have more anti-HLA antibodies, higher MFI titles, IgG subclasses were detected in all of these patients and they had more positive C3d antibodies. The most prevalent IgG subclass detected in this population was IgG1 (n = 351, 95.6%). **Conclusions:** In general, the IgG subclasses and the C3d assay were not able to detect or characterize the anti-HLA antibodies in most of the cases. Remarkably, in all the patients with history of previous kidney transplant IgG subclasses were identified and 90% of them yielded a positive C3d assay for complement activation. The ability of the assay to detect IgG subclasses and C3d positive antibodies was related with the MFI of the anti-HLA antibodies, which were significantly higher in patients with previous kidney transplant.

Keywords: Kidney transplant, HLA antibodies, IgG subclasses, C3d assay.

HLA con perlas de antígeno único (SAB, por sus siglas en inglés) (Luminex), subclase de inmunoglobulinas de los Acs y capacidad de fijación de complemento de los mismos mediante C3d. ***Resultados:*** *Se estudiaron 89 sueros/89 pacientes, con edad promedio de 33.3 años (± 13.8) y 60.2% hombres (n = 54). El 20.4% tenían historia de trasplante previo (n = 18), 17.2% embarazo (n = 15) y 60.2% transfusiones (n = 54). Fueron detectados 1,771 anticuerpos anti-HLA, 367 (20.1%) con alguna subclase de IgG y 216 (12.1%) con fijación de complemento (C3d+). Los factores relacionados a la detección de subclase de IgG fueron: SAB MFI > 4,000 (OR 15.9, 95% IC 11.2-22.4, p = < 0.01), positividad para C3d (OR 3.4, 95% IC 2.4-4.9, p < 0.01) y Acs HLA-B (OR 2.3, 95% IC 1.7-3.2, p < 0.01). Los factores asociados con positividad para C3d: SAB MFI (OR 1.15, 95% IC 1.10-1.21, p < 0.01), Acs HLA-DQ (OR 4.37, 95% IC 2.21-8.66, p < 0.01), Acs HLA-DR (OR 10.24, 95% IC 5.07-20.65, p < 0.01), Acs HLA-B (OR 0.35, 95% IC 0.18-0.67, p < 0.01), detección de subclase de IgG (OR 15.8, 95% IC 7.79-32.2, p < 0.01). Los pacientes con historia de trasplante previo presentaron una mayor cantidad de anticuerpos anti-HLA, mayor MFI, en todos se detectaron subclases de IgG y una mayor cantidad de anticuerpos fijadores de complemento. La subclase de IgG más prevalente fue IgG1 (n = 351, 95.6%).* ***Conclusiones:*** *En general, la detección de subclases de IgG y la capacidad de fijación de complemento C3d de los Acs anti-HLA fue poco frecuente. Sin embargo, en todos los pacientes con antecedente de trasplante previo se identificaron subclases de IgG y en el 90% positividad para C3d. La presencia de subclases de IgG y C3d se relacionó con la MFI de los Acs anti-HLA, significativamente más elevados en pacientes con trasplante previo.*

Palabras clave: *Trasplante renal, anticuerpos anti-HLA, subclases de IgG, ensayo C3d.*

INTRODUCTION

Whether preformed or developed *de novo* post-transplant, antibodies against HLA (HLA-Abs) antigenic specificities have been demonstrated to be the major immunological obstacle for successful kidney transplantation. The degree of sensitization of the potential kidney transplant recipient may delay kidney transplant and in some cases preclude it.[1-5] The existence of preformed donor specific HLA-Abs (DSA) foster the risk of immunological humoral mediated rejection events and participates in a decisive manner in the pathogenic mechanisms leading to chronic injury and graft loss.[6]

The importance to delineate the characteristics that confer pathogenesis and influence transplant outcomes encouraged transplant community to explore HLA-DSA complement binding capacity and immunoglobulin G (IgG) subclass composition.[7-9]

Advances in the development and evaluation of assays allowing to detect complement binding capacity includes C1q, C4d and C3d.[7,10,11] A large cohort study showed C1q binding DSAs to be associated with significant higher risk of antibody-mediated rejection, severe graft injury and graft loss.[7] Likewise, studies conducted using C3d assay demonstrated C3d binding DSA to be associated with a higher risk of graft loss independent of DSA-MFI, and to be a better predictor of graft loss

than C1q binding DSA.[11] Preformed DSAs able to bind C4d have been reported to predict antibody-mediated rejection and graft loss.[10]

The other relevant marker added to the formula intended to delineate the immunological risk is the IgG subclasses of circulating immunodominant HLA-DSA (iDSA). A recent study by Lefaucheur et al analyzing 125 kidney transplant recipients (TR) with DSA detected in the first year post transplant revealed IgG3 iDSA and C1q-binding iDSA strongly and independently associated with allograft failure.[9] Moreover, Viglietti et al conducted a prospective study in 851 kidney TR systematically screened for DSA and its characterization (MFI titer, C1q-binding capacity, and IgG subclasses) at transplant, 1 and 2 years post-transplant and at the time of post-transplant clinical events.[8] They showed that the addition of IgG3 or C1q-binding anti-HLA DSA status to the conventional approach of the DSA strength improved the performance in assessing the individual risk for allograft loss in > 60% of patients.[8]

Previously, we conducted a study to document the association between flow cytometry cross-match assay (FXM) result and the results obtained in the analysis of the C1q-SAB assay in a group of potential kidney transplant recipients with DSA against some HLA antigenic specificities of their potential living donors and a negative CDC cross-match in all cases. The analysis of the data disclosed that in patients with this profile, factors associated to a positive FXM were the presence of C1q + Abs (p = 0.004) and the MFI by LABScreen® SAB DSA (p = 0.007). 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, the most sensitive (92%, 79.1-100).[12] By using the same data proceeding from the study population, we performed an analysis oriented to describe the patient characteristics associated to C1q + as well as those of the Abs per se when associated to C1q binding. The analysis disclosed anti-HLA-DQ antibodies as highly and independently related to the C1q-binding capacity of the HLA antibodies.[13]

More recently, we compared the association and predictive capacity of DSA MFI, complement fixing capacity through the C3d assay, and IgG subclasses determination in the prediction of FXM result in a group of 93 donor/recipient pairs. The study revealed that neither C3d nor the IgG subclasses detection alone had an adequate predictive capacity for the FXM; however, in the absence of IgG subclass detection and a DSA-MFI < 2,300, the probability of a negative FXM was nearly 94%.[14]

The purpose of this report is to describe the characteristics of the documented anti-HLA antibodies for both: their capacity to fix complement (C3d assay) and the possibility to identify the IgG subclass in this patient population of 89 potential kidney transplant recipients.

MATERIAL AND METHODS

This is an observational, cross-sectional and descriptive study. We included sera from 89 sensitized patients that were being evaluated for living donor kidney transplant. HLA typing as well as class I and class II anti-HLA antibodies were performed. All of them presented HLA-DSA against their potential donors. For this study, cryopreserved serum samples from these patients were analyzed in a blinded fashion for the presence of C3d-binding donor-specific anti-HLA antibodies, as well as the IgG subclass composition. All the samples corresponded to the same date the patients were evaluated for pre-transplant PRA.

HLA typing

Briefly, DNA was purified using the BD tract Genomic DNA Isolation Kit (Maxim Biotech Inc. San Francisco, CA). The samples were amplified by polymerase chain reaction in a Verify 96 Well Thermal cycler (Applied Biosystems, Foster City, CA). The kidney donor and recipient HLA were determined using Micro SSP HLA DNA typing Trays (One Lambda Inc. Canoga Park,

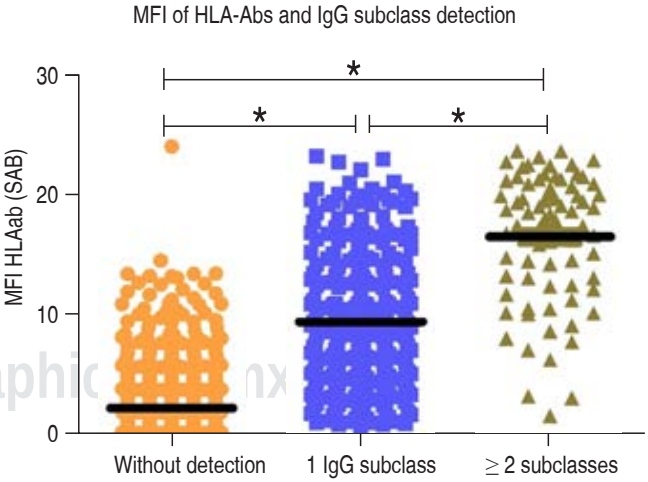

Figure 1: Correlation between the MFI of HLA-Abs (n = 1,673) detected by SAB and the capacity of the assay for IgG subclasses detection. The group with 2 or more IgG subclasses detection was the one with higher MFI by SAB. Y axis MFI HLA-Abs expressed in thousand per units.

Table 1: HLA-Abs characteristics divided by class I and class II.

	All Abs (n = 1,771)	IgG subclasses detection (n = 367)	Positive C3d assay (n = 216)
	n (%)	n (%)	n (%)
HLA-Ab class I	**857 (48.3)**	**214 (58.3)**	**45 (20.8)**
HLA A	291 (16.43)	69 (18.8)	23 (10.6)
HLA B	566 (31.96)	145 (39.51)	22 (10.1)
HLA-Ab class II	**636 (35.91)**	**116 (31.6)**	**161 (74.5)**
HLA DP	156 (8.81)	1 (0.27)	12 (5.56)
HLA DQ	170 (9.6)	63 (17.17)	52 (24.07)
HLA DR	310 (17.5)	52 (14.17)	97 (44.9)

CA) and HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 were reported for both donors and recipients.

Antibody assessment

HLA-Abs determination was performed by using Luminex LABScreen® Single Antigen Beads (LABScreen® SAB), class I and class II (One Lambda), and were systematically obtained as part of the patient's immunological risk profile. Before HLA-Abs determination sera was treated with dithiothreitol at a final concentration of 0.005 M, at 37 °C during 30 minutes followed by centrifugation at 3,000 g for 1 minute; then, the antibody detection using antigen coated beads was performed.[15]

HLA-Abs results were obtained in every patient in the Histocompatibility Laboratory database. For this analysis, HLA-Abs ≥ 400 MFI was considered positive.

C3d-binding anti-HLA antibody assay

Sera are pretreated with LIFECODES Serum Cleaner®, 40 µL of LSA beads are added to each of the assigned wells, and then 10 µL of patient or control serum are added and mixed. Sera is incubated for 30 minutes and washed four times; then, a phycoerythrin conjugated anti-human C3d antibody is added. After another incubation, 200 µL of wash buffer are added to each well to resuspend beads and collect data with the Luminex® platform. The signal intensity from each bead is compared to the signal intensity of negative control sera. All antibodies detected were included independently of their signal intensity.

IgG subclass analysis

For this analysis the modified single antigen flow beads (SAFB) assay described by Hönger et al was used.[16] Briefly, we used 6 µL of the patient serum and 1.5 µL of SAFB suspension (LABScreen® SA) supplemented with 20 µL of wash buffer (One Lambda). Next, 170 µL wash buffer (One Lambda) was added and the plate was centrifuged for 5 min at 1300 rpm. Two more washing steps were performed. Then, 25 µL of appropriately diluted PE-labeled IgG1-4 subclass reporter antibody (concentration: anti-IgG1 = 1.3 µg/mL, anti-IgG2 = 1.3 µg/mL, anti-IgG3 = 10.6 µg/mL, anti-IgG4 = 0.68 µg/mL) was added and incubated for 30 min. The SAFB results were obtained in the Luminex100™ analyzer. The IgG1-4 subclasses reporter antibody were monoclonal antibodies specific for IgG1-4 subclasses IgG1 clone 4E3, IgG2 clone 31-7-4, IgG3 clone HP6050, IgG4 clone HP6025 (Abcam, Cambridge, MA). As a negative control to establish a MFI cut/off for this study a commercial reagent was used, provided by One Lambda. All antibodies detected were included independently of their signal intensity.

Statistical analysis

Descriptive statistics were used according to the type of variable analyzed. 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 χ^2 or Fisher's exact test. Continuous variables were analyzed with Student's t-test or Mann-Whitney's U test.

The STATA version 11.1 statistical package and Excel 2013 were used to analyze the data.

RESULTS

Characterization of HLA-antibodies by SAB, C3d assay and IgG subclasses

In sera from 89 sensitized patients, we found 1,684 different HLA-Abs by LABScreen® SAB. Additionally, using C3d assay we detected another 63 additional HLA-Abs, and other 24 HLA-Abs by IgG subclasses, then we analyze a total of 1,771 HLA-Abs from 89 sera with a median of 12 per patient (IQR 6-25).

The IgG subclasses assay showed 367 abs with at least one IgG subclass, the most commonly detected was IgG1 alone or associated with other subclasses (351, 95.6%); only 7 IgG2 and 9 IgG3 were found isolated from IgG1. IgG4 was identified in 17 abs, always in combination with IgG1. Although, the assay was not able to detect any IgG subclass for most of the abs detected [1,404 (79.2%) abs without IgG subclass detection], we decided to analyze the difference in IgG subclasses detection by dividing the population of abs in 3 groups: without IgG subclass detection, 1 IgG subclass detected or ≥ 2 IgG subclasses detected *(Figure 1)*. This analysis showed that the higher the MFI by SAB, the higher the possibility of detecting at least one IgG subclass. The difference in the mean MFI for each group was significant ($p < 0.001$).

A ROC curve was traced to find the cutoff MFI by SAB that would predict the detection of IgG subclasses the best, with an area under the curve of 0.92. A MFI > 4,000 of the HLA-SAB was related with the detection of IgG subclasses with a sensibility of 86.1% (CI 95% 82.4-89.7) and specificity of 80.7% (CI 95% 78.6-82.8).

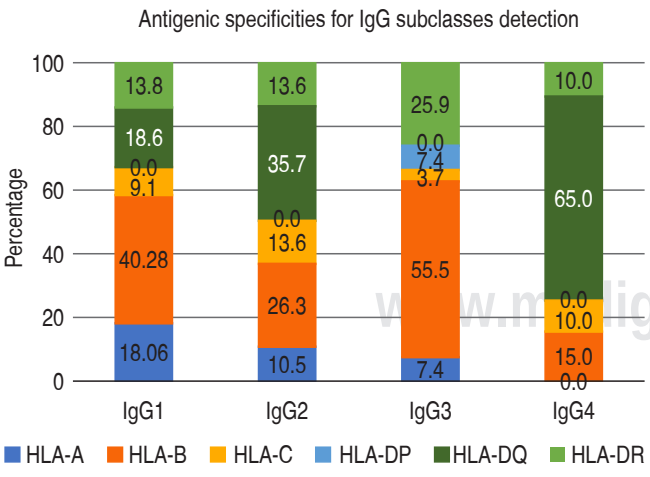

Figure 2: Proportion of detected IgG subclasses according to HLA antigenic specificity.

Table 2: General characteristics.

Patients	N = 89
Male, n (%)	54 (60.2)
Age (years), m ± SD	33.3 ± 13.8
Blood transfusion, n (%)	54 (60.2)
Previous transplant, n (%)	18 (20.4)
Pregnancies, n (%)	15 (17.2)
Class I PRA, med (IQR)	15% (2-20)
Class II PRA, med (IQR)	12% (5-19)
C3d+ HLA-Abs, n (%)	32 (35.9)
IgG subclass detection (%)	43 (48.3)

PRA = panel reactive antibodies; med = median; IQR = interquartilic range ; HLA-Abs = HLA antibodies.

The C3d assay exposed 216 complement-binding abs; 128 of these were identified in the IgG subclasses also. *Table 1* depicts a more detailed analysis of class I and class II HLA-Abs separately and grouped according to its IgG detection and C3d-binding capacity.

A multivariate analysis using different models showed that the best predictors of IgG subclasses detection were: single antigen MFI > 4,000 (OR 15.9, 95% CI 11.2-22.4, p = < 0.01), C3d positivity (OR 3.4, 95% CI 2.4-4.9, p < 0.01), HLA-Bab (OR 2.3, 95% CI 1.7-3.2, p < 0.01).

Comparatively, the multivariate analysis for C3d positivity were: MFI Single Antigen: OR 1.15, 95% CI 1.10-1.21, p < 0.01, HLA-DQab: OR 4.37, 95% CI 2.21-8.66, p < 0.01, HLA-DRab: OR 10.24, 95% CI 5.07-20.65, p < 0.01, HLA-Bab: OR 0.35, 95% CI 0.18-0.67, p > 0.01, IgG subclass detection OR 15.8, 95% CI 7.79-32.2, p < 0.01.

Distribution of HLA antibodies for IgG subclasses detection

The HLA-Abs against class II antigenic specificities, particularly those against HLA-DQ antigens, corresponded to most of the IgG2 and IgG4 subclasses identified. On the contrary, HLA-Abs against class I antigenic specificities were identified mostly as IgG1 and IgG3. Antibodies against HLA-DP were found only as IgG3 subclass *(Figure 2)*.

Antibodies analysis based on patient related factors

The result of every assay was then related to the each individual patient and its sensitization history. Eighty-

nine (89) patients were included; their average age was 33.3 (± 13.8) and males were more prevalent (n = 54, 60.2%). General characteristics of the cohort are described in *Table 2*.

Antibodies analysis by history of sensitization events

The objective of this analysis was to describe the immune profile for the different sensitization events, we included 88 potential TR patients; one of the sera was excluded for having both previous pregnancies and history of a previous kidney transplant. The patients were divided into 4 groups *(Table 3)* according to their history of sensitization events. Only 22 patients had no history of previous HLA alloimmunization exposure; the transfusion group (n = 35) presented this as the only past immunizing event. From the group of previous kidney transplant (n = 17), 76.4% had history of transfusions, while only 35.7% from the pregnancy group (n = 14) had this antecedent.

The group with a previous kidney transplant presented a higher PRA class II value, and larger amount of class II HLA-Abs with a greater MFI value. The presence of HLA-Abs was more prevalent in this set of patients (29.1 ± 18.9), the HLA-Abs against -DQ and -DR antigenic specificities were the ones with the highest MFI values (10,190 and 7,653 respectively).

Patients' factors related to the presence of IgG subclasses

Analyzing the difference between the patients in whom IgG subclasses were detected against those without detection we found out that in forty-three (48.3%) of the 89 samples we were able to detect at least one IgG subclass. Individuals with a history of previous kidney transplant, higher PRA Class I & II, and a greater number of HLA-Abs and its mean MFI were found to be related to the detection of at least one IgG subclass *(Table 4)*.

Previous kidney transplant relation to C3d-binding abs and IgG subclasses detection

We investigated which sensitizing event was related the most to a superior detection rate of IgG1, IgG2, IgG3 and IgG4, as well as for complement-binding

Table 3: Immunologic characteristics for different sensitization events.

	No events (n = 22)	Previous transplant (n = 17)	Pregnancy (n = 14)	Transfusion (n = 35)	
	m ± SD	m ± SD	m ± SD	m ± SD	p
%PRA class I	7.1 ± 8.7	23.6 ± 23.9	7.2 ± 6.9	17.3 ± 20.9	0.08
%PRA class II	7.4 ± 8.9	23.9 ± 14.9	8.3 ± 5.9	13.2 ± 12.4	< 0.01
N of HLA-Abs	10.4 ± 7.4	29.1 ± 18.9	10.9 ± 7.4	21.2 ± 19.1	< 0.01
MFI HLA-Abs	1,704 ± 638	6811 ± 3354	2,283 ± 1,542	2,516 ± 1,970	< 0.01
HLA-Abs class I	5.9 ± 6.2	20.1 ± 20	6.4 ± 5.8	14.4 ± 16.8	0.08
MFI HLA-Abs class I	1,971 ± 782	3,755 ± 2,785	3,281 ± 1,907	2,888 ± 2,028	0.28
HLA-Abs class II	4.5 ± 5	9 ± 7.4	4.4 ± 2.6	6.7 ± 5.5	0.06
MFI HLA-Abs class II	1,469 ± 662	9,348 ± 3,782	1,876 ± 1,299	1,900 ± 2,228	< 0.01
HLA-Abs A	1.7 ± 4.1	4.8 ± 4.8	2.3 ± 2.5	3.4 ± 4.5	0.052
HLA-Abs B	1.31 ± 1.5	10.6 ± 12.8	2.3 ± 3.8	8.1 ± 11.2	0.028
HLA-Abs C	2.8 ± 3.6	4.6 ± 4.9	1.6 ± 3.1	2.8 ± 3.6	0.31
HLA-Abs DQ	1.3 ±1.6	1.3 ± 2.5	0.7 ± 1.1	2.2 ± 2.3	0.08
HLA-Abs DR	1 ± 1.4	3.2 ± 1.9	1.3 ± 1.3	1.4 ± 1.4	< 0.01
MFI HLA-Abs DQ	1,396.2 ± 848	10,190 ± 6,067	2,044 ± 1,410	2,633 ± 3,347	< 0.01
MFI HLA-Abs DR	1,290 ± 472	7,653 ± 5,479	1,783 ± 1,647	1,788 ± 2,288	< 0.01
C3d+ HLA-Abs, n (%)	3 (13.6)	16 (94.1)	5 (35.7)	7 (18.4)	< 0.01
C3d+ HLA-Abs class I, n (%)	0	5 (29.4)	0	2 (5.2)	< 0.01
C3d+ HLA-Abs class II, n (%)	3 (13.6)	15 (88.2)	5 (35.7)	5 (13.1)	< 0.01
IgG subclasses detection (%)	5 (22.7)	17 (100)	8 (57.1)	14 (36.8)	< 0.01
IgG1 (%)	1 (4.5)	17 (100)	6 (42.8)	12 (31.5)	< 0.01

PRA = panel reactive antibodies; MFI = median fluorescence intensity; m = median; HLA-Abs = HLA antibodies.

Table 4: Risk factors for the presence of IgG subclasses.

	IgG subclasses detection (n = 43)	Without IgG subclasses detection (n = 46)	
	m ± SD	m ± SD	p
TR age	34.4 ± 12.2	32.1 ± 15.1	0.44
Previous transplant, n (%)	17 (39.5)	1 (2.1)	< 0.01
Pregnancy, n (%)	9 (20.9)	6 (13)	1.0
Transfusions, n (%)	29 (67.4)	25 (54.3)	0.21
%PRA class I	24.1 ± 23.8	6.8 ± 7.2	< 0.01
%PRA class II	17.2 ± 15.1	9 ± 7.9	< 0.01
No. of HLA-Abs	26.7 ± 20.6	11.3 ± 7.8	< 0.01
MFI HLA-Abs	4,709 ± 3,058	1,583 ± 673	< 0.01
HLA-Abs class I	19.5 ± 2.8	5.9 ± 0.8	< 0.01
HLA-Abs class II	7.1 ± 1.1	5.4 ± 0.6	0.14

TR = transplant recipient; PRA = panel reactive antibodies; MFI = median fluorescence intensity; m = median; HLA-Abs = HLA antibodies; No. = number.

antibodies (C3d+ HLA-Abs). The groups described in section *Antibodies analysis by history of sensitization events* were used, meaning history of previous kidney transplant (n = 17), pregnancy (n = 14) and transfusion (n = 35). As explained in *Figure 3*, those patients with a previous kidney transplant had more complement-binding abs detection (C3d+) and greater IgG detection for every subclass except IgG3; this was detected more frequently in patients with pregnancy history.

Due to the importance of certain HLA-Abs antigenic specificities in the outcomes of a transplant, we did a more detailed analysis of the most frequent antibodies found in this cohort. The class I antigenic specificity that exhibited more relevance for this set of patients was HLA-B antibodies; all the HLA-B antigenic specificities were more prevalent in the group of patients with previous kidney transplant than in the other groups. HLA-B51, -B52, -B59 and -B63 were the exception, these were more frequent in the transfusions only group. Nine of the 16 HLA-B antigenic specificities detected were not present in the group with no sensitization events *(Figure 4A)*.

Among the class II HLA antigenic specificities, the assays revealed HLA-DQ as the most relevant in the groups with any sensitizing event. Patients with previous kidney transplant exhibited a significantly higher detection rate of almost every HLA-DQ antigenic specificity analyzed, especially those against HLA-DQ2, -DQ6 and -DQ9 *(Figure 4B)*.

DISCUSSION

The purpose of our group in searching for specific characteristics of the HLA antibodies looked for an alternative to the need of FXM in those patients who have HLA-DSA with the potential donor and a negative CDC cross-match which constitute an indication for a FXM test. The shortage of histocompatibility laboratories in our country is a limitation for the complete immunologic risk assessment in patients with the aforementioned profile. In consequence, the first step of this research was to compare the association and predictive capacity of DSA-MFI, complement fixing capacity through the C3d assay, and IgG subclasses determination in the prediction of FXM result in a group of 93 donor/recipient pairs. The study revealed that neither C3d nor the IgG subclasses detection alone had an adequate predictive capacity for the FXM; however, in the absence of IgG subclass detection and a DSA-MFI < 2,300, the probability of a negative FXM was nearly 94%.[14]

The results herein presented describe the characteristics (HLA-Abs class, SAB MFI, C3d complement fixing capacity, and IgG subclass) of all the anti-HLA antibodies documented in the sera from 89 of the original 93 potential kidney transplant recipients. An extensive analysis based on the patient's history of immunizing events, individually and combined, was performed to elucidate the characteristics indicated. It is worth mentioning that all the assays were performed in neat serum therefore we cannot ensure that the prozone effect (inhibitory factors) could have influenced the MFI value of some Ab specificities.[17]

Interestingly, even in patients without any previous sensitizing event there was detection of HLA-Abs, possibly corresponding to natural antibodies.[18] Even though the MFI of the HLA-Abs detected in this sub-

group of patients was the lowest of the cohort, C3d positivity and IgG subclass detection occurred in 6 and 5 samples, respectively.

Overall, according to the results obtained in this analysis, it is evident that the most robust sensitized event identified in this group of patients corresponded to a previous kidney transplant, finding that is consistent with previous reports.[16,19-21] Likewise, the main factor related to both detection of IgG subclass and C3d positivity corresponded to the SA MFI. This fact was particularly represented for class II HLA-Abs with a mean MFI for DQ and DR above 10,000 and 7,000, respectively. Several centers have independently reported a high prevalence of HLA-DQ DSA in association with a significant risk for acute rejection and graft loss.[22,23]

Furthermore, the number of positive HLA-Abs for the C3d assay was significantly higher again for class II HLA-Abs in those with a history of a previous transplant, attaining almost 90% of these Abs in the cohort presented. The relevance of the SA MFI for C3d positivity was evidenced in the multivariate analysis as were also the presence of HLA-DQab, HLA-DRab, for class II antigens, and HLA-B ab for class I antigens, and IgG subclass detection.

In clinical studies of transplant recipients, the HLA ab documented with positivity to the C3d binding-capacity of DSA at the time of antibody mediated rejection (AMR) diagnosis allows for identification of patients at risk for allograft loss;[11] other study found detection of complement binding activity using both C1q and C3d assays to be a further prognostic marker for predicting AMR and allograft outcome in *de novo* DSA+ kidney transplant patients.[24] Certainly, this information belongs to patients already transplanted. However, research performed in pretransplant sera have shown contradictory results. It is interesting to mention the findings of Kamburova et al where the C3d-fixing ability of pretransplant DSA was not associated with increased risk for graft failure.[25] More recently Choi et al found preformed C3d positive DSAs significantly associated with a higher incidence and risk of AMR, and identification of the C3d binding-activity of preformed DSAs before and early after kidney transplant was important for both, predicting the persistence of preformed DSAs and the risk of AMR induced by the presence of preformed DSAs.[25] It is also interesting to mention that for class II antibodies, 42.6% of the HLA antibodies were C3d-positive, and their median MFI was 10,341 whereas that of the C3d-negative antibodies was 1,711.[26] Sicard et al also observed a correlation between C3d-binding capacity of antibodies with a higher number and a higher MFI of DSAs.[11] Our finding regarding the SAB MFI for class II C3d positive antibodies is in agreement to the previous observations. A high predictive capacity of 10240 IgG MFI for C1q positivity, and IgG MFI of 7,629 for C3d positivity has shown 84% sensitivity and 97% specificity.[27] This findings are similar to that reported by Wiebe et al,[28] Courant et al[29] recently reported that both C1q and C3d positivity could be predicted with an IgG MFI > 3,800 in EDTA-treated serum samples. Therefore, while the MFI threshold is inconsistent, multiple studies could predict complement-fixing Luminex results by the MFI level in the regular Luminex assay.

According to our findings and comparing the patients with a previous kidney transplant to patients with other sensitizing events it is clear that the higher the HLA-Abs MFI, the higher the possibility to obtain a C3d positive assay and to detect the HLA-Abs IgG subclass. Moreover, in a previous communication of our group studying pretransplant sera from 55 sensitized patients,[13] 80% of them with a previous transplant had C1q+ HLA-Abs, an association that has been reported in other series as ranging between 73 and 92%.[17,30,31] In our previous study, this was the main past event associated with the presence of C1q+ HLA-Abs. It was remarkable in that study the absence of association to other immunizing events (blood transfusion and pregnancy) and C1q binding capacity of HLA-Abs.[13]

The HLA-Abs IgG subclass detection was universal in the sera proceeding from patients with a previous kidney transplant in this study. This fact adds

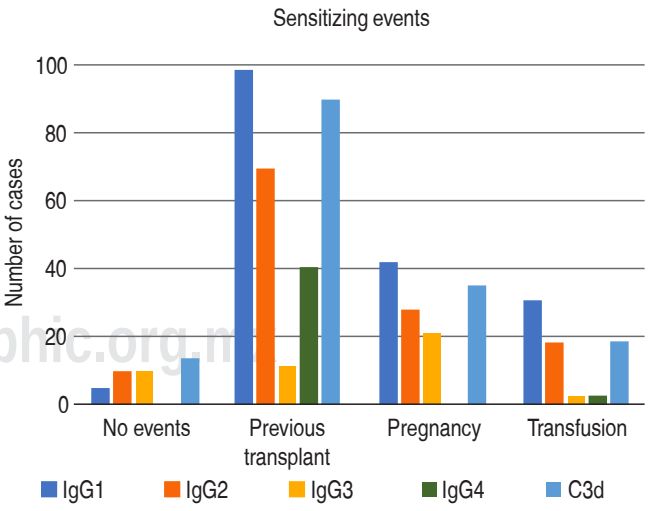

Figure 3: Distribution of IgG subclasses and complement-binding antibodies detection according to previous sensitizing events.

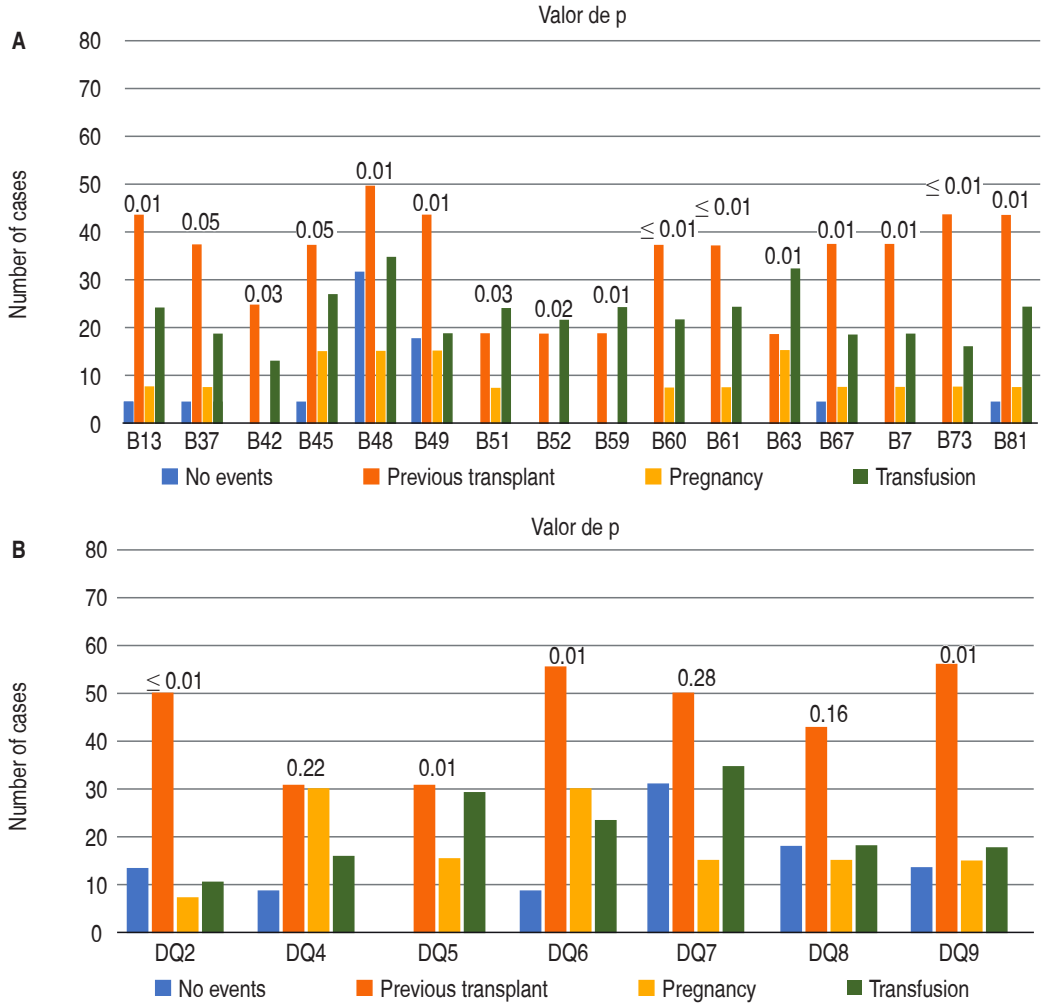

Figure 4: Distribution of different HLA antibodies specificities according to previous sensitization events. **A)** HLA-B antibodies. **B)** HLA-DQ antibodies.

the possibility to detect the IgG subclass according to the HLA-Abs MFI. The more commonly detected IgG subclass was IgG1 followed by IgG2, IgG4, and to a lesser extend IgG3; this order occurred regardless the sensitizing event. The difference observed among the groups *(Figure 3)* pertain to the IgG detection frequency according to the sensitizing event. When comparing patient's factors related to IgG subclasses detection versus no detection significant differences were found for having a previous transplant, number of HLA-Abs, HLA-Abs MFI, %PRA for class I and II, and class I HLA-Abs. However, on multivariate analysis using different models the best predictors of IgG subclasses detection were SA MFI > 4,000, C3d positivity, and HLA-B abs. Our findings resembles those recently published by Navas et al demonstrating a close relationship between the circulating antibody strength, the presence of a mixture of IgG subclasses, beyond the quasy omnipresent IgG1, and the complement-binding ability measured by C1q.[32]

Important to mention are the results obtained in this study in relation to the low frequency detected for both IgG subclass identification and C3d positivity which corresponded to 20.7% and 12.2%, respectively, of the total 1,771 HLA-Abs detected by the three methods used. Whether methodological factors could took place, at least in part in these results, cannot be ruled out despite the methods followed were exactly as the manufacturer indicate. Of note, a previous study regarding IgG subclass analysis only included sera samples

from patients with HLA-DSA MFI above than 2,000 because their initial experience demonstrated that HLA-DSA with less than 2,000 MFI were often negative in the IgG subclass analysis.[33] This finding make sense with our results where 47% (n = 837) of the HLA-Abs had less than 2,000 MFI and only in 16 (2%) HLA-Abs IgG subclass was detected; in contrast, at HLA-Abs MFI level above 2000 IgG subclass was determined for 351 (37.5%) HLA-Abs, lower indeed however than would have been be expected.

CONCLUSIONS

In conclusion, even though the detection of IgG subclasses (20.7%) and the complement, fixing capacity (C3d positivity, 12.2%) of the HLA-Abs for the entire cohort was low, IgG subclass detection was universal in those patients with a previous kidney transplant and C3d positivity attained almost 90% on them. Both, IgG subclass detection and C3d positivity of the HLA-Abs were associated with the SAB MFI, significantly higher in patients with a previous transplant event. This is the first study on its kind in our population and seems pertinent to insist in the characterization of the HLA-Abs though SAB assay for the patients awaiting kidney transplantation whether living related or deceased, due to the implications of preformed Abs in the faith of the graft.

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