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ORIGINAL ARTICLE

Determining donor-specific antibody C1q-binding ability improves the prediction of antibody-mediated rejection in human leucocyte antigen-incompatible kidney transplantation

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SUMMARY

Detrimental impact of preformed donor-specific antibodies (DSAs) against human leucocyte antigens on outcomes after kidney transplantation are well documented, however, the value of their capacity to bind complement for predicting antibody-mediated rejection (AMR) and graft survival still needs to be confirmed. We aimed to study DSA characteristics (strength and C1q binding) that might distinguish harmful DSA from clinically irrelevant ones. We retrospectively studied 60 kidney-transplanted patients with preformed DSA detected by single antigen bead (SAB) assays (IgG and C1q kits), from a cohort of 517 kidney graft recipients (124 with detectable anti-HLA antibodies). Patients were divided into DSA strength (MFI < vs. ≥ 15 000) and C1q-binding ability. AMR frequency was high (30%) and it increased with DSA strength ($P = 0.002$) and C1q+ DSA ($P < 0.001$). The performance of DSA C1q-binding ability as a predictor of AMR was better than DSA strength (diagnostic odds ratio 16.3 vs. 6.4, respectively). Furthermore, a multivariable logistic regression showed that C1q+ DSA was a risk factor for AMR (OR = 16.80, $P = 0.001$), while high MFI DSAs were not. Graft survival was lower in high MFI C1q+ DSA in comparison with patients with C1q− high or low MFI DSA (at 6 years, 38%, 83% and 80%, respectively; $P = 0.001$). Both DSA strength and C1q-binding ability assessment seem valuable for improving pretransplant risk assessment. Since DSA C1q-binding ability was a better predictor of AMR and correlated with graft survival, C1q-SAB may be a particularly useful tool.

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Key words
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Introduction

Preformed donor-specific antibodies (DSAs) against human leucocyte antigens (HLAs) have been associated with the occurrence of antibody-mediated rejection (AMR) and decreased kidney graft survival, even in the presence of a negative cytotoxic and/or flow cytometric crossmatch [1]. Nevertheless, several papers have shown

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that not all DSAs are deleterious to the graft [2,3]. In the last decade, the use of solid-phase assays improved greatly our ability to detect and identify anti-HLA antibodies but not without shortcomings given their excessive sensitivity and inability to accurately predict clinical events [4]. The availability of parameters able to distinguish deleterious DSA from irrelevant ones would be very important for pretransplant risk stratification, particularly when deciding whether a transplant should be performed and under which immunosuppression. Several authors have shown that DSA strength and HLA class could be considered for that purpose [5–7].

Graft injury driven by DSA occurs mainly through the activation of classical complement pathway [8,9]. Thus, determining which anti-HLA antibodies can bind complement (even after a negative cytotoxic crossmatch) may identify DSA of clinical relevance. A variety of studies employed Luminex-based single antigen bead (SAB) assays for *in vitro* C4d deposition detection (a marker of complement activation) in order to identify clinically impactful DSA in kidney transplantation with contradictory results [10–12]. More recently, an assay detecting the attachment of exogenous recombinant C1q to SABs incubated with heat-inactivated patient sera has been developed. The importance of preformed C1q-binding DSA in kidney transplantation has been scarcely studied and, until now, no significant impact on post-transplant outcomes (AMR or graft loss) has been demonstrated [7,13,14].

Hence, we designed a retrospective study in order to better understand the impact of preformed DSA strength and its C1q-binding ability in the outcomes (AMR and graft survival) of patients transplanted with a negative cytotoxic crossmatch but in the presence of DSA.

Materials and methods

Study design and patient selection

All 517 consecutive adult patients who received a kidney transplant in our unit between 2007 and 2012 were analysed. The presence of anti-HLA antibodies in pretransplant sera was detected in 124 patients (24%) before being transplanted. Nevertheless, the detection of preformed DSA by SAB assay at that time was incomplete (Fig. 1), with only 18 out of 124 screen-positive patients being detected to have DSA at transplant. Hence, transplantation proceeded mostly based on a negative T and B lymphocyte cytotoxic crossmatch (standard NIH technique, not enhanced with anti-human globulin) in current and peak sera.

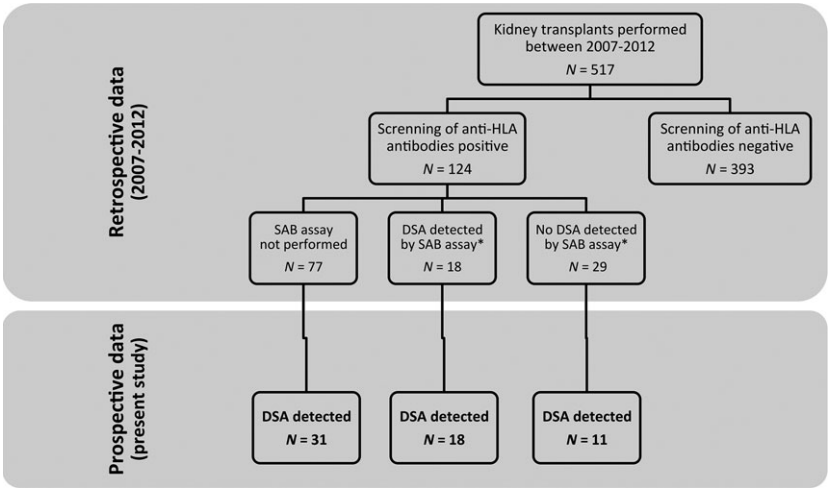

Figure 1 Study algorithm for the detection of preformed donor-specific antibodies (DSAs) by single antigen bead (SAB) assay in patients transplanted between 2007 and 2012. Retrospective data were known at the time of transplantation, when 124 patients had a positive screen, but only 18 patients recognized to have preformed DSAs. Prospective data result from the present study, in which pretransplant sera from all screen-positive patients (n = 124) were reanalysed by SAB assay (as described in the Materials and methods section) and donor typing for HLA-Cw and HLA-DQ loci was performed if anti-HLA antibodies against them were detected. Consequently, preformed DSAs were detected in 60 patients at the present study (in bold). *In 18 patients of these groups, the study by SAB assay before transplant was incomplete (anti-HLA antibody screening was positive for both HLA classes, but SAB assay was only performed for one of them). In all cases, donors were typed only for HLA-A, HLA-B and HLA-DR loci at the time of transplant. HLA, human leucocyte antigens.

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The recognition that at least some of these allosensitized patients presented a high immunological risk not fully accounted for at the time of their transplant led us to repeat a SAB assay for this study in all 124 screen-positive patients. Moreover, in patients with anti-HLA antibodies against HLA-Cw or HLA-DQ antigens, donors were presently typed for the respective *locus* (at time of transplant, only HLA-A, HLA-B and HLA-DR were typed). These analyses allowed us to detect 42 further patients transplanted with preformed DSA, a status previously unknown, besides 18 patients already known to have DSA at transplant, thus defining the study population ($n = 60$). Comparison of baseline characteristics and major post-transplant outcomes in patients with a positive screening before transplant, according to the detection of DSA at the present study, is shown as Table S1. Finally, pretransplant sera from all 60 patients were further analysed for detection of C1q-binding DSA using IgG and C1q SAB kits. No data on flow cytometric crossmatches were available for this study. The Institutional Review Board at Centro Hospitalar do Porto approved this study.

Anti-HLA antibody screening and specification

Pretransplant anti-HLA IgG antibodies were screened in patient sera collected every 3 months while on the waiting list, by multiplex microsphere based on Luminex Xmap® Technology (LABScreen® Mixed kit; One Lambda, Canoga Park, CA, USA). Screening for anti-HLA antibodies before transplant has been done per protocol in our unit since 2006.

Patients with a pretransplant positive screening for anti-HLA antibodies were retrospectively selected. In all of them, SAB assays (LabScreen Single Antigen Beads®; One Lambda Canoga Park, CA, USA) were prospectively performed in the same pretransplant (screen positive) sera within the context of this study, in order to determinate thoroughly the specificity of anti-HLA antibodies. In brief, patients' sera were incubated for 30 min with beads coated with single HLAs produced by recombinant technology. After three washes, the samples were incubated for 30 min with 100 µl of 1:100 phycoerythrin-conjugated goat anti-human IgG (One Lambda Inc.). After two final washes, the MFI of each bead was measured using LABScan™ 100 flow analyzer (Luminex®, Austin, TX, USA). To account for a possible complement interference or prozone effect, all samples were treated with ethylenediaminetetraacetic acid (EDTA). The analysis was performed using HLA FUSION® software (One Lambda Inc.), and a cut-off for a positive reaction was set at a normalized MFI value of ≥1000.

Detection of complement binding anti-HLA antibodies

Briefly, pretransplant serum was heat-inactivated (56 °C for 30 min) and spiked with purified human C1q in HEPES buffer (C1qScreen^R, One Lambda, Canoga Park, CA, USA) to ensure equal functional amounts of C1q per sample. SABs were added to the mixture and incubated for 20 min at room temperature, followed by addition of phycoerythrin-conjugated anti-human C1q. Beads were washed twice and analysed on a LABScan 100 flow analyzer. Antibodies were assigned as positive at a MFI raw value of ≥500 [14].

DSA assignment and characterization

Donor and recipient were typed before transplant in *loci* HLA-A*, HLA-B* and HLA-DR* using polymerase chain reaction amplification with specific sequence primers (SSP; Olerup SSP® low-resolution HLA typing kits, Stockholm, Sweden). Donor and recipient HLA-Cw* and HLA-DQ* antigens were also typed for this study by SSP DNA typing, when the recipient was sensitized against antigens from these *loci*. High resolution was performed in those cases in which it was necessary to establish whether the anti-HLA antibodies were DSAs. Donor typing in *locus* HLA-DP was not available.

In every patient, DSA antigenic targets were identified through the comparison of donor–recipient HLA mismatch to the antibody profile in each patient. For every individual DSA, the reported strength is based on the MFI of one SAB. In cases where more than one bead corresponding to the donor type was present within the panel, we recorded the corresponding bead with the highest MFI level. In case of more than one DSA against different HLAs, we took two approaches by defining DSA strength as the MFI of the highest level DSA (MFI_{max}) or as the sum of all individual DSA MFI values (MFI_{sum}).

We detected 138 DSAs in studied patients ($n = 60$), of which 16 (12%) were C1q binding. Eighty-seven DSAs were against HLA-I and 51 against HLA-II antigens. By HLA *loci*, 37 DSAs were against HLA-A, 43 against HLA-B, seven against HLA-Cw, 32 against HLA-DR and 19 against HLA-DQ antigens. Finally, 17 patients had DSA anti-DQ and five anti-Cw, with eight of them having DSA solely against these *loci*.

Induction protocol and maintenance immunosuppression

Induction therapy was used in all patients with an anti-IL-2 receptor monoclonal antibody [basiliximab; Novartis® (Novartis Europharm Limited, Camberley, UK) 20 mg twice at day 0 and 4] or a polyclonal antithymocyte globulin [ATG Fresenius® (Fresenius Biotech GmbH, Grafelfing, Germany) 3 mg/kg for 5–7 days]. ATG was primarily used in patients with previous transplant and/or those with high (>20%) cytotoxic panel-reactive antibodies (PRA). All patients had similar triple maintenance immunosuppression, consisting of oral tacrolimus, mycophenolate mofetil and prednisolone. No immunosuppression minimization strategy was implemented in these patients.

Eight patients known to have preformed DSA underwent a desensitization protocol (in all patients, sera used for DSA detection were collected before desensitization). Two patients received intravenous immunoglobulin (IvIg) 2 g/kg at transplant (0.5 g/kg immediately before transplant, and at days 1, 2 and 3) and 1 month after transplant (1 g/kg in two consecutive days). Three patients received similar dose of IvIg and underwent plasmapheresis every other day (first session immediately before transplant, for a total of 6–9 sessions) and three other patients received additionally a dose of rituximab (375 mg/m^2) at day 3 post-transplant.

Data collection and outcomes

Data regarding recipient and donor characteristics, and pre- and post-transplantation variables were collected retrospectively. Delayed graft function (DGF) was defined as dialysis requirement in the first week post-transplant. Graft biopsies were performed for cause only, when in the presence of prolonged DGF, a rise in serum creatinine by more than 20% compared with previous measurements and/or increased levels of proteinuria. Estimated glomerular filtration rate (eGFR) was evaluated using the 2006 MDRD equation [15]. All patients were followed up from time of transplant until death, graft failure (defined as return to dialysis or retransplant) or 31 December 2015. Graft survival was analysed considering graft failure censored for death with a functioning graft.

Rejection diagnosis and treatment

Graft rejection was defined as biopsy-proven rejection (specimens were evaluated by light microscopy and immunofluorescence staining for C4d) and classified according to Banff classification as updated in 2009 [16] (the current classification at time of observed acute rejection episodes). So, only C4d-positive AMR was considered and treated as such. Mild acute cellular rejection (ACR Banff grade I) was treated with pulse steroids (500 mg methylprednisolone for 3 days) and increased maintenance immunosuppression. All other ACRs were treated with ATG. All patients with AMR were treated with plasmapheresis every other day and IvIg 100 mg/kg after each session; per protocol, the number of plasmapheresis sessions was 4. After the last plasmapheresis session, every patient received high-dose IvIg (2 g/kg) divided into four daily doses; a similar dose of IvIg (2 g/kg) was repeated 1 month later. Eleven patients received, additionally, one dose of rituximab (375 mg/m^2). Patients presenting with chronic active AMR received no specific treatment.

Statistical analysis

Continuous data were described using mean (standard deviation) or median (interquartile range) and categorical data were expressed as numbers (frequencies). The distributions of continuous variables were analysed using Kolmogorov–Smirnov test. Categorical data including demographic, clinical and immunological features were compared using Pearson chi-square test (chi-square for trend test was used in the presence of a variable with three ordinal categories) or Fisher's exact test, as appropriate. Continuous variables were compared with Student's *t*-test or Mann–Whitney *U*-test, as appropriate. Both MFI_{max} and MFI_{sum} were explored as predictors of AMR by receiver operating characteristic (ROC) analysis, with MFI_{sum} presenting an area under the curve (AUC) of 0.742 (95% confidence interval: 0.604–0.881) and MFI_{max} of 0.722 (95% confidence interval: 0.583–0.861). No significant difference between the two AUC was found ($P = 0.186$). Additionally, these analyses were repeated 1000 times with the use of bootstrap samples in order to quantify the amount of overestimation. The AUC estimates obtained were the same for both variables, with bias-corrected 95% confidence intervals being slightly wider for MFI_{max} (MFI_{max}: 0.565–0.851; MFI_{sum}: 0.599–0.861). Hence, MFI_{sum} was selected for our analysis (henceforth referred only as MFI).

Given the nonparametric nature of MFI data, we decided to categorize the studied population in two groups considering DSA strength, based on the observation that the most discriminative MFI value (highest

Youden's index) for AMR prediction according to ROC analysis (AUC 0.74) was 15 000. Sensitivity, specificity, positive and negative likelihood ratios, and diagnostic odds ratio (and their respective 95% confidence interval) were calculated to study the performance of DSA strength and C1q-binding ability as predictors of AMR [17]. Risk factors for AMR were explored by univariate and multivariable (using a backward elimination method, with a P-value < 0.05 necessary for retention in the model) logistic regression. The model used for the multivariable analysis included only those variables presenting a univariate P-value < 0.1. Graft survival curves were visualized using Kaplan–Meier method, with comparison between patients' groups being done by log-rank test.

A two-sided P-value of <0.05 was considered as statistically significant. Statistical calculations were performed using SPSS, version 23.0 (SPSS Inc., Chicago, IL, USA) and STATA/MP, version 14.1 (Stata Corp, College Station, TX, USA).

Results

Baseline characteristics

Patients were divided according to DSA strength and C1q-binding ability groups and their characteristics are compared in Table 1. Presensitizing events were more common in patients with stronger and C1q+ DSA. Previous transplantation was more common in patients with C1q+ DSA versus than in those with C1q− DSA (77% vs. 34%; $P = 0.006$). Alternatively, earlier blood transfusions were more frequent in patients with high MFI DSA (68% vs. 37%, $P = 0.019$). Furthermore, median time on dialysis was higher in both patients with stronger DSA (11.3 vs. 9.0 years, $P = 0.089$) and C1q+ DSA (13.1 vs. 9.0 years, $P = 0.176$). No difference in donor characteristics was detected between groups.

Immunological data were expectedly distinct between DSA strength groups, with patients with high MFI having more frequently cytotoxic PRA ≥15% (73% vs. 26%, $P < 0.001$), higher number of DSA (3 vs. 2, $P < 0.001$) and C1q+ DSA (46% vs. 8%, $P = 0.002$). Median MFI was 24 900 and 5200 in the high and low DSA strength groups, respectively. Similarly, DSA number and MFI were significantly higher in patients with C1q+ DSA than in those with C1q− DSA (number 4 vs. 2, $P = 0.001$; MFI 24 300 vs. 7600, $P = 0.001$, respectively). ATG induction was more frequent in both patients with strong (86% vs. 53%, $P = 0.008$) and C1q+ (92% vs. 57%, $P = 0.023$) DSA. Few patients ($n = 8$) received a desensitization regimen, since a detailed analysis of DSA presence and characteristics at the time of transplant was missing in many of them.

Overall clinical outcomes

Post-transplant outcomes are detailed in Table 2. Graft biopsies were performed in 40 patients (in the first year post-transplant in 70%), with all patients with C1q+ DSA undergoing graft histological examination. Acute AMR overall frequency was high (30%) and it increased with DSA strength (16% in low and 55% in high MFI groups, $P = 0.002$) and, even more, with C1q+ (17% in C1q− and 77% in C1q+ patients, $P < 0.001$). Both pure AMR ($n = 10$) and mixed AMR ($n = 8$) were observed. All episodes of AMR occurred early after transplant (median number of days 12, ranging from 4 to 20 days), with no difference regarding time to AMR being noticed between groups. Eight patients experienced ACR-only episodes. Additional comparison of baseline characteristics and post-transplant outcomes, considering only patients with strong DSA ($n = 22$) according to C1q-binding status, is presented as Table S2.

Graft function at 1 year post-transplant and at the end of follow-up was lower in the C1q+ than in C1q− patients (median eGFR 37 vs. 52 ml/min, $P = 0.027$; eGFR 5 vs. 48 ml/min, $P = 0.078$, respectively). No difference in graft function was observed between DSA MFI groups in both time points. Proteinuria at 1 year was more common in patients with C1q+ DSA (55%) than in those with C1q− DSA (12%) ($P = 0.006$).

C1q-binding DSA

C1q-binding DSAs were detected in 13 (22%) patients, with 16 C1q-binding DSA beads being identified against antigens present at HLA *locus* A/B/DR/DQ (10 against HLA-II and six against HLA-I antigens). AMR occurred in 10 (77%) and censored graft failure in 7 (54%) patients with C1q+ DSA. Detailed information about immunological data and outcomes of patients with C1q-binding DSA is given in Table 3.

Predictors and risk factors for AMR

The performance of DSA characteristics (strength and C1q-binding ability) as predictors of AMR is detailed in Table 4. C1q+ DSA presented a lower sensitivity and higher specificity than DSA strength (56% vs. 67% and 93% vs. 76%, respectively) for the diagnosis of AMR. The probability of AMR occurrence in patients with

Table 1. Baseline characteristics according to DSA strength and C1q-binding groups.

	MFI < 15k *N* = 38	MFI ≥ 15k *N* = 22	*P*	C1q– DSA *N* = 47	C1q+ DSA *N* = 13	***P***
Recipient						
Age (years), mean ± SD	47.9 ± 11.4	48.6 ± 10.2	0.791	48.2 ± 10.7	47.8 ± 12.2	0.920
Female gender, *n* (%)	26 (32)	13 (59)	0.465	32 (68)	7 (54)	0.349
HCV positive, *n* (%)	4 (11)	2 (9)	1	5 (11)	1 (8)	1
Retransplant, *n* (%)	13 (34)	13 (59)	0.061	16 (34)	10 (77)	0.006
Previous blood transfusions, *n* (%)	14 (37)	15 (68)	0.019	20 (43)	9 (69)	0.088
Previous pregnancies, *n* (%)*	18 (69)	8 (62)	0.725	22 (69)	4 (57)	0.666
Years on dialysis, median (IQR)	9.0 (4.3–15.1)	11.3 (7.2–18.2)	0.089	9.0 (4.5–15.6)	13.1 (6.9–19.6)	0.176
CKD aetiology, *n* (%)						
Chronic glomerulonephritis	16 (42)	11 (50)	0.761	19 (40)	8 (62)	0.242
Diabetes mellitus	5 (13)	3 (14)		5 (11)	3 (23)	
Polycystic kidney disease	6 (16)	1 (5)		7 (15)	0	
Chronic tubulointerstitial nephritis	4 (11)	2 (9)		5 (11)	1 (8)	
Unknown	7 (18)	5 (23)		11 (23)	1 (8)	
Donor						
Age (years), mean ± SD	49.2 ± 15.4	47.6 ± 12.8	0.653	48.7 ± 14.1	48.4 ± 16.1	0.946
Female gender, *n* (%)	11 (29)	5 (23)	0.600	11 (23)	5 (39)	0.303
Living donor, *n* (%)	4 (11)	0	0.286	2 (4)	2 (15)	0.202
Donor–recipient CMV status, *n* (%)						
–/–	1 (3)	0	0.747	1 (2)	0	0.895
+/–	2 (5)	1 (5)		2 (4)	1 (8)	
–/+	8 (21)	3 (14)		9 (19)	2 (15)	
+/+	27 (71)	18 (82)		35 (75)	10 (77)	
Pretransplant immunological data						
Cytotoxic peak PRA ≥ 15%, *n* (%)	10 (26)	16 (73)	<0.001	18 (38)	8 (62)	0.134
DSA HLA class, *n* (%)						
I	21 (55)	7 (32)	0.144	24 (51)	4 (31)	0.264
II	7 (18)	4 (18)		9 (19)	2 (15)	
I + II	10 (26)	11 (50)		14 (30)	7 (54)	
DSA number, median (IQR)	2 (1–2)	3 (2–4)	<0.001	2 (1–2)	4 (2–5)	0.001
DSA MFI*1000, median (IQR)	5.2 (2.3–10.6)	24.9 (19.7–30.6)	–	7.6 (2.6–15.4)	24.3 (14.8–28.4)	0.001
C1q-binding DSA, *n* (%)	3 (8)	10 (46)	0.002	–	–	–
Transplant						
ABDR HLA mismatches, mean ± SD	3.61 ± 1.44	4.00 ± 0.82	0.232	3.68 ± 1.34	4.0 ± 0.91	0.553
ATG induction, *n* (%)	20 (53)	19 (86)	0.008	27 (57)	12 (92)	0.023
Desensitized, *n* (%)	3 (8)	5 (23)	0.129	4 (9)	4 (31)	0.059
IvIg only, *n*	1	1		1	1	
IvIg + PP, *n*	1	2		2	1	
IvIg + PP + Rtx, *n*	1	2		1	2	

DSAs, donor-specific antibodies; MFI, mean fluorescence intensity; IQR, interquartile range; SD, standard deviation; CKD, chronic kidney disease; PRA, panel-reactive antibodies; HCV, hepatitis C virus; CMV, cytomegalovirus; HLA, human leucocyte antigen; ATG, antithymocyte globulin; IvIg, Intravenous immunoglobulin; PP, plasmapheresis; Rtx, rituximab.

*Analysis considering women only (*n* = 39).

Table 2. Post-transplant outcomes according to DSA strength and C1q-binding groups.

	MFI < 15k *N* = 38	MFI ≥ 15k *N* = 22	*P*	C1q− DSA *N* = 47	C1q+ DSA *N* = 13	*P*
Delayed graft function, *n* (%)	12 (32)	9 (41)	0.465	16 (34)	5 (39)	0.755
Patients with ≥1 graft biopsy, *n* (%)	22 (58)	18 (82)	0.058	27 (57)	13 (100)	0.003
Number/patient, median (IQR)	1 (0–1)	1 (1–2)	0.022	1 (0–1)	2 (1–3)	0.001
AMR, *n* (%)	6 (16)	12 (55)	0.002	8 (17)	10 (77)	<0.001
Pure AMR, *n* (%)	3 (8)	7 (32)	0.029	2 (4)	8 (62)	<0.001
Mixed (AMR + ACR), *n* (%)	3 (8)	5 (23)	0.129	6 (13)	2 (15)	1
Days to AMR*, median [min.–max.]	14 [4–20]	11 [7–17]	0.397	12 [4–20]	12 [7–18]	0.964
ACR-only, *n* (%)	6 (16)	2 (9)	0.698	8 (17)	0	0.182
1 year eGFR (ml/min)†, median (IQR)	51.6 (38.3–62.6)	50.0 (33.9–68.8)	0.879	51.8 (42.6–68.5)	36.5 (25.8–55.5)	0.027
1 year ProtU (>0.3 g/g)‡, *n* (%)	6 (18)	5 (26)	0.496	5 (12)	6 (55)	0.006
Final eGFR (ml/min)†, median (IQR)	43.7 (25.6–58.3)	48.1 (5.0–64.0)	0.838	48.1 (27.5–58.3)	5.0 (5.0–61.1)	0.078
Final ProtU (>0.3 g/g)§, *n* (%)	8 (27)	1 (8)	0.237	8 (21)	1 (20)	1
Censored graft failure¶, *n* (%)	5 (13)	8 (36)	0.070**	6 (13)	7 (54)	<0.001**
Patient death, *n* (%)	2 (5)††	2 (9)††	0.616**	2 (4)††	2 (15)††	0.180**
Years of follow-up, median (IQR)‡‡	4.6 (3.3–6.1)	4.3 (2.7–6.6)	0.896	4.9 (3.6–6.6)	2.8 (2.1–4.2)	0.012

DSAs, donor-specific antibodies; MFI, mean fluorescence intensity; AMR, acute antibody-mediated rejection; ACR, acute cellular rejection; min., minimum; max., maximum; eGFR, estimated glomerular filtration rate; IQR, interquartile range; ProtU, proteinuria.

*Considering only patients that experienced AMR (*N* = 18).

†*N* = 56. Patients on dialysis were assumed to have an eGFR of 5 ml/min and were included in nonparametric statistical analysis.

‡Available in 53 patients, whose graft remained functioning at 1 year post-transplant.

§Available in 43 patients, whose graft remained functioning at the end of follow-up.

¶Causes in the MFI < 15k group: three cases of unspecified chronic graft dysfunction and two of chronic active AMR; in the MFI ≥ 15k group: four cases of chronic active AMR, three of acute AMR and one of unspecified chronic graft dysfunction. Causes in the C1q− DSA group: three cases of chronic active AMR, and three of unspecified chronic graft dysfunction; in the C1q+ DSA group: three cases of chronic active AMR, three of acute AMR and one of unspecified chronic graft dysfunction.

**Log-rank test.

††Causes: infection (one case) and neoplasia (one case).

‡‡Years until patient death, graft failure or end of follow-up.

Table 3. Immunological data and outcomes of patients with C1q-binding DSA.

Patient no.	C1q-binding beads			IgG-DSA number	IgG-MFI sum*	AMR	Days to AMR	Outcome†	Follow-up (years)
	Specificity	IgG-MFI	C1q-MFI						
1	DR1	8295	1954	5	39 765	Pure AMR	8	HD	2.7
	DR11	12 474	9517						
	DR12	14 710	9994						
2	A3	18 994	5540	5	34 504	Pure AMR	11	1.22	3.2
3	DQ6	22 208	15 049	4	25 393	Pure AMR	12	1.17	8.9
4	DQ5	11 582	1648	4	19 849	Pure AMR	13	HD	3.6
5	A68	29 316	23 049	1	29 316	Pure AMR	9	HD	2.7
6	DQ5	15 613	1777	4	24 259	Pure AMR	17	HD	0.1
7	A2	4284	735	3	8950	Mixed	7	HD	2.2
	DQ6	2995	3485			(AMR + ACR)			
8	DR4	5434	2415	2	8080	Pure AMR	18	1.95	4.8
9	DQ6	10 583	3330	2	14 343	No rejection	–	1.03	3.3
10	A3	22 836	2035	4	27 429	No rejection	–	1.10	2.8
11	DR14	10 078	3600	4	15 262	Pure AMR	12	D	0.8
12	B49	14 078	6442	2	20 518	Mixed	11	HD	6.4
						(AMR + ACR)			
13	B52	14 534	1652	5	24 387	No rejection	–	HD	2.0

DSAs, donor-specific antibodies; MFI, mean fluorescence intensity; AMR, acute antibody-mediated rejection; HD, haemodialysis; ACR, acute cellular rejection; D, death.

*For each patient, the sum of MFI of all detected DSA beads was calculated.

†Three alternative outcomes are presented: patient death (D) with a functioning graft during follow-up, return to dialysis (HD) or, in patients with a graft still functioning, last serum creatinine.

Table 4. Summary statistics (and 95% confidence intervals) describing the predictive capacity for AMR of DSA strength (MFI < vs. ≥ 15 000) and C1q-binding ability.

	DSA strength	C1q-binding DSA
Sensitivity (%)	66.7 (41.2–85.6)	55.6 (31.3–77.6)
Specificity (%)	76.2 (60.2–87.4)	92.9 (79.4–98.1)
Positive LR	2.80 (1.49–5.27)	7.78 (2.42–24.96)
Negative LR	0.44 (0.22–0.85)	0.48 (0.28–0.81)
Diagnostic OR	6.40 (1.91–21.47)	16.25 (3.63–72.67)

AMR, antibody-mediated rejection; DSAs, donor-specific antibodies; MFI, mean fluorescence intensity; LR, likelihood ratio; OR, odds ratio.

C1q+ DSA was 16.3 higher than its occurrence in those with C1q− DSA, while the chance of AMR was only 6.4 higher in patients with high versus low MFI DSA, reflecting higher discriminative ability of the former versus the latter as a predictor of AMR.

In Table 5, we present the logistic regression analysis of risk factors for AMR occurrence. In the multivariable analysis, we identified peak PRA ≥15% (OR = 6.03, $P = 0.029$), DSA against HLA class II (OR = 14.03 vs. DSA against HLA class I, $P = 0.018$) and C1q-binding DSA (OR = 17.00, $P = 0.001$) as significant risk factors for AMR.

Censored graft survival

Thirteen patients experienced death-censored graft failure and mean graft survival time was 7.3 years (95% CI: 6.5–8.0). C1q-binding DSA (Fig. 2a) was associated with significantly lower graft survival (at 6 years, graft survival was 81% in C1q− and 44% in C1q+ DSA

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Table 5. Analysis of risk factors for AMR occurrence by logistic regression.

	Univariate OR (95% CI)	*P*	Multivariable OR* (95% CI)	*P*
Recipient				
Age	0.97 (0.92–1.02)	0.231		
Female (versus male)	0.79 (0.25–2.47)	0.680		
Donor				
Age	0.98 (0.95–1.02)	0.402		
Female (versus male)	1.60 (0.48–5.37)	0.447		
Retransplant	2.03 (0.66–6.22)	0.215		
Cytotoxic peak PRA ≥ 15%	**4.00 (1.24–12.91)**	**0.020**	6.03 (1.20–30.33)	0.029
ABDR HLA MM	**1.52 (0.93–2.48)**	**0.078**		
ATG induction	2.38 (0.67–8.48)	0.181		
Desensitization	2.71 (0.60–12.35)	0.197		
DGF	2.50 (0.80–7.83)	0.115		
DSA HLA class		**0.058**		0.057
I	**Reference**		Reference	
II	**5.00 (1.02–24.53)**	**0.047**	14.03 (1.56–126.12)	0.018
I + II	**4.50 (1.15–17.65)**	**0.031**	4.95 (0.81–30.42)	0.084
DSA MFI ≥ 15 000	**6.40 (1.91–21.47)**	**0.003**		
C1q+ DSA	**16.25 (3.63–72.67)**	**<0.001**	16.80 (3.18–88.85)	0.001

AMR, antibody-mediated rejection; CI, confidence interval; PRA, panel-reactive antibodies; HLA, human leucocyte antigen; MM, mismatches; ATG, antithymocyte globulin; DGF, delayed graft function; DSAs, donor-specific antibodies; MFI, mean fluorescence intensity.

*Multivariable model included only those variables presenting a univariate *P*-value < 0.1 (in bold). Significant risk factors were identified with the use of backward elimination, with a *P*-value < 0.05 needed for retention in the model.

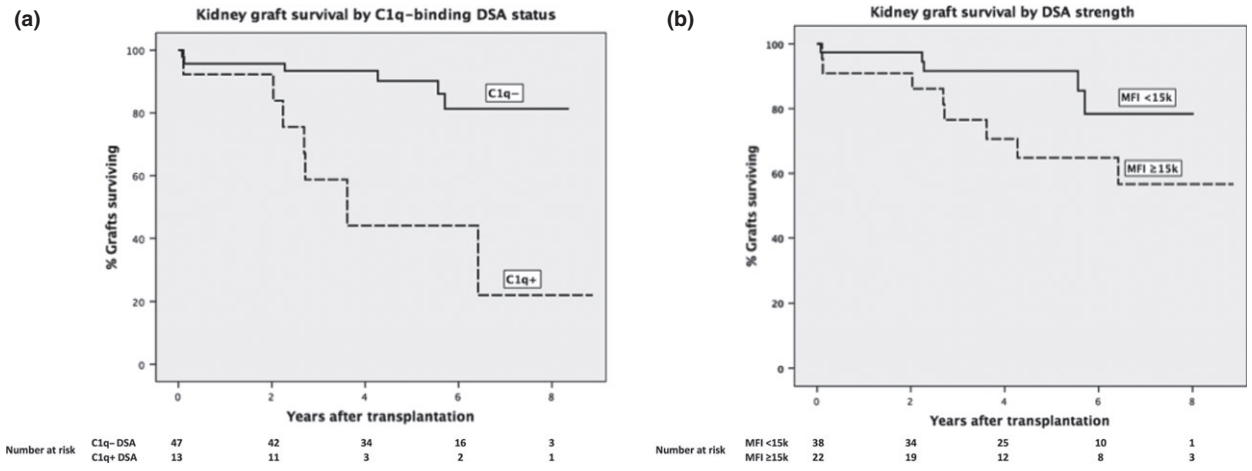

Figure 2 Death-censored kidney graft survival curves according to donor-specific antibody (DSA) characteristics. (a) Death-censored graft survival by C1q-binding DSA status [solid line, patients without C1q-binding DSA (n = 47); dashed line, patients with C1q-binding DSA (n = 13); $P < 0.001$]. (b) Death-censored graft survival by DSA strength [solid line, patients with low (<15k) MFI DSA (n = 38); dashed line, patients with high (≥15k) MFI DSA (n = 22); $P = 0.070$].

patients; $P < 0.001$). DSA strength (Fig. 2b) was non-significantly correlated with lower graft survival (at 6 years, graft survival was 78% in low MFI and 65% in high MFI DSA patients; $P = 0.070$). Patients with high MFI C1q-binding DSA (Fig. 3a) had shorter graft survival (38%) than those with high MFI non-C1q-binding DSA (83%) or with low MFI non-C1q-binding DSA (80%), at 6 years ($P = 0.001$). AMR occurrence (Fig. 3b) in the presence of C1q+ DSA was associated with poorer graft survival (42%) than in those

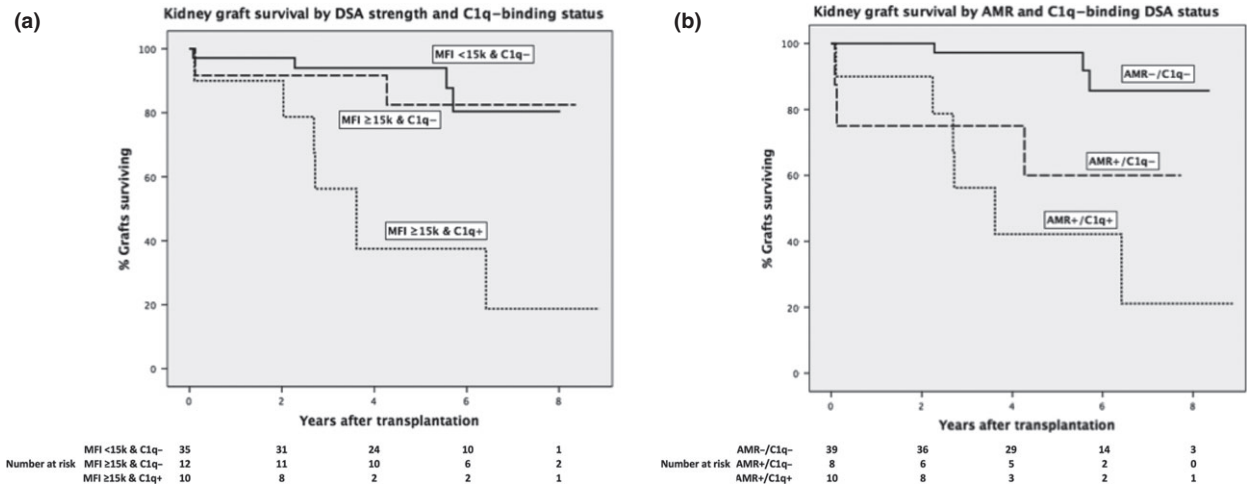

Figure 3 Death-censored kidney graft survival curves according to donor-specific antibody (DSA) strength or AMR occurrence stratified for DSA C1q-binding status. (a) Death-censored graft survival by DSA strength and C1q-binding status [solid line, patients with DSA MFI < 15k and non-C1q binding (n = 35); dashed line, patients with DSA MFI ≥ 15k and non-C1q binding (n = 12); dotted line, patients with DSA MFI ≥ 15k and C1q binding (n = 10); overall P = 0.001; pairwise comparisons: MFI < 15k and C1q− versus MFI ≥ 15k and C1q− P = 0.844, MFI < 15k and C1q− versus MFI ≥ 15k and C1q+ P < 0.001, MFI ≥ 15k and C1q− versus MFI ≥ 15k and C1q+ P = 0.011; MFI < 15k and C1q+ group was not considered in the statistical analysis given the small number involved (n = 3)]. (b) Death-censored graft survival by AMR and C1q-binding DSA status [solid line, patients with no AMR nor C1q-binding DSA (n = 39); dashed line, patients with AMR but no C1q-binding DSA (n = 8); dotted line, patients with AMR and C1q-binding DSA (n = 10); overall P < 0.001; pairwise comparisons: AMR−/C1q− versus AMR+/C1q− P = 0.027, AMR−/C1q− versus AMR+/C1q+ P < 0.001, AMR+/C1q− versus AMR+/C1q+ P = 0.359; AMR−/C1q+ group was not considered in the statistical analysis given the small number involved (n = 3)].

experiencing AMR in the absence of C1q+ DSA (60%) or those without AMR nor C1q+ DSA (86%), at 6 years (P < 0.001).

Discussion

Our study shows that improved pretransplant immunological risk stratification in patients transplanted with preformed DSA (and with a negative cytotoxic crossmatch) is feasible through the analysis of DSA characteristics. We showed that adverse events after transplant were predicted by the presence of strong DSA, particularly if C1q binding, independently from the type of immunosuppression used. Also, we noticed that a majority of patients in our study cohort had an uneventful post-transplant course.

The prevalence of AMR was high (30%) and a subsequent increase in the risk of graft failure was observed. Identical outcomes have been reported in kidney graft recipients with similar immunological risk [5,13,18,19]. Lefaucheur *et al.* [5]. demonstrated an AMR prevalence of 25% in patients with preformed DSA and negative current and remote cytotoxic crossmatch, with a relative risk for graft loss in patients who experienced AMR of 4.1 in comparison with those who did not. Another group showed an AMR incidence at 1 year of 35% in a group of ATG-induced kidney graft recipients with DSA [18]. More recently, it was reported that 15 out of 60 high-risk patients (DSA MFI sum > 6000) experienced AMR, with most receiving ATG induction and roughly half of them being additionally treated with rituximab and/or IvIg [19]. In our cohort, 65% of patients were induced with ATG and 13% underwent a desensitization protocol.

Importantly, 42 (70%) patients did not experience AMR and those remaining with graft functioning at the end of follow-up (n = 43) had a median graft function of 52 ml/min and low prevalence of proteinuria (21%). Many of these patients (72%) belonged to the low strength DSA group and most of them (91%) had no C1q+ DSA before transplant. DSA+ kidney graft recipients (if DSA MFI <5000 for HLA-A/HLA-B/HLA-DR and <10 000 for HLA-DQ in the highest ranked bead) with a low positive flow cytometric crossmatch and receiving ATG plus IvIg induction were shown to have similar patient and graft survival, and acute rejection rates in comparison with DSA- patients [20]. Nevertheless, HLA-incompatible kidney transplantation has been associated with a high prevalence of chronic active AMR [21,22]. A Japanese group showed, in 26 patients, transplanted in the presence of DSA and with a negative cytotoxic crossmatch, that underwent desensitization

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with rituximab/splenectomy and double filtration plasmapheresis, a very low frequency of AMR ($n = 2$) but a prevalence of chronic active AMR of 35% at 1 year [23].

Most cases of AMR occurred in patients with high MFI DSA (67%) and/or with C1q+ DSA (56%). In these groups, patients presented high MFI values (median values around 25 000), uncommonly seen in negative cytotoxic crossmatch kidney transplantation [5, 14, 19, 20, 23–25]. Nevertheless, we emphasize that these values correspond to the cumulative MFI of all detected DSA beads in each patient, while many similar studies[5,12,14,20] report the immunodominant DSA MFI (highest ranked bead) that is comparatively and expectedly lower. Also, differently from most referred studies, SAB assay was always performed in our study after treatment with EDTA in order to circumvent the prozone effect, which preferably affects samples containing high levels of HLA-reactive IgG and of antibody-triggered complement split product deposition, leading to false-low or false-negative IgG-MFI levels [26–28]. Gloor *et al.* [25]. reported cumulative MFI values of up to 65 000 and 50 000 in patients transplanted with a positive cytotoxic (anti-human globulin-enhanced) crossmatch or in patients with a high (>300 MCS) positive flow cytometric crossmatch (and a negative cytotoxic crossmatch), respectively. Unfortunately, the issue of which approach should be used when transplanting these patients with strong DSA (or if even they should transplanted) cannot be addressed by our data. Still, we show that ATG induction or the use of desensitization strategies had no impact on AMR occurrence.

We report a low frequency of C1q-binding DSA, present in 13 (22%) patients and corresponding to 12% of all detected DSA. Prevalence of C1q-binding DSA in patients transplanted in the presence of DSA has been reported between 14% and 64% [7,13,14]. Importantly, many of them belonged to strong DSA group (median 24 300, IQR 14 800–28 400), as expected given the known relationship between DSA MFI and C1q-binding ability [29,30]. Therefore, we tried to determine whether DSA C1q-binding ability could improve our pretransplant immunological risk assessment beyond its strength. First, in a comparative analysis of the predictive performance of each DSA characteristic, C1q-binding ability was shown to be a better predictor of AMR than strength. Then, in the multivariable logistic regression, only C1q-binding ability was a significant risk factor for AMR. Moreover, a significantly lower graft function and higher prevalence of proteinuria in C1q+ versus C1q− patients at 1 year post-transplant were observed, while no differences were found between DSA strength groups. Finally, regarding censored graft survival, a poorer survival in patients with high MFI C1q-binding DSA versus those with high MFI non-C1q-binding DSA was observed (log-rank $P = 0.011$). Hence, we believe that our results demonstrate that DSA C1q-binding ability adds valuable information in the pretransplant setting. These are important findings since the published data had shown no impact of preformed C1q-binding DSA on AMR occurrence [7,13]. Similarly, Loupy *et al.* [14] reported that preformed C1q-binding DSA was associated with graft loss, although only the persistence (from pretransplant) or *de novo* presence of C1q-binding DSA at 1 year was an independent predictor of graft loss.

This study has also limitations. First, the lack of available information about flow cytometric crossmatch results may affect its clinical application, particularly in highly sensitized patients [31]. Second, no protocol biopsies were performed in our cohort, an important tool in the management of HLA-incompatible kidney transplantation [32]. Third, the known limitations of SAB assay and their reported MFI values should be considered while interpreting our results [33]. Fourth, induction treatment and desensitization strategies were not homogeneous between the groups. Nevertheless, they showed no impact on the analyses of post-transplant outcomes. Lastly, possible DSAs against HLA-DP were not detected, since donor typing in this *locus* was not available.

In summary, our study demonstrates that the detection of DSA C1q-binding ability may improve pretransplant risk assessment beyond its strength. An uneventful post-transplant course was common in the low strength and/or non-C1q-binding DSA groups, in whom few patients were desensitized. Finally, preformed C1q-binding DSA in comparison with DSA strength was shown to be a better predictor of AMR and more strongly associated with graft failure. These observations are important, particularly for those centres that perform HLA-incompatible kidney transplantation in order to improve the definition of unacceptable antigens and to help in the decisions about patient transplantability or desensitization strategies.

Authorship

JM: involved in study concept and design, acquisition of data and patient recruitment, statistical analysis, analysis and interpretation of data and manuscript drafting. ST: involved in technical support and laboratory

analysis and analysis and interpretation of data. LD: involved in acquisition of data and patient recruitment, analysis and interpretation of data and critical revision of the manuscript for important intellectual content. LSM: involved in acquisition of data and patient recruitment and critical revision of the manuscript for important intellectual content. IF: involved in statistical analysis and analysis and interpretation of data. IB: involved in critical revision of the manuscript for important intellectual content. AC-H: involved in acquisition of data and patient recruitment, critical revision of the manuscript for important intellectual content and study supervision. AC: involved in study supervision.

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Conflicts of interest

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Comparison of baseline characteristics and post-transplant outcomes in patients with positive screening for anti-HLA antibodies before transplant (n = 124), according with the detection of DSA at the present study.

Table S2. Comparison of baseline characteristics and post-transplant outcomes in patients with strong (MFI ≥ 15k) DSA (n = 22), according with the detection of C1q-binding DSA.

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