ORIGINAL ARTICLE

Failure to remove *de novo* donor-specific HLA antibodies is influenced by antibody properties and identifies kidney recipients with late antibody-mediated rejection destined to graft loss – a retrospective study

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SUMMARY

Current research is focusing on identifying bioclinical parameters for risk stratification of renal allograft loss, largely due to antibody-mediated rejection (AMR). We retrospectively investigated graft outcome predictors in 24 unsensitized pediatric kidney recipients developing HLA *de novo* donor-specific antibodies (*dn*DSAs), and treated for late AMR with plasmapheresis + low-dose IVIG + Rituximab or high-dose IVIG + Rituximab. Renal function and DSA properties were assessed before and longitudinally post treatment. The estimated GFR (eGFR) decline after treatment was dependent on a negative % eGFR variation in the year preceding treatment ($P = 0.021$) but not on eGFR at treatment ($P = 0.74$). At a median follow-up of 36 months from AMR diagnosis, 10 patients lost their graft. Altered eGFR ($P < 0.001$) and presence of C3d-binding DSAs ($P = 0.005$) at treatment, and failure to remove DSAs ($P = 0.01$) were negatively associated with graft survival in the univariable analysis. Given the relevance of DSA removal for therapeutic success, we analyzed antibody properties dictating resistance to anti-humoral treatment. In the multivariable analysis, C3d-binding ability ($P < 0.05$), but not C1q-binding, and high mean fluorescence intensity ($P < 0.05$) were independent factors characterizing DSAs scarcely susceptible to removal. The poor prognosis of late AMR is related to deterioration of graft function prior to treatment and failure to remove C3d binding and/or high-MFI DSAs.

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Key words
antibody-mediated rejection, anti-humoral therapy, complement-binding DSA, *de novo* donor-specific anti-HLA antibodies, pediatric kidney transplantation

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Introduction

A rapidly expanding body of studies has indicated that the humoral alloimmune response, mediated by donor-specific HLA antibodies (DSAs), is a detrimental factor initiating antibody-mediated rejection (AMR) and reducing long-term kidney allograft survival [1–3]. Sensitized recipients with preformed HLA antibodies are historically known as a cohort at higher risk of AMR [4,5]. In the last years, evidence has indicated that also a proportion of the low-risk, previously nonsensitized, kidney recipients will develop post-transplant *de novo* DSAs (*dn*DSAs), possibly leading to late AMR and graft loss [1,2,6–9].

The prognosis of untreated late AMR is unfavorable [10,11]. The therapeutic approach to AMR includes different strategies aimed at removing existing antibodies and inhibiting their redevelopment, such as plasmapheresis, intravenous immunoglobulins (IVIG), the anti-B cell monoclonal antibody (MAb) rituximab, and the plasmacell-targeting agent bortezomib, or inhibiting tissue damage mediators, such as the anti-C5 MAb eculizumab and anti-C1s MAb BIVV009 [12,13]. These modalities have shown some degree of success in the treatment of early acute AMR [14]. However, late AMR has proven a more difficult condition to treat [12], likely due to the inflammatory lesions occurring in a milieu of chronic tissue damage.

Current evidence in the setting of late AMR is based on few, generally noncontrolled studies, that report stabilization/amelioration of graft function in a proportion of patients [11,15–19]. However, due to the heterogeneity and/or exiguity of cohorts, use of historical controls, different treatment protocols and limited follow-up, the results are inconclusive, and poorly informative on response predictors. Several studies have demonstrated a correlation between graft outcome and biological characteristics of DSAs, such as Ig isotype [20], HLA class antibody specificity [21,22], mean fluorescence intensity (MFI) [8,23,24], complement-binding ability [25–27] and graft homing [28,29]. Analysis of these antibody biological properties could also provide meaningful insight into the response to anti-humoral treatment in late AMR. In particular, pre- and post-treatment variations of DSA properties, such as antibody strength or ability to fix complement, are the parameters that have been found associated with outcome in kidney recipients treated for AMR [30–32]. These studies included large proportions of patients with early acute AMR, thus, the role of these predictors in late AMR is unclear and needs to be further assessed.

In a cohort of nonsensitized, first transplant recipients receiving Ab removal/down-modulation treatment for late AMR, we analyzed predictors of graft outcome, including *dn*DSA removal. In addition, we analyzed susceptibility to removal of *dn*DSAs, to define DSA biologic characteristics useful for risk stratification.

Patients and methods

Patients and transplant procedure

This retrospective study was conducted on the 24 consecutive patients, among 128 nonsensitized recipients undergoing a complement dependent cytotoxicity-negative first kidney graft at the Genova Transplant Center between 07/2002 and 12/2014, who were positive for *dn*DSA post transplant and diagnosed with late AMR (defined as an active or chronic AMR due to *de novo* DSAs, occurring beyond the first post-transplant year).

Graft biopsies were performed for clinical indication, including the presence of functional deterioration, proteinuria, and, since 2010, serum *dn*DSA development (DSA MFI > 1000). Biopsies were histologically graded following Banff criteria [33]. C4d staining was performed on frozen sections by indirect immunofluorescence.

Pretransplant sera were analyzed for the presence of HLA-Ab by solid phase assays using a Luminex platform [7,27]. Patients received induction with basiliximab, and a triple drug immunosuppressive regimen including a calcineurine inhibitor, mycophenolate mofetil and prednisone. Graft function was estimated by calculating GFR (eGFR) using the Schwartz or MDRD formula, as appropriate [34,35].

All recipients were longitudinally followed up for the presence of post-transplant HLA *dn*DSAs by Luminex technology [27]. Sera were collected at 1, 3, 6, 12 months in the first post-transplant year, and annually thereafter. Samples obtained until 01/2010 belonged to a unique source of sera analyzed retrospectively for HLA-Ab, while from 02/2010 all samples were collected and analyzed prospectively. Among the 128 nonsensitized recipients, six patients had transient *dn*DSAs while 44 patients were consistently found positive for *dn*DSAs. Among the latter, 24 were diagnosed with late AMR.

The study was approved by the Institutional Review Board of Fondazione Ca' Granda, Ospedale Maggiore, Milano (n.867/2014).

HLA typing

Genomic DNA was extracted from whole blood samples using an automated DNA extractor. Recipient low-resolution HLA-A*, HLA-B*, HLA-DRB1*, HLA-DQB1*

typing was performed with a microarray bead-based technique (Lambda Array Beads Multi-Analyte System LABMAS, One Lambda, Canoga Park, CA, USA). HLA typing of donors was performed by both serology (Immucor GTI Diagnostics, Waukesha, WI, USA; Canoga Park, CA, USA) and polymerase chain reaction – sequence-specific primers (PCR-SSP) (ABDR SSP Combi Tray, Olerup SSP, Saltsjöbaden, Sweden). When recipient sera were found to display *de novo* antibodies directed against histocompatibility antigens, such as HLA class I C and/or DQA1*, or DP alleles, both recipient and donor were retrospectively typed for the relevant loci at high resolution with sequence-based typing (SBT) [22].

Characterization of HLA antibodies

Anti-HLA class I and class II IgG Ab were tested by LABScreen Mixed kit and single-antigen bead (SAB) assays (One Lambda, CA, USA) (21). Single-antigen results >1000 MFI cut-off value were considered positive. Sera were pretreated by disodium-EDTA (Sigma-Aldrich, Milan, Italy) [27].

Heat-inactivated sera were tested with C1qScreen™ (One Lambda) for identification of complement-binding Ab [25]. Antibody positivity was assigned at >500 MFI.

Serum samples were analyzed for the presence of C3d-binding DSAs with the single-antigen flow bead technology (Immucor Lifecode Transplant Diagnostics, Nijlen, Belgium) [26,27].

Anti-humoral therapy

Late AMR positive patients were treated with an anti-humoral protocol consisting in a combination of high IVIG (2 g/kg b.w.) in six patients, or, since 2010, plasmapheresis [four procedures + 100 mg/kg body weight (b.w.) IVIG after each procedure] in 18 patients, followed in all 24 patients by anti-CD20 MAb (Rituximab 375 mg/m^2 body surface).

Patients with graft dysfunction (clinical AMR) received two treatment courses within 3 months, while those with subclinical AMR received one treatment course. Patients with concomitant features of cell-mediated tubulo-interstial inflammation were administered additional steroid pulses. Standard immunosuppression was modified to include Tacrolimus in all *dn*DSA-positive patients, aiming at a trough level range between 5 and 7 ng/ml. Anti-humoral treatment was administered within 1–4 weeks from biopsy. Complement-binding capability was tested at least once before and after patient treatment.

Twelve kidney transplant (KTx) recipients with superimposable transplant characteristics and treated for post-transplant *dn*DSAs, in the absence of histological and clinical signs of AMR (preemptive cohort, Table S1) were also considered for DSA removal analysis. In this preemptive group anti-humoral treatment did not include anti-CD20, but only PEX + low-dose IVIG (four patients) or high IVIG (four patients) or both (four patients).

Criteria of response to anti-humoral treatment

*dn*DSA removal was defined according to functional criteria, based on complement-binding capability. In detail, for complement-binding positive DSAs: from C3d+/C1q+ to C3d−/C1q+ or C3d−/C1q−; from C3d−/C1q+ to C3d−/C1q−; for complement-binding negative DSAs, reduction in MFI < 1000. Necessarily, when DSAs were present that did not bind complement, response to treatment was considered as MFI reduction below positivity threshold.

In addition, criteria based on change in Ab strength, represented by MFI categories [36], were also tested. In detail, we analyzed (i) strength category shift: MFI >10 000 to MFI from 5000 to 10 000; from MFI comprised between 5000 and 10 000 to MFI < 5000; from MFI comprised between 5000 and 1000 to negative; (ii) MFI relative change value, calculated according to a previously described formula (MFI at 1 year from treatment initiation — MFI at treatment/MFI at treatment), and reported as a continuous variable [32].

Statistical analysis

Relative eGFR change 1 year pre- and post-treatment was compared with a paired Student's *t*-test. We fitted a linear random effect model to assess eGFR changes over time. We tested whether the changes over time depended either on the pretreatment relative variation and/or the presence of eGFR <50 ml/min/1.73 m^2 pretreatment, by including the interaction with time of these two variables in the model. We estimated event-free survival with the Kaplan–Meier method and compared it between risk groups with the log rank test. For graft failure, censoring event was death with functioning graft. Patients who did not experience graft failure were censored at the end of follow-up. We estimated hazard ratios and 95% confidence intervals with a Cox model. To determine differences among groups, we compared categorical variables by Fisher's exact test. We estimated

odds ratios and 95% confidence intervals with logistic models. Logistic regression was employed for the multivariable analysis. Two-sided P-values < 0.05 were considered statistically significant. Stata14 (Stata Corporation, College Station, TX, USA) was used for computation.

Results

Histological and immunologic features of the AMR cohort

Among the 24 patients diagnosed with late AMR, 13 had graft dysfunction (eGFR loss ≥20%) in the 12 months preceding AMR diagnosis, and 11 were found with stable renal function (subclinical late AMR) (Table 1). Late AMR was observed at a median of 4.7 (range 1.5–11.7) years after transplantation. Twenty-one patients had positive staining for C4d in peritubular capillaries. Transplant glomerulopathy was present in 11 subjects. Some patients presented with concomitant features of cell-mediated tubulo-interstial inflammation, however, none fulfilled the Banff criteria for a diagnosis of acute T-cell mediated rejection ≥ type IA. dnDSAs were exclusively directed to HLA class II in 11 patients, and to HLA class I in three patients, while 10 recipients had antibodies to both HLA classes. A total of 53 dnDSAs were observed in the 24 patients (mean: 2.2/patient). Considering HLA class II Abs, anti-DR were found in five cases, anti-DQ in 21, and anti-DP in one case. HLA class I Abs were directed to HLA-A in 14 cases, HLA-B in 9, and HLA-C in three cases. MFI peak values (median and range) at AMR diagnosis were 12 792 (1163–25 100) and 2650 (1000–24 079) for class II and class I, respectively.

All but four of the 24 immunodominant DSAs were C1q-positive at the time of AMR diagnosis, while C3d-binding was observed in 16 of the 20 C1q-positive immunodominant DSAs. No DSAs were C3d-positive/C1q-negative.

At onset, 14 DSAs were C1q-positive, and seven were C3d-binding DSAs, with a total median MFI of 7113 (vs. 12 453 at treatment initiation). In the time span between the first detection and treatment initiation, six more patients showed immunodominant DSA C1q-binding capability acquisition, and nine developed C3d-positivity (Table 2). At both DSA onset and anti-humoral treatment start, DSA features were comparable in clinical and subclinical AMR (Table 2). Indeed, considering patients with subclinical AMR, it is relevant to note how, in the time span between the first DSA detection and treatment initiation, two of the five patients (40%) that had complement-negative DSAs at first detection developed C1q-binding ability, and two of the seven patients (29%) that had C3d-negative DSAs at first detection developed C3d-binding ability.

Table 1. Demographics of study population.

	AMR patients $n = 24$
Characteristics at Tx	
Recipient	
Male sex	15
Age (median, IQ range)	14.0 (6.5–17.0)
Donor	
Male sex	16
Age (median, IQ range)	12.0 (5.0–17.0)
Deceased	22
Total HLA A,B,DR,DQ mismatches (mean n ±SD)	4.39 (±1.03)
HLA-DR,DQ mismatches (mean n ±SD)	2.00 (±0.88)
HLA-DQ mismatches (mean n ±SD)	1.00 (±0.66)
Immunosuppressive therapy	
CyA/Tac	20/4
Delayed graft function	2
T-cell mediated rejection ≥1A	3
Histological BANFF scores of baseline biopsy (mean ± SD)	
i + t	1.21 (±0.78)
ptc + g	2.04 (±1.12)
cg	0.58 (±0.72)
ci + ct	2.29 (±0.99)
C4d	2.62 (±0.87)

Tx: transplant; i: interstitial inflammation; t: tubulitis; ptc: peritubular capillaritis; g: glomerulitis; c: chronic.

Graft function and AMR treatment outcome

Median observation time from AMR diagnosis was 36 months (range 12–91). eGFR values of each patient from 1 year prior to AMR diagnosis throughout the follow-up are reported in Fig. 1. Mean deterioration of graft function in the 12 months preceding therapeutic intervention for the whole cohort was −16(±22)%. In detail, eGFR declined from 84 ml/min/1.73 m^2 at 12 months prior to anti-humoral therapy to 69 ml/min/1.73 m^2 at treatment baseline. At the time of AMR treatment initiation, five of the 24 patients had an eGFR <50 ml/min/1.73 m^2. During the first year post-AMR therapy, a further decrease in mean eGFR, with % graft function deterioration going from −15(±21) to −21 (±24), was observed, that progressed in the following

Table 2. Immunodominant DSA characteristics in the treated patients.

	AMR		Clinical AMR		Subclinical AMR		
	n abs	%	*n* abs	%	*n* abs	%	*P* value
Total Antibodies	24		13		11		
HLA Class I	6	25	4	31	2	18	0.65
HLA Class II	18	75	9	69	9	82	0.65
HLADQ	15	63	7	54	8	73	0.42
C1q+ at onset	14	58	8	61	6	54	1.00
C3d+ at onset	7	29	3	23	4	36	0.66
Mean fluorescence intensity (MFI) at onset	7113		6902		7323		0.52
C1q+ at treatment	20	83	12	92	8	73	0.30
C3d+ at treatment	16	67	10	77	6	54	0.39
MFI at treatment	12 453		15 525		11 600		0.82

years, as graft function worsened [mean δ-eGFR at 24 and 36 months: −15(±33)% and −19(±36)%, respectively] ($P < 0.01$). The eGFR decline over time was dependent on a negative % eGFR variation in the 12 months preceding treatment ($P = 0.021$), but not on the eGFR at treatment baseline ($P = 0.74$).

We evaluated the impact of removal/down-modulation therapy on renal function. In the absence of a control group, we analyzed the correlation between the eGFR slope in the 12 months preceding treatment and that of the first 12 months post treatment, to detect any slope change induced by therapy. A difference in slope of 6.3 (−1.1 to 13.7; $P = 0.09$) was observed between pre- and post-treatment eGFR, independent of eGFR at baseline, with greater post-treatment decrease, suggesting an overall absence of an effect mediated by anti-humoral therapy at 1-year follow-up.

Ten of the 24 patients (42%) lost the graft due to AMR at a median time of 25 months (12–91) from baseline. All the remaining patients show an eGFR ≥50 ml/min/1.73 m^2 at follow-up (Fig. 1). The 10 graft losses were observed in the clinical AMR group. Interestingly, two of the three patients in the clinical AMR group who stabilized their renal function showed a stabilization/improvement in the humoral histology scores (Table S2). Considering the subclinical AMR cohort, we observed amelioration/stabilization of humoral histology scores in three of the five patients who underwent a post-treatment control biopsy (Table S2).

Following anti-humoral treatment, 12 of 24 patients showed MFI strength decrease according to the categories described by Vo *et al.* [36] at 1-year follow-up. A response in terms of *dn*DSA removal according to functional criteria based on complement-binding

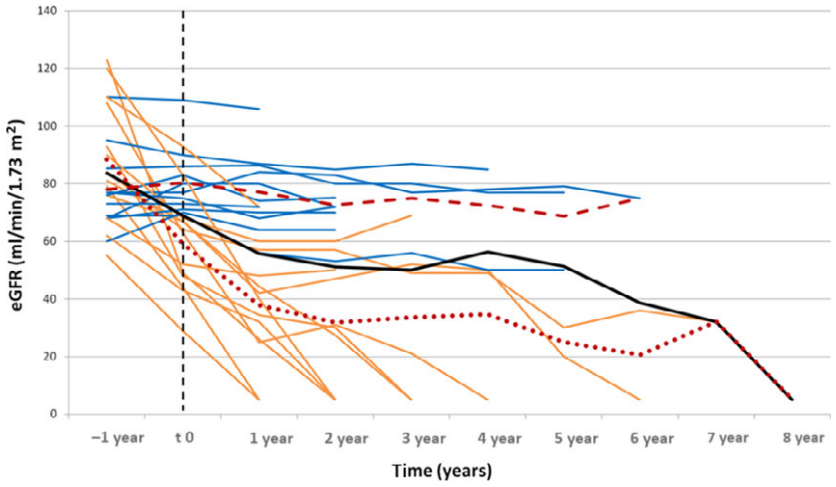

Figure 1 Renal function in 24 pediatric renal transplant recipients with chronic late AMR 1 year before and during the follow-up after anti-humoral therapy. Individual eGFR values (yellow lines: clinical AMR; blue lines: subclinical AMR) before and after initiation (t0) of anti-humoral therapy. Mean eGFR values for the whole serie (black line) and for the two subgroups (clinical AMR: dotted red line; subclinical AMR: dashed red line) are also reported. Graft function was estimated by calculating GFR (eGFR) using the Schwartz or MDRD formula, as appropriate.

capability, was observed in eight of 24 patients (33%). The rate of removal was comparable when considering the clinical and subclinical cohorts (31 vs. 36%). Actual reversal of immunodominant DSA complement-binding ability was detected in five patients (one from C3d+C1q+ to complement negative, one from C3d+C1q+ to C3d−C1q+, and three from C3d−C1q+ to complement negative), while three cases were complement-negative DSAs with MFI lowering below the 1000 threshold. DSA removal remained stable in six of the eight patients at a median follow-up of 24 months (range 17–42). Of the remaining two patients, one showed a rebound after initial DSA clearance responsive to subsequent standard anti-humoral treatment, and remains with good renal function at +80 months, while the other regained DSA C3d positivity and progressed to graft loss despite further standard treatment. Nonresponders received repeated standard treatment cycles that produced no benefit in terms of DSA removal.

We evaluated the role of clinical, histological, and immunological factors at the time of AMR diagnosis, and of anti-humoral treatment, on graft loss (Table 3). In the univariable analysis, % eGFR decline in the 12 months preceding treatment, eGFR < 50 ml/min/1.73 m^2 at diagnosis and C3d-binding ability of DSAs were found negatively associated with outcome, while immunodominant DSA removal, defined by functional criteria but not by MFI variation, and the use of plasmapheresis positively correlated with graft survival (Table 3).

DSA characteristics associated to removal in DSA-positive patients undergoing anti-humoral treatment

We then proceeded to analyze DSA biological properties associated to removal susceptibility in our AMR and preemptive cohorts (Table 4). For this purpose, all *dn*DSAs detected in the AMR and preemptive cohorts were analyzed. All DSA removal evaluations were performed employing exclusively the complement-based functional criteria.

In the univariable analysis, the HLA antigen specificity associated to poor Ab removal was class II DQ. In addition, complement-binding capability characterized DSAs endowed with resistance to removal/down-modulation treatment. In line with the complement findings, DSAs with higher Ab strength, that we previously demonstrated to be associated with complement-binding property [27] were poorly removed (Table 4A).

To analyze the hierarchy associated to poor antibody removal, a multivariable analysis was carried out. As all parameters evaluated in the univariable analysis resulted significantly associated to removal, but could not be all inserted in the multivariable assessment due to the small sample size, we favored a model that allowed to better dissect a possible independent role of HLA DQ specificity in DSA removal susceptibility.

We found that both MFI >10 000 and C3d-binding ability, but not HLA DQ DSA specificity, were independently associated with resistance to removal (Table 4B). Of note, when dissecting the respective role of C1q and C3d binding properties, we observed that C1q-positive DSAs that resulted negative for C3d binding were almost as easily removed as complement negative (Fig. 2).

Discussion

We analyzed the effects of anti-humoral treatment in a cohort of unsensitized, pediatric first kidney graft recipients experiencing late AMR after development of post-transplant *dn*HLA-DSAs. The main findings of the study were (i) DSA removal, measured as down-modulation of complement-binding capability, positively correlated with graft survival in kidney recipients with late AMR; (ii) DSAs endowed with C3d-binding ability, but not those binding only C1q, and DSAs with MFI > 10 000, were associated with poor susceptibility to removal.

To date, the benefits of treating late AMR are uncertain [11,15–19,37]. In our late AMR cohort, treatment including IVIG and rituximab, associated or not with plasmapheresis, was mostly unsuccessful in influencing graft function deterioration in patients with clinical AMR, as 10 of 13 patients lost their graft. This outcome is in line with published series [17,19,38,39], although median eGFR in our cohort was higher than in most published studies, and tacrolimus target trough levels were generally attained in the post anti-humoral treatment phase. Accordingly, graft loss in our cohort was not only associated with eGFR at treatment, but also independently correlated with renal function deterioration in the year preceding diagnosis. Indeed, patients treated with subclinical AMR, an entity previously described as associated with suboptimal graft outcome [40], maintained good renal function throughout the median 3.6 year follow-up. In the absence of an untreated control group, we cannot ascribe this clinical outcome to anti-humoral therapy; however, it is noteworthy that none of the patients in the subclinical AMR group showed significant reduction in graft function, and in follow-up biopsies a stabilization/amelioration of humoral histological scores was observed in some patients.

Table 3. Risk of developing graft loss as a function of individual clinical parameters at the time of AMR diagnosis or of response to treatment.

Variables	Patients (*n*)	HR*	95% CI	*P* value
Clinical factors				
eGFR at AMR diagnosis				
≥50 ml/min/1.73 m^2	19			
<50 ml/min/1.73 m^2	5	30.11	3.40–266.61	**<0.001**
eGFR slope -12 m to diagnosis†		0.92	0.88–0.97	**<0.001**
Histological factors				
i + t ≥ 2				
No	17			
Yes	7	2.61	0.65–10.54	0.18
MI (g + ptc ≥ 2)				
No	8			
Yes	16	1.24	0.31–5.01	0.76
Interstitial fibrosis and tubular atrophy score ≥3				
No	19			
Yes	5	2.28	0.60 – 8.70	0.24
TG				
No	13			
Yes	11	1.52	0.40–5.82	0.54
Immunologic factors				
*dn*DSA class I + II‡				
No	14			
Yes	10	0.70	0.18–2.67	0.59
C3d-binding *dn*DSA				
No	8			
Yes	16	NE	NE	**0.005**
*dn*DSA mean fluorescence intensity (MFI) peak preAMR		1.00	0.99–1.01	0.27
*dn*DSA MFI (RIS score§ peak pre anti-humoral treatment)		1.06	0.99–1.13	0.10
Therapy-related factors				
Pex in treatment regimen				
No	6			
Yes	18	0.11	0.02–0.46	**0.003**
*dn*DSA removal (MFI-based [36])				
No	12			
Yes	12	0.32	0.07–1.53	0.12
*dn*DSA removal (MFI-relative change [32])		3.98	0.53–29.60	0.16
*dn*DSA removal (functional criteria)				
No	16			
Yes	8	NE	NE	**0.01**

NE, not evaluable.

*Hazard ratio (HR) and 95% confidence interval, quantified by Cox proportional-hazard models were computed.

†Time-dependent variable.

‡Patients with both class I and II *dn*DSAs were compared with patients showing single class DSA.

§The DSA-RIS was calculated by giving 0 points for no DSA, two points for each weak DSA (MFI 2500-5000), five points for each moderate DSA (MFI 5000-10 000), and 10 points for each strong DSA (MFI >10 000), according to Ref. 36).

Statistically significant values are marked in bold.

In addition to kidney function deterioration, poor DSA control was a factor associated with reduced graft survival in our late AMR cohort, an observation so far demonstrated in the early acute AMR setting [30]. Indeed, the rate of immunodominant DSA removal in the patients who lost the graft was 10% compared to 50% in the patients who did not progress, and the three patients with clinical late AMR whose graft function stabilized all showed imunodominant *dn*DSA clearance. The significant correlation of DSA removal with graft outcome was only

Table 4. *dn*DSAs characteristics associated to susceptibility to removal. (A) Univariable analysis. (B) multivariable analysis.

(A) DSA properties	Not removed	Removed	Total *n*	*P* value	Odds ratio	95% confidence interval
HLA Class II	21	14	35	0.003	4.9	1.7–13.8
HLA Class I	8	26	34			
HLA DQ+	17	8	25	0.002	5.7	1.9–16.5
HLA DQ−*	12	32	44			
C1q+	23	21	44	0.025	3.5	1.2–10.3
C1q−	6	19	25			
C3d+	17	2	19	<0.001	26.9	5.4–133.7
C3d−	12	38	50			
Mean fluorescence intensity (MFI) >10 000	19	4	23	<0.001	17.1	4.7–61.9
MFI ≤ 10 000	10	36	46			

(B) DSA properties	P value	Odds ratio	95% confidence interval
HLA DQ+	0.413	1.8	0.4–7.3
HLA DQ−*			
C1q+	0.710	0.8	0.2–3.1
C1q−			
C3d+	<0.05	10.1	1.5–68.3
C3d−			
Mean fluorescence intensity (MFI) >10 000	<0.05	5.7	1.2–27.1
MFI ≤ 10 000			

The analysis includes all DSAs (n = 69) from the 24 patients with AMR (n = 53) and those (n = 16) from 12 kidney transplant recipients treated with the same anti-humoral protocol for DSA positivity in the absence of AMR (preemptive treatment cohort).

*All HLA DSA specificities other than HLA DQ.

observed for removal measured on a functional level. Although we cannot rule out a sample size bias, this observation may indicate that a functional approach could be a more reliable tool to predict response to anti-humoral therapy than the mere MFI value reduction [31].

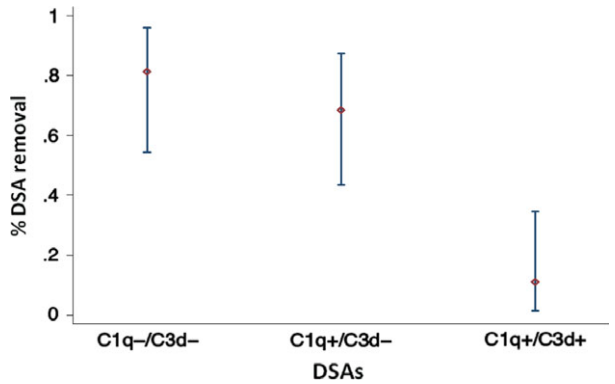

Figure 2 Susceptibility to removal according to DSA ability to bind complement. % DSA removal according to DSA ability to bind complement fractions (no complement binding: C1q−/C3d−; C1q only: C1q+/C3d−; C3d and C1q: C1q+/C3d+) was analyzed by a nonparametric test for trends (P < 0.0001). Results are reported as mean and 95% confidence interval.

Indeed, in some of our patients, low-MFI DSAs could bind C3d and induce graft damage and loss. The concept of the need for accurate predictors of antibody removal has been also underscored by the recent demonstration that SAB titration analysis provides a better estimation of responsiveness to treatment than % delta change in MFI levels for IgG and C1q tests [41]. For treatment efficacy monitoring, DSA complement-binding assessment could represent an economically feasible option in comparison with the more expensive titration approach.

When considering the effect of different treatment modalities on AMR outcome, we found that plasmapheresis was significantly associated with better graft survival. This observation is likely related to the removal effect obtained with this procedure [42–44]; however, its immunomodulating effect on DSA production may also have played a role [45]. Beside graft function and DSA removal, the only other factor correlated with outcome in our AMR cohort was DSA positivity for C3d binding, a property that has recently emerged as a strong predictor of kidney graft loss [26,27,46].

Our AMR cohort was characterized by a contained degree of chronicity, as IFTA score >2 and transplant

glomerulopathy were present in only 21% and 46% of the patients, respectively, likely due to the use of kidneys from young donors and timely execution of graft biopsy. These features, in addition to the relatively small cohort size, could account for the absence of correlation between chronicity histology scores and graft outcome.

Considering the relevance of DSA removal in conditioning graft outcome, we analyzed DSA characteristics dictating susceptibility to anti-humoral treatment. As reported in the literature [16], we observed that DSAs with high MFI were more resistant to removal. However, the antibody feature more likely to condition poor DSA removal in our cohort was complement-binding activity. In particular, the multivariable analysis evidenced that C3d-positive, but not C1q-positive, antibodies were resistant to removal. This observation may account for the discrepancies reported in the literature on the role of complement-binding DSAs on kidney graft outcome [25–27,47,48], as only a portion of C1q-positive DSAs are also endowed with C3d binding ability [27]. Our findings provide further evidence to support the notion that C3d-fixing *dn*DSAs are better fit to stratify risk of graft loss after kidney transplantation.

From our data, patients with C3d-positive and high-MFI DSAs that do not respond to standard anti-humoral therapy deserve additional treatments within experimental clinical trials. In the case of responders maintaining detectable DSAs, follow-up data need to be extended to assess the opportunity of additional standard therapy aimed at obtaining DSA clearance.

Since the small sample size of this study, conducted in a pediatric cohort, and differential exposure of patients to treatments limit the strength of our conclusions, larger cohort studies are needed to address the important question of preeminent predictors of response to anti-humoral therapy.

While it is common practice to intervene in case of late AMR, treatment of patients with confirmed positive DSAs in the absence of histological damage is still object of debate. To avoid unnecessary treatment, a risk stratification based on DSA biological characteristics is being postulated, and a number of studies are focusing on identification of a suitably performant profile [49]. However, emerging observations indicate a progressive acquisition of DSA damaging properties, such as complement-binding activity and MFI strength [27]; as a consequence, a longitudinal change in risk profile can be hypothesized. In this light, an early stratification may not adequately identify the high risk patients. Thus, early or preemptive [50] intervention strategies could be the appropriate choice to contrast graft insult, once transient DSAs have been ruled out. This approach will have to be tested in prospective, controlled studies, auspicably stratified for DSA biological properties.

Authorship

MCi, AN, MCa, FG, PC: conceived and designed the study, analyzed results, and wrote the manuscript. ATa, SBa, AI, AG, MR, LC: processed samples and executed immunological analysis. IF, AM, ATr, SBo, EV: enrolled patients, provided clinical care, collected patient data and commented on the manuscript. CK: performed statistical analysis. GG, GMG: supervised the study and critically revised the manuscript.

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Conflict of interest

The authors have declared no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Characteristics of the 12 patients treated preemptively for *dn*DSA positivity.

Table S2. Histological findings in the three patients with clinical late AMR that stabilized renal function after anti-humoral treatments, and in the five subclinical AMR patients who underwent a follow-up biopsy after treatment.

REFERENCES

1. Lee PC, Zhu L, Terasaki PI, Everly MJ. HLA-specific antibodies developed in the first year posttransplant are predictive of chronic rejection and renal graft loss. *Transplantation* 2009; **88**: 568.
2. Hidalgo LG, Campbell PM, Sis B, *et al.* *De novo* donor-specific antibody at the time of kidney transplant biopsy associates with microvascular pathology and late graft failure. *Am J Transplant* 2009; **9**: 2532.
3. Loupy A, Hill GS, Jordan SC. The impact of donor-specific anti-HLA antibodies on late kidney allograft failure. *Nat Rev Nephrol* 2012; **8**: 348.
4. Burns JM, Cornell LD, Perry DK, *et al.* Alloantibody levels and acute humoral rejection early after positive crossmatch kidney transplantation. *Am J Transplant* 2008; **8**: 2684.
5. Lefaucheur C, Loupy A, Hill GS, *et al.* Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation. *J Am Soc Nephrol* 2010; **21**: 1398.
6. Wiebe C, Gibson IW, Blydt-Hansen TD, *et al.* Evolution and clinical pathologic correlations of *de novo* donor-specific HLA antibody post kidney transplant. *Am J Transplant* 2012; **12**: 1157.
7. Ginevri F, Nocera A, Comoli P, *et al.* Posttransplant *de novo* donor-specific HLA antibodies identify pediatric kidney recipients at risk for late antibody-mediated rejection. *Am J Transplant* 2012; **12**: 3355.
8. Everly MJ, Rebellato LM, Haisch CE, *et al.* Incidence and impact of *de novo* donor-specific alloantibody in primary renal allografts. *Transplantation* 2013; **95**: 410.
9. Kim JJ, Balasubramanian R, Michaelides G, *et al.* The clinical spectrum of *de novo* donor-specific antibodies in pediatric renal transplant recipients. *Am J Transplant* 2014; **14**: 2350.
10. David-Neto E, Prado E, Beutel A, *et al.* C4d-positive chronic rejection: a frequent entity with a poor outcome. *Transplantation* 2007; **84**: 1391.
11. Wiebe C, Gibson IW, Blydt-Hansen TD, *et al.* Rates and determinants of progression to graft failure in kidney allograft recipients with *de novo* donor-specific antibody. *Am J Transplant* 2015; **15**: 2921.
12. Djamali A, Kaufman DB, Ellis TM, Zhong W, Matas A, Samaniego M. Diagnosis and management of antibody-mediated rejection: current status and novel approaches. *Am J Transplant* 2014; **14**: 255.
13. Eskandary F, Jilma B, Mühlbacher J, *et al.* Anti-C1s monoclonal antibody BIVV009 in late antibody-mediated kidney allograft rejection – results from a first-in-patient phase 1 trial. *Am J Transplant* 2018; **18**: 916.
14. Davis S, Cooper JE. Acute antibody-mediated rejection in kidney transplant recipients. *Transplant Rev (Orlando)* 2017; **31**: 47.
15. Fehr T, Rűsi B, Fischer A, Hopfer H, Wűthrich RP, Gaspert A. Rituximab and intravenous immunoglobulin treatment of chronic antibody-mediated kidney allograft rejection. *Transplantation* 2009; **87**: 1837.
16. Everly MJ, Terasaki PI, Trivedi HL. Durability of antibody removal following proteasome inhibitor-based therapy. *Transplantation* 2012; **93**: 572.
17. Billing H, Rieger S, Süsal C, *et al.* IVIG and rituximab for treatment of chronic antibody-mediated rejection: a prospective study in paediatric renal transplantation with a 2-year follow-up. *Transpl Int* 2012; **25**: 1165.
18. Redfield RR, Ellis TM, Zhong W, *et al.* Current outcomes of chronic active antibody mediated rejection - A large single center retrospective review using the updated BANFF 2013 criteria. *Hum Immunol* 2016; **77**: 346.
19. Kulkarni S, Kirkiles-Smith NC, Deng YH, *et al.* Eculizumab therapy for chronic antibody-mediated injury in kidney transplant recipients: a pilot randomized controlled trial. *Am J Transplant* 2017; **17**: 682.
20. Lefaucheur C, Viglietti D, Bentlejewski C, *et al.* IgG donor-specific anti-human HLA antibody subclasses and kidney allograft antibody-mediated injury. *J Am Soc Nephrol* 2016; **27**: 293.
21. DeVos JM, Gaber AO, Knight RJ, *et al.* Donor-specific HLA-DQ antibodies may contribute to poor graft outcome after renal transplantation. *Kidney Int* 2012; **82**: 598.
22. Tagliamacco A, Cioni M, Comoli P, *et al.* DQ molecules are the principal stimulators of *de novo* donor-specific antibodies in nonsensitized pediatric recipients receiving a first kidney transplant. *Transpl Int* 2014; **27**: 667.
23. Tambur AR, Herrera ND, Haarberg KM, *et al.* Assessing antibody strength: comparison of MFI, C1q, and titer information. *Am J Transplant* 2015; **15**: 2421.
24. Viglietti D, Loupy A, Vernerey D, *et al.* Value of donor-specific anti-HLA antibody monitoring and characterization for risk stratification of kidney allograft loss. *J Am Soc Nephrol* 2017; **28**: 702.
25. Loupy A, Lefaucheur C, Vernerey D, *et al.* Complement-binding anti-HLA antibodies and kidney-allograft survival. *N Engl J Med* 2013; **369**: 1215.
26. Sicard A, Ducreux S, Rabeyrin M, *et al.* Detection of C3d-binding donor-specific anti-HLA antibodies at diagnosis of humoral rejection predicts renal graft loss. *J Am Soc Nephrol* 2015; **26**: 457.
27. Comoli P, Cioni M, Tagliamacco A, *et al.* Acquisition of C3d-binding activity by *de novo* donor-specific HLA antibodies correlates with graft loss in nonsensitized pediatric kidney recipients. *Am J Transplant* 2016; **16**: 2106.
28. Bachelet T, Couzi L, Lepreux S, *et al.* Kidney intragraft donor-specific antibodies as determinant of antibody-mediated lesions and poor graft outcome. *Am J Transplant* 2013; **13**: 2855.
29. Nocera A, Tagliamacco A, Cioni M, *et al.* Kidney intragraft homing of *de novo* donor-specific HLA antibodies is an essential step of antibody-mediated damage but not per se predictive of graft loss. *Am J Transplant* 2017; **17**: 692.
30. Everly MJ, Rebellato LM, Ozawa M, *et al.* Beyond histology: lowering human leukocyte antigen antibody to improve renal allograft survival in acute rejection. *Transplantation* 2010; **89**: 962.
31. Ramon DS, Huang Y, Zhao L, *et al.* Use of complement binding assays to assess the efficacy of antibody mediated rejection therapy and prediction of graft survival in kidney transplantation. *Hum Immunol* 2017; **78**: 57.
32. Viglietti D, Loupy A, Aubert O, *et al.* Dynamic prognostic score to predict kidney allograft survival in patients with antibody-mediated rejection. *J Am Soc Nephrol* 2018; **29**: 606.
33. Haas M, Sis B, Racusen LC, *et al.* Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant* 2014; **14**: 272.
34. Schwartz GJ, Munoz A, Schneider MF, *et al.* New equations to estimate GFR in children with CKD. *J Am Soc Nephrol* 2009; **20**: 629.
35. Shaffi K, Uhlig K, Perrone RD, *et al.* Performance of creatinine-based GFR estimating equations in solid-organ transplant recipients. *Am J Kidney Dis* 2014; **63**: 1007.
36. Vo AA, Sinha A, Haas M, *et al.* Factors predicting risk for antibody-mediated

rejection and graft loss in highly human leukocyte antigen sensitized patients transplanted after desensitization. *Transplantation* 2015; **99**: 1423.
37. Gupta G, Abu Jawdeh BG, Racusen LC, *et al.* Late antibody-mediated rejection in renal allografts: outcome after conventional and novel therapies. *Transplantation* 2014; **97**: 1240.
38. Smith RN, Malik F, Goes N, *et al.* Partial therapeutic response to Rituximab for the treatment of chronic alloantibody mediated rejection of kidney allografts. *Transpl Immunol* 2012; **27**: 107.
39. Kizilbash S, Claes D, Ashoor I, *et al.* Bortezomib in the treatment of antibody-mediated rejection in pediatric kidney transplant recipients: a multicenter Midwest Pediatric Nephrology Consortium study. *Pediatr Transplant* 2017; **21**: e12873.
40. Orandi BJ, Chow EH, Hsu A, *et al.* Quantifying renal allograft loss following early antibody-mediated rejection. *Am J Transplant* 2015; **15**: 489.
41. Tambur AR, Glotz D, Herrera ND, *et al.* Can solid phase assays be better utilized to measure efficacy of antibody removal therapies? *Hum Immunol* 2016; **77**: 624.
42. Stegall MD, Gloor J, Winters JL, Moore SB, Degoey S. A comparison of plasmapheresis versus high-dose IVIG desensitization in renal allograft recipients with high levels of donor specific alloantibody. *Am J Transplant* 2006; **6**: 346.
43. Yamada C, Ramon DS, Cascalho M, *et al.* Efficacy of plasmapheresis on donor-specific antibody reduction by HLA specificity in post-kidney transplant recipients. *Transfusion* 2015; **55**: 727.
44. Kim M, Martin ST, Townsend KR, Gabardi S. Antibody-mediated rejection in kidney transplantation: a review of pathophysiology, diagnosis, and treatment options. *Pharmacotherapy* 2014; **34**: 733.
45. Sadeghi M, Daniel V, Wang H, *et al.* Plasmapheresis adjusts inflammatory responses in potential kidney transplant recipients. *Transplantation* 2013; **95**: 1021.
46. Kim JJ, Shaw O, Martin C, *et al.* Clinical risk stratification of paediatric renal transplant recipients using C1q and C3d fixing of *de novo* donor-specific antibodies. *Pediatr Nephrol* 2018; **33**: 167.
47. Messina M, Ariaudo C, Praticò Barbato L, *et al.* Relationship among C1q-fixing *de novo* donor specific antibodies, C4d deposition and renal outcome in transplant glomerulopathy. *Transpl Immunol* 2015; **33**: 7.
48. Wiebe C, Gareau AJ, Pochinco D, *et al.* Evaluation of C1q status and titer of *de novo* donor-specific antibodies as predictors of allograft survival. *Am J Transplant* 2017; **17**: 703.
49. Lefaucheur C, Viglietti D, Mangiola M, Loupy A, Zeevi A. From humoral theory to performant risk stratification in kidney transplantation. *J Immunol Res* 2017; **2017**: 5201098.
50. Matignon M, Pilon C, Commereuc M, *et al.* Intravenous immunoglobulin therapy in kidney transplant recipients with *de novo* DSA: results of an observational study. *PLoS ONE* 2017; **12**: e0178572.