ORIGINAL ARTICLE

De novo donor-specific anti-HLA antibodies after kidney transplantation are associated with impaired graft outcome independently of their C1q-binding ability

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SUMMARY

Many aspects of post-transplant monitoring of donor-specific (DSA) and non-donor-specific (nDSA) anti-HLA antibodies on renal allograft survival are still unclear. Differentiating them by their ability to bind C1q may offer a better risk assessment. We retrospectively investigated the clinical relevance of *de novo* C1q-binding anti-HLA antibodies on graft outcome in 611 renal transplant recipients. Acute rejection (AR), renal function, and graft survival were assessed within a mean follow-up of 6.66 years. Post-transplant 6.5% patients developed *de novo* DSA and 11.5% *de novo* nDSA. DSA (60.0%; $P < 0.0001$) but not nDSA (34.1%, $P = 0.4788$) increased rate of AR as compared with controls (27.4%). C1q-binding anti-HLA antibodies did not alter rate of AR in both groups. Renal function was only significantly diminished in patients with DSAC1q$^+$. However, DSA significantly impaired 5-year graft survival (65.2%; $P < 0.0001$) in comparison with nDSA (86.7%; $P = 0.0054$) and controls (90.7%). While graft survival did not differ between DSAC1q$^-$ and DSAC1q$^+$ recipients, 5-year allograft survival was reduced in nDSAC1q$^+$ (80.9%) versus nDSAC1q$^-$ (90.7%, $P = 0.0251$). *De novo* DSA independently of their ability to bind C1q are associated with diminished graft survival.

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Introduction

De novo donor-specific anti-HLA antibodies (DSA) emerge after transplantation in 7–30% of nonimmunized recipients when screened with single-antigen bead assay (SAB) [1–6]. *De novo* DSA are associated with antibody-mediated rejections [4,7–9], chronic graft dysfunction [4], and diminished allograft survival [5,10]. They often precede rejection and/or graft loss suggesting they play a pathognomonic role in graft injury and could therefore be used as a potential prognostic biomarker [11–13]. However, not every renal allograft recipient with *de novo* DSA detected in SAB assay develops an acute rejection episode as SAB assays

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are highly sensitive and do not determine the cytotoxic potential of these antibodies. The current challenge is to identify those patients with DSA at immunologic risk to be able to guide the individual therapy. Some approaches to do so include the determination of the mean fluorescent intensity (MFI) of anti-HLA antibodies in SAB [7,12,14], the IgG subclass [15] and/or the complement-binding capacity of anti-HLA antibodies. [16–18]. In particular, the binding of the complement fraction C1q, which is the first step in the activation of the classic complement cascade, is thought to be a potential marker to assess the cytotoxic potential of these antibodies. However, the clinical importance of C1q-binding *de novo* DSA in predicting graft outcome remains controversial in that some studies identified C1q-binding DSA as a more accurate marker in predicting acute rejection and graft failure [19] while others did not [17]. Furthermore, the impact of the complement-binding capacity of non-donor-specific anti-HLA antibodies (nDSA) is unclear. Therefore, we aimed to evaluate the impact of C1q-binding anti-HLA antibodies, either donor-specific or non-donor-specific, following kidney transplantation at our center.

Methods

Study design and patients

We conducted a retrospective cohort study including all patients (n = 732) who received a renal transplantation between January 2005 and December 2011 at our center and of whom data were acquired until 31/12/2014. One hundred and twenty-one patients were excluded from our analysis for various reasons (missing serum sample, incomplete data, primary nonfunction, recipient age <18 years). A flowchart of the study population is shown in Fig. 1. The remaining patients (n = 611) had been screened for the presence of anti-HLA antibodies before transplantation and during the routine follow-up by means of Luminex® (LabScreen LSA; One lambda, Canoga Park, CA, USA). Furthermore, all detected anti-HLA antibodies were screened for their C1q-binding ability. Patients without *de novo* anti-HLA antibodies after transplantation served as control group. All patients had a negative T- and B-cell CDC cross-match at time of transplantation.

HLA-typing

HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3, HLA-DRB4, HLA-DRB5, and HLA-DQB1 of recipients and donors were determined twice by DNA typing using an SSP (Olerup SSP AB, Stockholm, Sweden) or SSO (LabType SS0; One lambda) approach on the Luminex platform.

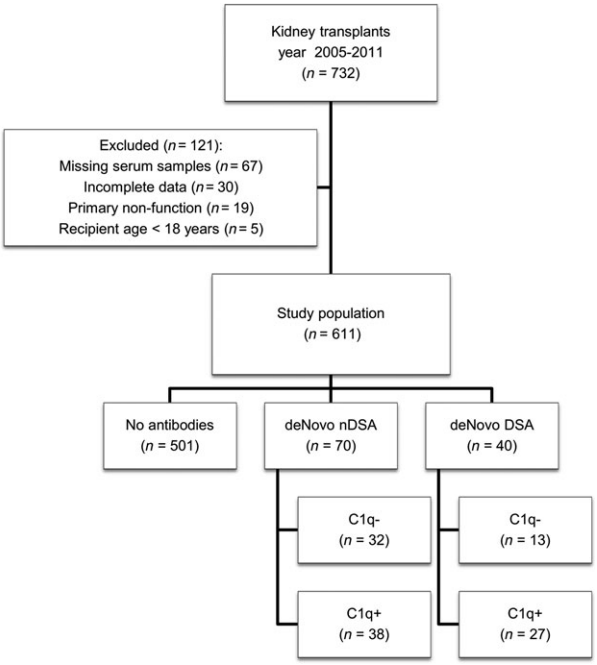

Figure 1 Flowchart of study population.

Anti-HLA antibody screening

Recipients were screened for the presence of anti-HLA antibodies against HLA-class I and II by means of Luminex® (Lifecodes Life Screen Deluxe, Immucor Inc., Norcross, GA, USA) every 3 month before transplantation and in yearly intervals during routine follow-up visits in our out-patient clinic and/or in case of graft dysfunction after transplantation. Positively screened patients (according to instructions of Lifecodes Life Screen Deluxe) were measured by single-antigen bead assay (LabScreen; One lambda) to identify permissible allele-level antigens. All sera were pretreated by heat inactivation (56 °C in a dry heat block for 30 min) and filtered to overcome the prozone effect. The antibody specificity in relation to the donor (donor-specific and non-donor-specific) was confirmed if the MFI was above 1000. The C1q-binding capacity of HLA antibodies was tested by C1q-SAB assay (LabScreen). Nonacceptable HLA antigens had been blocked before transplantation, when the patient had high-level antibodies (MFI > 3000) against the specific HLA antigen according to the consensus paper of Susal *et al.* [20] or if the patient had anti-

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HLA antibodies against repeated mismatches or antipaternal antigens (independent of the MFI).

Biopsies/Rejections

Allograft biopsies were taken in case of a sudden loss of graft function or when patients developed a BK viremia. Rejection was classified according to the diagnostic criteria proposed at the 2007 and 2011 Banff Conferences [21,22]. C4d staining was routinely performed in paraffin sections of all biopsies. C4d-positive staining in peritubular capillaries (PTC) was evaluated semi-quantitatively as follows: minimal (<10% of PTC), focal (11–50% of PTC), and diffuse (>50% of PTC). The diagnosis of BK nephropathy was based on histologic features in combination with positive staining for SV40.

Statistics

Statistical analyses were performed in R version 3.2.1 [23]. Descriptive statistics (number of cases, percentages for categorical variables, median and/or mean ± standard deviation (SD) for metric variables) were used to characterize the study population. To investigate differences between the five groups, the ANOVA test was used for comparing the means in metric variables, and the Pearson's chi-squared test for comparing proportions in categorical variables, with *P*-values based on simulation in case of small cell [24] counts. To investigate differences between two selected groups, the Fisher's exact test [25] was used for categorical variables and the t-tests for metric variables.

Regression models were used to analyze the relationship of the two dependent variables: (i) acute rejection (yes versus no) and (ii) graft survival (time in years with censored observations) with the independent variables: recipient age (years) and gender (female versus male), type of transplant (kidney alone, kidney plus pancreas, kidney plus others), retransplantation (yes versus no), *de novo* anti-HLA antibodies (donor specific, C1q binding), donor age (years), and gender (female versus male), HLA mismatch (*n*), repeated mismatch (yes versus no), cold ischemia time (hours), DGF (yes versus no), type of induction therapy, type of immunosuppressive regiment. For graft survival, creatinine levels at years 1, 2, and 3 were additionally considered as independent variables.

To analyze the relationship between the independent and dependent variables, two different models were used: (i) a logit model for acute rejection and (ii) a Cox proportional hazards model for graft survival. First, the relationship between acute rejections and graft survival with each independent variable was analyzed. For each model, we used the cases with valid responses on the respective independent variable. Missing data were as follows: MM in one patient, cold ischemia time in six patients, DGF in 21 cases. In patients with graft loss within the first 3 years, serum creatinine was stated with 10 mg/dl to indicate renal malfunction at the different time points thereafter. For all remaining independent variables, values were known for all patients.

The relationship of each dependent variable with independent variables was verified by a multivariate model. For this purpose, a stepwise variable selection procedure based on Akaike information criterion (AIC) to select the final model was used. Missing values were replaced by the median of the variable (i.e., the missing MM value in one patient by 3 and the missing values for cold ischemia time in six patients by 10.34).

Kaplan–Meier curves [26] were used to visualize graft survival in the different groups. The log-rank test [27] was used to test for differences in survival time between groups.

Results

Baseline characteristics of study population

In total, 611 patients undergoing renal transplantation were included in the main analysis. 81.7% of all patients received their first transplant. Only 17.8% had preformed anti-HLA antibodies prior of transplantation. Five distinct populations were identified, according to the presence or absence of *de novo* donor-specific (DSA) or non-donor-specific (nDSA) anti-HLA antibodies and their ability to bind the complement product C1q ($C1q^+$ or $C1q^-$). Five hundred and one patients without circulating *de novo* anti-HLA antibodies served as control group. Table 1 show the characteristics of the five groups at the time of transplantation. The mean ± SD (median) follow-up within our patient population was 6.66 ± 1.96 (6.56) years. The follow-up was comparable between all five groups [controls: 6.55 ± 1.99 (6.54) years, $nDSAC1q^-$ 6.64 ± 1.75 (6.67) years, $nDSAC1q^+$ 6.28 ± 1.67 (5.86) years, $DSAC1q^-$ 6.71 ± 2.22 (7.51) years, $nDSAC1q^+$: 7.32 ± 1.9 (7.82) years]. No significant differences were noted in recipient age, type of transplantation, HLA mismatches, CDC-PRA (%), donor age and sex, and number of deceased donors between the groups. The number of female recipients was significantly higher in the $nDSAC1q^-$ group, while the number of prior transplants and pretransplant anti-HLA antibodies was significantly higher

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Table 1. Baseline characteristics of study population according to the presence or absence of *de novo* nDSA or DSA and their ability to bind C1q ($C1q^-$ or $C1q^+$). For categorical variables, the number of cases and the corresponding percentages are reported, while for metric variables, mean ± standard deviation is reported.

	No HLA Ab *N* = 501	*De novo* nDSAC1q$^-$ *N* = 32	*De novo* nDSAC1q$^+$ *N* = 38	*De novo* DSAC1q$^-$ *N* = 13	*De novo* DSAC1q$^+$ *N* = 27	*P*-value
Recipient						
Sex, female (%)	187 (37.33)	21 (65.62)	14 (36.84)	6 (46.15)	8 (29.63)	0.0217†
Age (years)	50.61 ± 12.81	52.25 ± 11.71	50.82 ± 11.78	48.00 ± 18.17	46.78 ± 11.50	0.4850*
Type of Tx						
Kidney (%)	427 (85.23)	30 (93.75)	35 (92.11)	11 (84.62)	24 (88.89)	0.8151‡
Kidney plus pancreas (%)	60 (11.98)	2 (6.25)	2 (5.26)	2 (15.38)	2 (7.41)	
Kidney plus other (%)	14 (2.79)	0 (0)	1 (2.63)	0 (0)	1 (3.70)	
Retransplantation (%)	60 (11.98)	12 (37.50)	30 (78.95)	2 (15.38)	8 (29.63)	0.0005‡
Donor						
Sex, female (%)	257 (51.30)	18 (56.25)	19 (50.00)	5 (38.46)	15 (55.56)	0.8457†
Age (years)	51.98 ± 16.88	53.03 ± 15.57	55.42 ± 13.37	49.00 ± 18.43	48.96 ± 14.49	0.5460*
Deceased donor (%)	393 (78.44)	27 (84.38)	32 (84.21)	10 (76.92)	24 (88.89)	0.5817‡
Cold ischemia time (hours)	10.68 ± 7.64	13.24 ± 8.61	11.34 ± 6.22	10.18 ± 7.81	12.80 ± 8.10	0.2610*
Immunosuppression						
Tac/MPA/Steroids	254 (50.7)	22 (68.75)	30 (78.95)	7 (53.85)	12 (44.44)	0.1379‡
CsA/MPA/Steroids	209 (41.42)	8 (25)	6 (15.79)	5 (38.46)	13 (48.15)	
MPASteroids	27 (5.39)	1 (3.12)	1 (2.63)	1 (7.69)	1 (3.7)	
Others	11 (2.2)	1 (3.12)	1 (2.63)	0 (0)	1 (3.7)	
Immunologic data						
Mismatch (*n*)	3.16 ± 1.68 3	2.66 ± 1.75 8	2.61 ± 1.52 8	3.23 ± 1.24 6.5	3.56 ± 1.19 7	
CDC-PRA prior to Tx (%)	3	8	8	6.5	7	
Anti-HLA ab prior to Tx						
None (%)	452 (90.22)	17 (53.12)	5 (13.16)	10 (76.92)	18 (66.67)	0.0005‡
nDSA (%)	46 (9.18)	11 (34.38)	29 (76.32)	1 (7.69)	6 (22.22)	
DSA (%)	3 (0.60)	4 (12.50)	4 (10.53)	2 (15.38)	3 (11.11)	
Anti-HLA ab class Post-Tx						
Class I	0	13 (40.60)	6 (15.80)	2 (15.40)	1 (4.00)	
Class II	0	12 (37.50)	18 (47.40)	6 (46.20)	21 (77.60)	
Class I and II	0	7 (21.90)	14 (36.80)	5 (38.40)	5 (18.40)	

To investigate whether the five groups differ in any of these baseline characteristics, different statistical tests were applied:

*ANOVA test for metric variables.

†Pearson's chi-squared test for categorical variables with sufficient group sizes.

‡Pearson's chi-squared test with simulated *P*-value for categorical variables with insufficient group sizes for the ordinary Pearson's chi-squared test. ab= antibody; Tx = transplantation

in the nDSAC1q$^+$ group. None of our patients with post-transplant *de novo* DSA had pretransplant DSA. Nine of 27 patients (33.3%) with *de novo* DSAC1q$^+$ also developed *de novo* nDSAC1q$^+$.

Rejection

In total, 356 (58.3%) patients underwent at least one biopsy [control: 280/501 (55.5%), *de novo* nDSAC1q$^-$: 18/32 (56%), *de novo* nDSAC1q$^+$: 27/38 (71%), *de novo* DSAC1q$^-$: 9/13 (69.2%), *de novo* DSAC1q$^+$: 21/27 (74%)]. The median time of biopsy after transplantation did not differ between the five groups. Acute clinical rejection developed in 183 patients: 175 (95.6%) patients had T-cell-mediated rejection including those with borderline changes and only eight (4.4%) patients had an antibody-mediated rejection (AMR). While the ratio of patients experiencing an acute rejection episode did not significantly differ between the control (27.4%) and the *de novo* nDSA group (31.4%; $P = 0.4788$), the risk of rejection significantly increased in patients with *de novo* DSA (60%, versus control $P < 0.0001$, versus nDSA $P = 0.0048$; Fig. 2a). The C1q-binding capacity of *de novo* anti-HLA antibodies enhanced the immunologic

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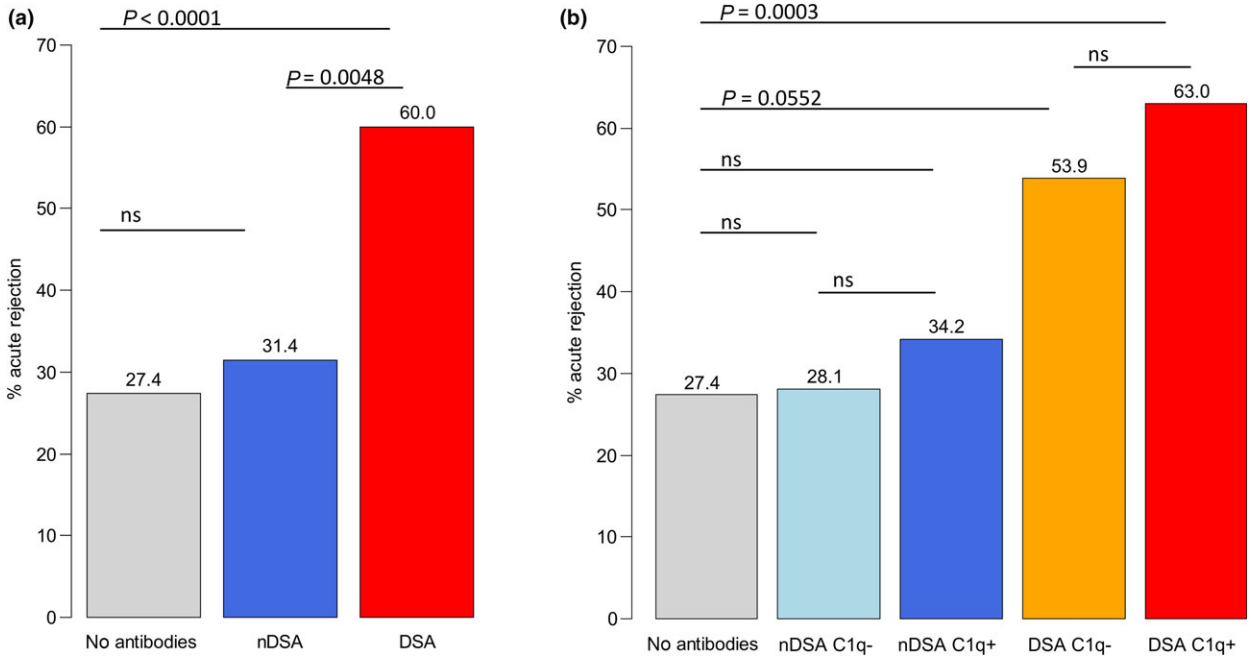

Figure 2 Ratio of biopsy-proven acute rejection episodes in control patients (no anti-HLA antibodies) as compared to (a) patients with *de novo* nDSA and DSA; or (b) patients with *de novo* nDSA C1q$^-$ or -C1q$^+$, *de novo* DSA C1q$^-$ or -C1q$^+$. *P*-values are based on Fisher's exact test.

potency of *de novo* nDSA and DSA, with patients developing *de novo* DSAC1q$^+$ having the highest risk of rejection. However, the effect was not statistically significant in either group (Fig. 2b).

In the univariate models as well as the multivariate model mismatch ($P < 0.0001$), *de novo* DSAC1q$^-$ ($P = 0.0469$) and *de novo* DSAC1q$^+$ ($P = 0.0007$) significantly increased the risk of graft rejection, while a combined kidney–pancreas transplantation ($P = 0.0112$) and a tacrolimus-based triple immunosuppression ($P = 0.0095$) significantly lowered the risk of rejection. Table 2

Graft dysfunction

Reflecting the highest rate of acute rejection episodes patients with *de novo* DSAC1q$^+$ had significantly more graft dysfunction as assessed by serum creatinine levels 3 years after transplantation. The mean ± SD (median) serum creatinine was 2.39 ± 2.43 (1.70) mg/dl in control, 1.94 ± 2.22 (1.30) mg/dl in nDSAC1q$^-$, 3.10 ± 3.23 (1.80) mg/dl in nDSAC1q$^+$, 2.62 ± 2.47 (1.70) mg/dl in DSAC1q$^-$, and 4.16 ± 3.34 (2.80) mg/dl in DSAC1q$^+$ patients. Graft function did not differ at time of referral from the hospital (i.e., day 14), 1 and 2 years after transplantation between the five groups.

Graft survival

Figure 3 shows kidney allograft survival according to anti-HLA antibody status. Graft loss developed in 74 of 611 patients (16%). The development of *de novo* DSA significantly reduced the 5-year graft survival (65.2%; $P < 0.0001$) while *de novo* nDSA had no influence (86.7%; $P = 0.2610$) as compared to control patients (90.7%). When patients were subsequently categorized according to their C1q-binding capacity, 5-year graft survival did not significantly differ between *de novo* nDSAC1q$^-$ and *de novo* nDSAC1q$^+$ (93.5% vs. 80.9%; $P = 0.0747$) and between *de novo* DSAC1q$^-$ and *de novo* DSAC1q$^+$ (76.9% vs. 59.7%; $P = 0.7810$). However, 5-year graft survival according to *de novo* donor-specific versus non-donor-specific antibodies and C1q-binding status significantly decreased from nDSAC1q$^-$ (93.5%; $P = 0.4860$) to nDSAC1q$^+$ (80.9%; $P = 0.0251$) to DSAC1q$^-$ (76.9%; $P = 0.0012$) to DSAC1q$^+$ (59.7%, $P < 0.0001$) as compared with controls (90.7%), with the last group having the highest risk for graft loss.

Factors influencing graft survival

The association of graft loss with clinical, immunologic, and histologic parameters analyzed in Cox proportional hazards modes and verified by a multivariate model by

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Table 2. Clinical, functional, and immunologic factors associated with acute rejection episodes (univariate and multivariate logit-models). Risk factors in the multivariate model were identified by a stepwise variable selection procedure based on AIC.

		Univariate models		Multivariate model	
	Number of patients	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Type of Tx					
Kidney	527	1.00			
Kidney plus pancreas	68	0.41 (0.20–0.76)	0.0083	0.39 (0.18–0.78)	0.0112
Kidney plus other	16	0.30 (0.05–1.09)	0.1140	0.29 (0.04–1.12)	0.1168
Retransplantation					
No	499	1.00			
Yes	112	1.19 (0.76–1.89)	0.4310		
Mismatch	610	1.16 (1.04–1.29)	0.0060	1.27 (1.13–1.44)	<0.0001
De novo anti-HLA antibody					
None	501	1.00			
nDSAC1q$^-$	32	1.04 (0.45–2.23)	0.9236	1.22 (0.51–2.71)	0.6281
nDSAC1q$^+$	38	1.38 (0.67–2.73)	0.3643	1.79 (0.84–3.69)	0.1193
DSAC1q$^-$	13	3.10 (1.01–9.78)	0.0454	3.22 (1.01–10.65)	0.0469
DSAC1q$^+$	27	4.52 (2.05–10.47)	0.0002	4.30 (1.89–10.37)	0.0007
DGF					
No	449	1.00			
Yes	141	1.39 (0.93–2.08)	0.1056		
Immunosuppression					
CsA/MPASteroids	241	1.00			
Tac/MPA/Steroids	325	0.58 (0.40–0.83)	0.0028	0.58 (0.39–0.87)	0.0095
MPASteroids	31	0.94 (0.42–2.02)	0.8752	0.74 (0.32–1.66)	0.4775
Others	14	0.13 (0.01–0.68)	0.0521	0.12 (0.01–0.66)	0.0485

CI, confidence interval.

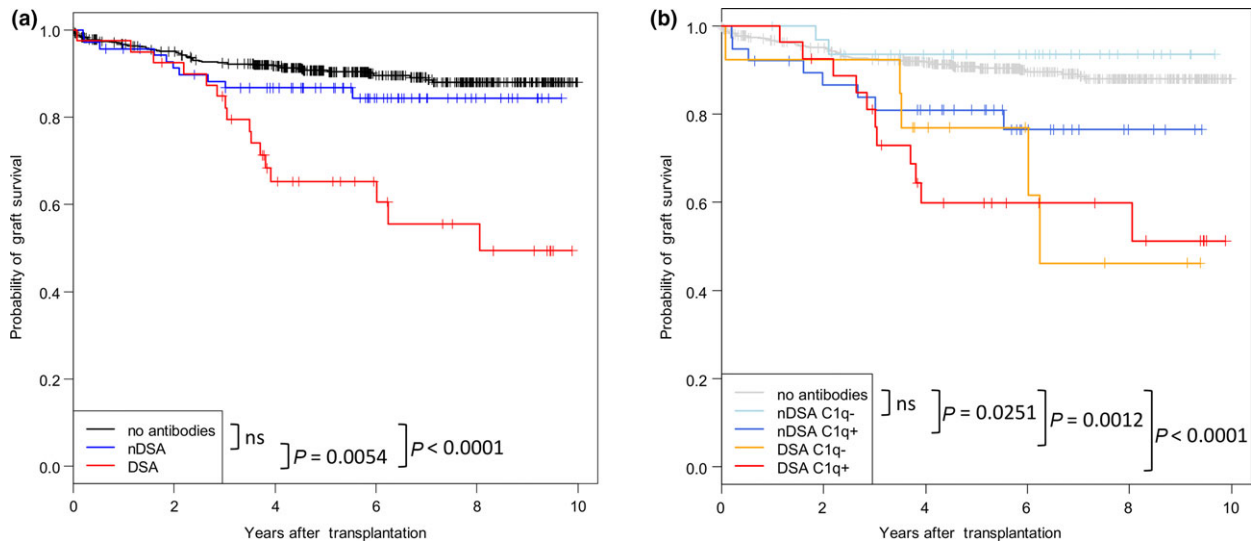

Figure 3 Kaplan–Meier curves comparing graft survival of control patients (no anti-HLA antibodies) with patients with *de novo* (a) nDSA and DSA; or (b) patients with *de novo* nDSA C1q$^-$ or -C1q$^+$, DSA C1q$^-$ or -C1q$^+$. *P*-values are based on the log-rank test.

a stepwise variable selection procedure based on AIC is shown in Table 3. The following parameters were significant predictors of graft loss in the univariate models: DGF ($P = 0.0018$), mismatch ($P = 0.0198$); retransplantation ($P = 0.0005$), acute rejection episodes ($P < 0.0001$); renal function at 1, 2, and 3 years post-transplant ($P < 0.0001$ each); *de novo* nDSAC1q$^+$ ($P = 0.0274$), *de novo* DSAC1q$^-$ ($P = 0.0029$), and *de*

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Table 3. Clinical, functional, and immunologic factors associated with allograft loss (univariate and multivariate Cox proportional hazard models). Risk factors in the multivariate model were identified by a stepwise variable selection procedure based on AIC.

		Univariate models		Multivariate model	
	Number of patients	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Type of Tx					
Kidney	527	1.00			
Kidney plus pancreas	68	0.51 (0.20–1.25)	0.1411		
Kidney plus other	16	0.48 (0.07–3.43)	0.4611		
Retransplantation					
No	499	1.00			
Yes	112	2.37 (1.46–3.85)	0.0005	1.94 (0.84–4.48)	0.1196
Mismatch					
Total	610	1.18 (1.03–1.36)	0.0198	1.28 (1.05–1.58)	0.0171
DR	610	1.40 (1.01–1.92)	0.0414		
De novo anti-HLA antibody					
None	501	1.00			
nDSAC1q$^-$	32	0.61 (0.15–2.50)	0.4910	0.60 (0.10–3.53)	0.5680
nDSAC1q$^+$	38	2.32 (1.10–4.90)	0.0274	1.02 (0.37–2.82)	0.9671
DSAC1q$^-$	13	4.05 (1.61–10.16)	0.0029	7.05 (2.14–23.22)	0.0013
DSAC1q$^+$	27	4.42 (2.30–8.51)	<0.0001	3.77 (1.40–10.16)	0.0086
DGF					
No	449	1.00			
Yes	141	2.14 (1.33–3.45)	0.0018	0.58 (0.28–1.20)	0.1433
Rejection					
No	428	1.0000			
Yes	183	5.28 (3.26–8.53)	<0.0001		
Renal function (creatinine mg/dl)					
1 year post-tx	573	2.69 (2.24–3.23)	0.0001	1.48 (1.23–1.79)	<0.0001
2 year post-tx	562	2.92 (2.35–3.64)	<0.0001	2.30 (1.70–3.13)	<0.0001
3 year post-tx	542	1.77 (1.66–1.89)	<0.0001	1.73 (1.53–1.95)	<0.0001
Immunosuppression					
CsA/MPA/Steroids	241	1.00			
Tac/MPA/Steroids	325	0.76 (0.47–1.24)	0.2729	0.51 (0.25–1.03)	0.0608
MPA/Steroids	31	1.75 (0.77–3.96)	0.1801	0.61 (0.18–2.08)	0.4282
Others	14	1.61 (0.49–5.25)	0.4298	0.11 (0.02–0.78)	0.0272

CI, confidence interval.

novo DSAC1q$^+$ ($P < 0.0001$). Renal function, mismatch, and *de novo* DSA independently of their C1q-binding capacity remained significant predictors of kidney allograft loss in a multivariate analysis, while *de novo* C1q-binding nDSA, DGF, and retransplantation did not. An immunosuppressive regiment with tacrolimus, MMF, and steroids improved graft survival in comparison with a triple therapy with cyclosporin, MMF, and steroids ($P = 0.0608$).

Correlation of MFI and C1q-binding capacity

The majority of *de novo* DSA (67.5%) and *de novo* nDSA (54.3%) were C1q positive. The mean MFI of the immune-dominant *de novo* DSA (antibody with the highest MFI level) was 11 627.5 ± 7266.9. The mean MFI of the immune-dominant *de novo* nDSA was 8171.4 ± 5703.98. Both, the MFI of *de novo* DSA (correlation coefficient 0.658, $P < 0.0001$) and the MFI of *de novo* nDSA (correlation coefficient 0.458, $P < 0.0001$) significantly correlated with their C1q-binding capacity (Fig. 4)

While the MFI of *de novo* nDSA did not alter allograft survival, the risk of allograft loss significantly increased with MFI in patients with *de novo* DSA. Five-year graft survival in *de novo* DSA with a MFI < 3000 (71.4%; $P = 0.1900$) was better than in those with MFI between 3000 and 10 000 (62.2%, $P < 0.0001$) and in those with MFI >10 000 (63.6%; $P < 0.0001$). *P*-values display the comparison with controls (5 year graft survival 90.7%; Fig. 5).

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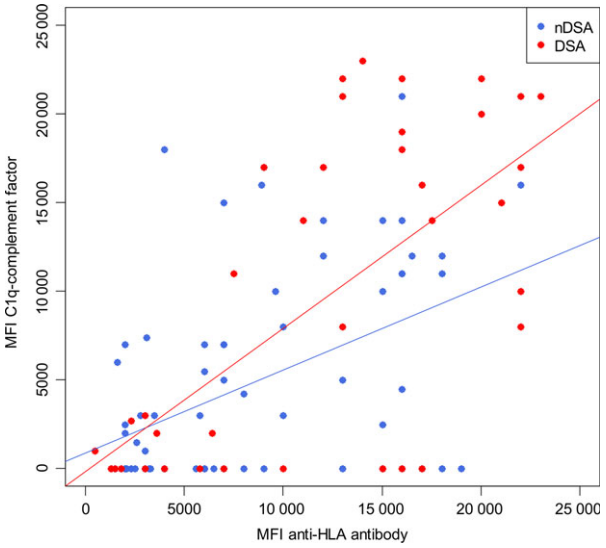

Figure 4 Correlation of peak MFI of *de novo* nDSA (gray line) and *de novo* DSA (black line) with the respective peak MFI of C1q.

Discussion

In this report, we present our experience in 611 patients that underwent kidney transplantation between January 2005 and December 2011 at our center and were screened for the presence of *de novo* DSA or *de novo* nDSA and their C1q complement-binding capacity by means of Luminex®. We observed that *de novo* DSA independent of their C1q-binding capacity were a significant risk factor of kidney allograft loss, while *de novo* nDSA only influenced allograft function, when they had the ability to bind C1q.

The incidence of *de novo* DSA (6.55%) and *de novo* nDSA (11.46%) in our study population was within the range of previously published reports (7–30%) [1–6]. We confirm the widely reported negative effect of *de novo* DSA on graft outcome [5,10,28]. However, our results are contrasting four studies, including the large series of Loupy *et al.* [16,19,29,30] in that allograft survival in our patient population was equally decreased in patients with *de novo* DSAC1q$^+$ and *de novo* DSAC1q$^-$. In accordance, Guidicelli *et al.* [17] demonstrated that the long-term graft function was similarly impaired in patients with *de novo* DSAC1q$^+$ and DSAC1q$^-$ antibodies although C1q-binding *de novo* DSA were associated with short-term graft loss appearing briefly after the development of DSA. The authors concluded that the duration of exposure to DSA but not their C1q-binding capacity *per se* is the pivotal risk factor for graft injury. The difference between our observation and the data by Loupy *et al.* [19] may be due to the longer median follow-up of 6.6 years as compared to 4.8 years in the French population.

Interestingly, *de novo* nDSAC1q$^+$ antibodies were associated with a significant risk of graft failure within our population. *De novo* nDSA have been shown to negatively influence graft outcome in some [3,31], but not all studies [2,5,28,32]. Suesal at al. for example showed that significantly more patients with graft loss had *de novo* nDSA as compared to a matched group of

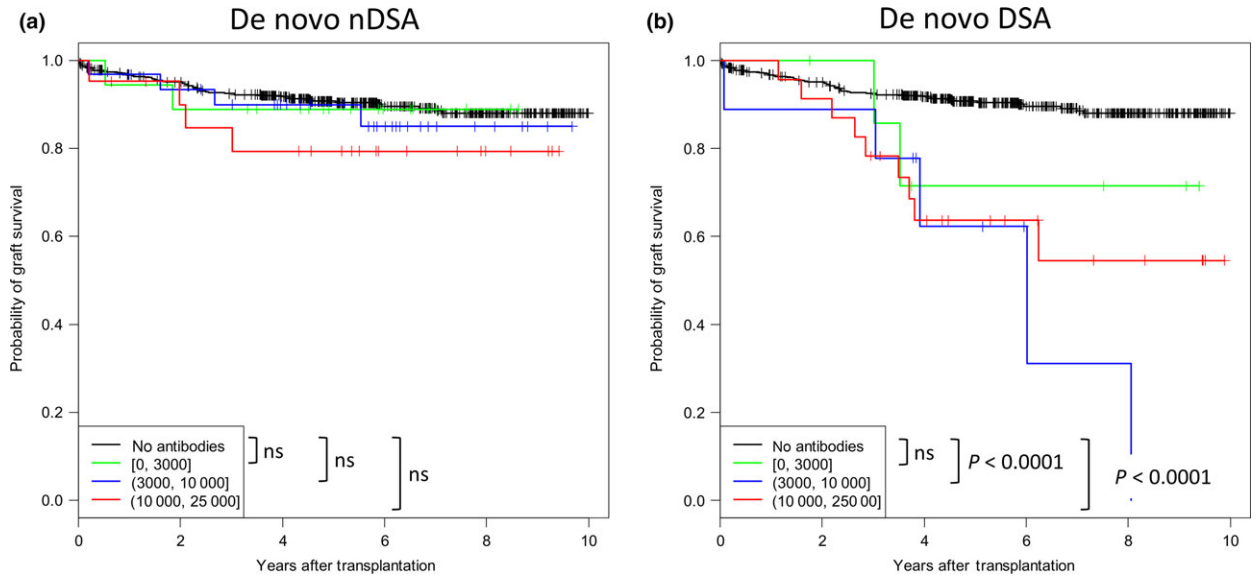

Figure 5 Kaplan–Meier curves comparing graft survival of control patients (no anti-HLA antibodies, n = 501) with patients (a) with *de novo* nDSA with MFI <3000 (n = 18), 3000–10 000 (n = 31), or MFI 10 000–25 000 (n = 21); or (b) with *de novo* DSA with MFI < 3000 (n = 8), 3000–10 000 (n = 9), or MFI 10 000–25 000 (n = 23). *P*-values are based on the log-rank test.

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patients with stable graft function. However, none of the above-mentioned studies evaluated the complement-binding ability of nDSA. The C1q-binding capacity of nDSA may be a sign of their enhanced immunogenicity and/or a sign of cross-reactivity with DSA. nDSA have been reported to be produced in parallel to DSA due to epitope sharing with mismatched donor antigens [33]. One could speculate that concomitant and cross-reactive nDSA can be detected in the serum of our patients while weak DSA are absorbed in the allograft or escape our diagnostic tools. Furthermore, we cannot rule out a prozone effect, which would result in false-negative results despite high-antibody titers. To abolish the prozone effect, all sera were treated with heat inactivation, which in our hands and studies of other working groups is as efficient as EDTA addition [34]. according to manufacturer's instructions, EDTA addition is not suitable for the Luminex C1q-binding assay because it may decrease weak MFI signals and increase background reactions. In addition, the C1q assay is not able to detect the presence of weak antibodies [35,36]. Patients with nDSAC1q$^+$ antibodies were more immunized before transplantation and/or were more frequently retransplanted. While pretransplant screening revealed predominantly nDSA antibodies within this group, we cannot rule out that some patients might have been misclassified because they might have had DSA with low MFI (< 1000) pretransplant.

In accordance with the decreased allograft survival, the development of *de novo* DSA independent of their ability to bind C1q was associated with a significant increase in acute rejection episodes. Messina *et al.* [37] showed that the majority of patients who developed transplant glomerulopathy, the late pathologic finding of AMR, had C1q negative DSA. In accordance, the predictive value of complement (C1q, C4d, or C3)-fixing DSA was moderate in 86 DSA-positive kidney transplant recipients subjected to protocol biopsies. The highest accuracy for predicting AMR was computed for peak IgG MFI (AMR 0.73; C4d+ AMR 0.71). However, combined analysis of antibody characteristics in multivariate models did not improve AMR prediction [38].

As previously described [16–18], we observed a significant correlation between the MFI of anti-HLA antibodies and their ability to bind C1q in *de novo* DSA and nDSA. The observation is in line with the study of Yell *et al.* [39] who found that the C1q-binding activity of DSA in patients with AMR largely reflects differences in antibody MFI values. However, while the MFI seems to be a good way to identify deleterious *de novo* DSA, it does not seem to discriminate between harmful and harmless *de novo* nDSA. These findings contradict the negative impact of C1q-binding nDSA on graft survival, again indicating that this group might have been immunized with low-level DSA prior to transplantation or *de novo* DSA escape our diagnostic tools.

Finally, we found two independent risk factors for *de novo* DSA. The first was the number of HLA mismatches, in particular the number of HLA-DRB1 mismatches, which has been widely recognized [4,5,10]. The second is a cyclosporine- versus tacrolimus-based immunosuppressive regiment indicating that tacrolimus might be more potent in inhibiting humoral alloresponses. Nonadherence [5,40], immunosuppression with less potency such as everolimus as compared to cyclosporin [41], cyclosporin as compared to tacrolimus [2], and azathioprine as compared to mycophenolate mofetil [29], as well as insufficient immunosuppression or drug minimization [42] have been shown to be associated with the development of anti-HLA antibodies. However, contradicting the work of Brokhof *et al.* [43] induction with ATG did not decrease the risk of developing *de novo* anti-HLA antibodies in our study.

The major limitation of our study is the retrospective single-center study design, the lack of early anti-HLA antibody measurements, missing protocol biopsies, and HLA-DP typing of the donors. Therefore, we can only hypothesize about the exact time point of development of DSA and the diagnosis of AMR might have been underrated. To address all these questions, prospective and multicenter studies are needed.

In summary, we demonstrated that *de novo* DSA independent of their C1q-binding capacity is a significant risk factor of kidney allograft loss, while nDSA only influence allograft function, when they have the ability to bind C1q.

Authorship

TK, AH, VL, MF, US, MG and MA: participated in research design. TK and AH: participated in writing the article. TK, AD, AH, MS, BM, JA and JW: participated in the performance of the research. CO, TK, AH and MC: participated in analyzing the data.

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Conflict of interest

The authors declare no conflict of interests.

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