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ORIGINAL ARTICLE

The evolution of histological changes suggestive of antibody-mediated injury, in the presence and absence of donor-specific anti-HLA antibodies

Maarten Coemans[1,2], Aleksandar Senev[1,3], Elisabet Van Loon[1,4], Evelyne Lerut[5], Ben Sprangers[1,4], Dirk Kuypers[1,4], Marie-Paule Emonds[1,3], Geert Verbeke[2] & Maarten Naesens[1,4]

1 Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium
2 Leuven Biostatistics and Statistical Bioinformatics Centre (L-BioStat), Department of Public Health and Primary Care, KU Leuven, Leuven, Belgium
3 Histocompatibility and Immunogenetics Laboratory, Belgian Red Cross-Flanders, Mechelen, Belgium
4 Department of Nephrology and Renal Transplantation, University Hospitals Leuven, Leuven, Belgium
5 Department of Imaging & Pathology, University Hospitals Leuven, Leuven, Belgium

Correspondence
Maarten Naesens, MD, PhD, Nephrology and Renal Transplantation Research Group, Department of Microbiology, Immunology and Transplantation, KU Leuven, Herestraat 49, 3000 Leuven, Belgium.
Tel.: +32 16 34 45 80;
fax: +32 16 34 45 99;
e-mail: maarten.naesens@kuleuven.be

SUMMARY

The interplay between donor-specific anti-HLA antibodies (HLA-DSA), histology of active antibody-mediated rejection ($aABMR_h$), transplant glomerulopathy (cg), and graft failure in kidney transplantation remains insufficiently understood. We performed a single-center cohort study ($n = 1000$) including 2761 protocol and 833 indication biopsies. Patients with pretransplant HLA-DSA were more prone to develop aABMRh (OR 22.7, 95% CI, 11.8–43.7, $P < 0.001$), cg (OR 5.76, 95% CI, 1.67–19.8, $P = 0.006$), and aABMRh/cg (OR 19.5, 95% CI, 10.6–35.9, $P < 0.001$). The negative impact of pre-transplant HLA-DSA on graft survival (HR 2.12, 95% CI, 1.41–3.20, $P < 0.001$) was partially mediated through aABMRh and cg occurrence. When adjusted for time-dependent HLA-DSA (HR 4.03, 95% CI, 2.21–7.15, $P = 0.002$), graft failure was only affected by $aABMR_h$ when cg was evident. In HLA-DSA negative patients, $aABMR_h$ was associated with impaired graft outcome only when evolving to cg (HR 1.32, 95% CI, 1.07–1.61, $P = 0.008$). We conclude that the kinetics of HLA-DSA are important to estimate the rate of graft failure, and that histological follow-up is necessary to discover, often subclinical, ABMR and cg. In the absence of HLA-DSA, patients experience similar histological lesions and the evolution to transplant glomerulopathy associates with impaired graft outcome.

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Key words
$aABMR_h$, HLA-DSA, joint modeling, kidney transplantation

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INTRODUCTION

Antibody-mediated rejection (ABMR) is a major threat for long-term kidney allograft survival [1–4]. ABMR is characterized by histological evidence of acute or chronic tissue injury, together with the evidence of current/recent antibody interaction with vascular endothelium and serologic evidence of donor-specific anti-HLA antibodies (HLA-DSA) [5]. ABMR is not a one-hit disease, but a disease process [6,7] with characteristic acute lesions that precede chronic lesions. This led to the phenotypic distinction between acute and chronic ABMR in the Banff classification [5]. The tissue injury of acute ABMR is primarily characterized by microvascular inflammation (glomerulitis and peritubular capillaritis), while transplant glomerulopathy is the major

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histological manifestation of chronic ABMR [8]. Transplant glomerulopathy is considered to be irreversible, and is an important determinant of graft failure, independent of the microcirculation inflammation [9,10].

ABMR is caused by the presence of circulating antibodies, primarily targeted to HLA molecules mismatched between donors and recipient. These antibodies bind to allogeneic targets expressed by the graft microvasculature. This initiates antibody-dependent cellular cytotoxicity (ADCC) and activation of the complement cascade, causing inflammation and microvascular inflammation [6]. HLA-DSA are time-dependent [11]: they can either be present prior to transplantation, because of a former transplantation, pregnancy, or blood transfusion, or arise *de novo* after transplantation because nonadherence or suboptimal immunosuppression in the presence of immunogenic mismatched HLA molecules. Both pre-transplant and *de novo* HLA-DSA are strong risk factors for kidney allograft failure [11,12]. The extent of their harm is suggested to depend on class, specificity, and temporal relationships, with reported differences in the literature between persisting versus resolving pre-transplant HLA-DSA, and pretransplant versus *de novo* HLA-DSA [11,13–18]. Nevertheless, detailed analysis of the temporal relation between the presence, occurrence, or resolution of HLA-DSA, the histological evolution, and graft failure is lacking.

In the absence of detectable HLA-DSA, patients can also develop the histological lesions of ABMR (aABMR$_h$) after kidney transplantation. Although the literature on this HLA-DSA negative aABMR$_h$ group is growing [19–21], the importance of this phenotype for outcome is not well understood. In comparison to HLA-DSA positive aABMR$_h$, HLA-DSA negative aABMR$_h$ patients seem less prone to graft failure [19]. Nonetheless, the histological evolution of this phenotype remains unstudied. More specifically, it is not known if HLA-DSA negative aABMR$_h$ also associates with transplant glomerulopathy and whether this phenotype also drives negative outcomes in the absence of HLA-DSA, as observed in the presence of HLA-DSA.

In this study, we describe the posttransplant evolution and impact of aABMR$_h$ and transplant glomerulopathy, in light of the presence or absence, and the kinetics of HLA-DSA.

Materials and methods

Patients and biopsies

All adult patients who received a kidney transplant at University Hospitals Leuven between March 2004 and February 2013 were eligible for the study (n = 1137). We excluded patients who received a combined organ transplantation (n = 113) or a kidney transplantation after another solid organ transplantation (n = 24). Clinical data were collected during routine clinical follow-up. We required full clinical data on the following variables: donor/recipient gender, donor/recipient age, donor type (donation after brain death, donation after circulatory death, living donor), transplantation rank (first versus repeat transplantation), recipient BMI, cold ischemia time, HLA-DSA, and the number of HLA antigen mismatches. Anti-HLA antibodies were determined pre- and post-transplantation in one histocompatibility laboratory, at day 0 and 3 months after transplantation, yearly and at the time of an indication biopsy, as described previously [11,22]. We assessed the presence of HLA-DSA according to a median fluorescence index (MFI) greater than 500, and, as recently proposed by the STAR working group [23], we also considered a different cut-off at MFI above 1400 for HLA-DSA presence (Supplemental Methods).

We included all posttransplant renal allograft biopsies (protocol + indication biopsies), performed up to 5 years after transplantation, until the time of data extraction (1st September 2019). Protocol kidney transplant biopsies were performed at time of transplantation, and at 3, 12, and 24 months after transplantation. In addition, patients who were transplanted before October 2005 were invited for a protocol biopsy performed at 48 months, patients transplanted before November 2008 for a protocol biopsy at 36 months, and patients transplanted before January 2010 for a protocol biopsy at 60 months. Histological lesions were semiquantitatively scored according to the Banff consensus [24] with a small deviation for C4d thresholds. More specifically, for C4d deposition in PTCs, we used the following semiquantitatively scores: C4d0–no staining (0%), C4d1–minimal staining (0–25% of PTCs), C4d2–focal staining (25–75% of PTCs), and C4d3–diffuse staining (>75% of PTCs). The diagnosis of the histological phenotypes was established retrospectively, using the Banff 2019 criteria [25]. Borderline changes were diagnosed as foci of tubulitis ($t > 0$) with minor interstitial inflammation (i1) or moderate-severe interstitial inflammation (i2 or i3) with mild (t1) tubulitis. We identified aABMR$_h$ by positivity for the two first Banff criteria for active ABMR according to the Banff 2019 classification [25,26], not taking into account donor-specific antibodies or gene expression changes. Transplant glomerulopathy was defined as a cg score greater than 0. The severity of the cg lesions was

semiquantitatively scored according to the Banff categories based on light microscopy. When $aABMR_h$ and/or cg were present, we considered the biopsy as "$aABMR_h$/cg" positive. Baseline immunosuppression consisted primarily of tacrolimus, mycophenolic acid, and corticosteroids, with addition of induction therapy in higher risk patients. No desensitization therapies for HLA antibodies were used. Only very few cases with ABMR received specific therapy, as presented previously [19], because of the retrospective rescoring of the biopsy specimens and the lack of access to efficacious therapies.

This study was approved by the Ethics Committee of the University Hospitals Leuven (S64006) and the clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul. In addition, we adhere to the Declaration of Helsinki.

Statistical analysis

We reported demographics as means with standard deviations for continuous variables and as frequencies and proportions for categorical ones. Univariable and multivariable Cox proportional hazard models quantified the effect of pretransplant HLA-DSA and of HLA-DSA as a time-varying covariate on graft survival. Graft failure was defined as the loss of kidney transplant function, i.e., return to dialysis or retransplantation, and in case of death with a functioning graft, we censored at the time of death. In the time-varying Cox model, as in all consecutive time-dependent models, patients who did not develop *de novo* HLA-DSA were censored at 2 years after their last HLA-DSA measurement. The posttransplant evolution of histological phenotypes according to the HLA-DSA status was analyzed using multivariable logistic mixed models resulting in subject-specific odds ratio estimates. In these models, to account for the time-varying nature of HLA-DSA, we additionally estimated within- and between-patients effects, according to the mean-centering approach as proposed by Neuhaus and Kalbfleisch [27]. In all multivariable models, the confounders adjusted for were donor and recipient age, donor and recipient sex, race, cold ischemia time, recipient body mass index, donor type (living; donation after circulatory death; donation after brain death), repeat transplantation, and the number of HLA-ABDR mismatches. We applied joint longitudinal-survival modeling to, first, evaluate the effect of $aABMR_h$, cg, and $aABMR_h$/cg on graft failure (Figure 1a) and, second, to assess mediation of the effect of pretransplant HLA-DSA on graft failure via the occurrence of these posttransplant phenotypes (Figure 1b) (Supplemental Methods). As the status of HLA-DSA was less variable in time, we chose not to model its evolution. Instead, we included HLA-DSA as an exogenous time-dependent variable in subsequent models (Figure 1c). The same joint longitudinal-survival technique was applied in order to estimate the cumulative effect, as area under the logit curve, of $aABMR_h$ on the first occurrence of cg. SAS 9.4 (SAS Institute, Cary, NC, USA) was used for all statistical analyses, except for the joint models, which were fitted via the JMbayes package [28] in R.

RESULTS

Demographics

In total, 1000 transplantations were included in the study, with a median follow-up of 7.87 years (Table 1 and Figure S1). In 108 transplantations (10.8%), pretransplant HLA-DSA were present. Of these, 40 (37.0%) had antibodies against HLA class I, 42 (38.9%) against HLA class II, and 26 (24.1%) against both. The majority of HLA-DSA had a median fluorescence intensity above 2000, while 25 patients (23.1%) had an MFI between 500 and 1400 and 14 (13.0%) between 1400 and 2000. In the pretransplant HLA-DSA group, there were significantly more females and repeat transplantations. In 59 cases, pretransplant HLA-DSA resolved spontaneously after transplantation (despite not having received specific therapy for removal of these DSA) while 47 patients developed *de novo* HLA-DSA during follow-up (before graft failure). The timing of these DSA occurrences was reported in Table S1. In total, 3594 biopsies were performed in 941 patients, with 833 indication biopsies and 2761 protocol biopsies (Figure S1).

HLA-DSA as risk factor for graft failure

Patients with pretransplant HLA-DSA experienced more graft failures than patients without, according to a hazard ratio (HR) of 2.78 (95% CI, 1.95–3.94, $P < 0.001$) and 2.12 (95% CI, 1.41–3.20, $P < 0.001$) in the univariable and multivariable Cox model respectively (Figure S2a). HLA-DSA as time-varying covariate associated with graft failure, evidenced by an instantaneous HR of 3.29 (95% CI, 2.25–4.81, $P < 0.001$) and 2.72 (95% CI, 1.81–4.08, $P < 0.001$) in univariable and multivariable models, respectively (Table S2). When applying the

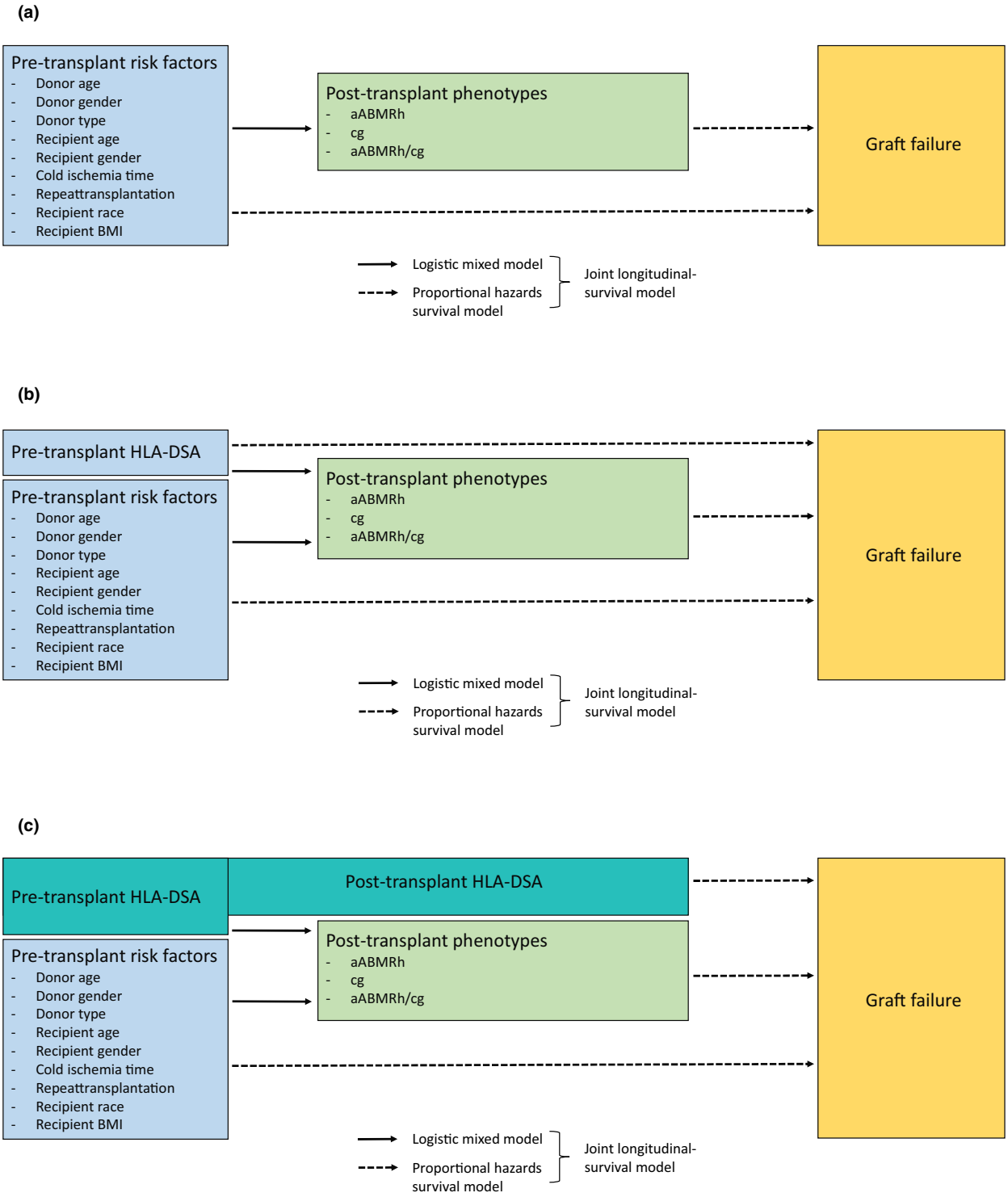

Figure 1 Conceptual schemes of the multivariable joint models for the association between the time-dependent histological phenotypes and death-censored graft failure, (a) excluding HLA-DSA, (b) including pretransplant HLA-DSA and (c) including time-varying HLA-DSA as a covariate. BMI = body mass index, aABMRh = histological picture of active antibody-mediated rejection, cg = transplant glomerulopathy, aABMRh/cg = histological picture of active antibody-mediated rejection and/or transplant glomerulopathy.

1400 MFI threshold for defining HLA-DSA positivity, comparable results were obtained. The univariable HR of pre-transplant HLA-DSA for graft failure (Figure S2b) amounted to 3.71 (95% CI, 2.60–5.32, $P < 0.001$) while the HR for time-varying HLA-DSA was 3.86 (95% CI, 2.62–5.67, $P < 0.001$).

aABMR$_h$ as risk factor for transplant glomerulopathy

We observed 121/3594 (3.4%) biopsies with cg in 88/941 (9.4%) patients while 401/3594 (11.2%) biopsies in 217/941 patients (23.1%) showed aABMR$_h$ (Table S3). At or before the first cg occurrence, 44 (50.0%) patients were diagnosed with aABMR$_h$. Of the patients who did not develop cg (n = 853), 171 (20.0%) ever experienced prior or concomitant aABMR$_h$ (Figure S3). On the basis of joint longitudinal-survival modeling, there was

Table 1. Demographic and clinical characteristics of 1000 renal-transplant donors and recipients at time of transplantation, according to pretransplant HLA-DSA status.

	Total (n = 1000)	No pretransplant HLA-DSA (n = 892)	Pretransplant HLA-DSA (n = 108)	P-value[¶]
Recipient demographics at time of transplantation				
Female sex – no. (%)	391 (39.1)	329 (36.9)	62 (57.4)	<0.001
Age – yr	53.7 ± 13.3	53.7 ± 13.2	53.9 ± 13.9	0.88
Repeat transplant – no. (%)	154 (15.4)	93 (10.4)	61 (56.5)	<0.001
Weight – kg	73.0 ± 14.7	73.3 ± 14.6	70.5 ± 14.9	0.06
Body mass index – (kg/m^2)	25.4 ± 4.5	25.4 ± 4.4	25.7 ± 5.0	0.48
Ethnicity				
Caucasian – no. (%)	984 (98.4)	879 (98.5)	105 (97.2)	0.40
African – no. (%)	12 (1.2)	10 (1.1)	2 (1.9)	0.38
Asian – no. (%)	3 (0.3)	2 (0.2)	1 (0.9)	0.29
Hispanic – no. (%)	1 (0.1)	1 (0.1)	0 (0.0)	1.00
No. of HLA-ABDR mismatches*	2.70 ± 1.31	2.69 ± 1.31	2.85 ± 1.24	0.21
A	0.96 ± 0.70	0.97 ± 0.70	0.94 ± 0.71	0.76
B	1.02 ± 0.64	1.01 ± 0.65	1.12 ± 0.59	0.10
DRB1	0.72 ± 0.56	0.71 ± 0.56	0.79 ± 0.56	.17
MFI*of the HLA-DSA				
500-1400	-	-	25 (23.1)	-
1400-2000	-	-	14 (13.0)	-
≥2000	-	-	69 (63.9)	-
Specificity of pre-transplant donor-specific HLA antibodies*				
Class I – no. (%) [MFI range]	-	-	40 (37.0) [589–12 257]	-
Class II – no. (%) [MFI range]	-	-	42 (38.9) [504–19 224]	-
Class I + II – no. (%) [MFI range]	-	-	26 (24.1) [984–13 285]	-
Immunosuppression regimen: TAC-MPA-CS*, n (%)	873 (87.3)	772 (86.6)	101 (93.5)	0.04
Induction therapy – n (%)	416 (41.6)	342 (38.3)	74 (68.5)	<0.001
Anti-CD25 – n (%)	363 (87.3)	304 (88.9)	59 (79.7)	0.03
Thymoglobulin – n (%)	25 (6.0)	15 (4.4)	10 (13.5)	0.003
Other – n (%)	28 (6.7)	23 (6.7)	5 (6.8)	0.99
Donor demographics				
Female sex – no. (%)	465 (46.5)	412 (46.2)	53 (49.1)	0.57
Age – yr	47.7 ± 14.8	47.8 ± 14.6	47.6 ± 16.8	0.92
Living donors – no. (%)	59 (5.9)	51 (5.7)	8 (7.4)	0.48
Donation after brain death – no. (%)	780 (78.0)	693 (77.7)	87 (80.6)	0.50
Donation after circulatory death – no. (%)	161 (16.1)	148 (16.6)	13 (12.0)	0.22
Cold ischemia time – hours	14.2 ± 5.7	14.1 ± 5.6	14.6 ± 5.8	0.45
Posttransplant data[†]				
Overall graft survival[‡]				
At 1 year – %	93.0	92.9	93.5	0.84
At 2 years – %	90.0	90.7	84.3	0.04
At 5 years – %	75.1	76.9	60.2	<0.001
Death-censored graft survival[§]				
At 1 year – %	95.2	95.3	94.4	0.70

Transplant International 2021; 34: 1824–1836

Table 1. Continued.

	Total (n = 1000)	No pretransplant HLA-DSA (n = 892)	Pretransplant HLA-DSA (n = 108)	P-value[¶]
At 2 years – %	93.9	94.5	89.7	0.06
At 5 years – %	89.0	90.9	72.9	<0.001

*HLA = Human Leukocyte Antigen, MFI = Median Fluorescence Intensity; TAC-MPA-CS = Tacrolimus-mycophenolic acid-corticosteroids.

[†]Based on complete follow-up

[‡]Overall graft survival = composite of graft failure and recipient death

[§]Death-censored graft survival = graft failure censored at time of recipient death with a functioning graft

[¶]P values are based on Chi-Square test for the categorical variables, on 2-sample t test for continuous variables and on the Log-Rank test for survival estimates.

a cumulative effect of aABMR$_h$ on the first occurrence of cg (HR 1.20, 95% CI, 1.12–1.30, P <0.001), even after controlling for confounders and for the effect of pretransplant HLA-DSA. In a sensitivity analysis restricted to the group of patients without pretransplant HLA-DSA (N = 837) and censored for *de novo* HLA-DSA occurrence, a similar effect was noted (HR 1.17, 95% CI, 1.06–1.32, P < 0.001), as was the case in the group with pretransplant HLA-DSA (N = 104) only (HR 1.14, 95% CI, 1.01–1.40, P = 0.03). On top of the pathophysiological argument that cg is related to repeated endothelial cell injury [7,29,30], these results evidence the temporal relation between aABMR$_h$ and cg, both in the presence and in the absence of HLA-DSA. In subsequent analyses, we consider aABMR$_h$ and/or cg together as aABMR$_h$/cg.

HLA-DSA as risk factor for aABMRh and transplant glomerulopathy

Based on the evaluation of all 3594 posttransplant biopsies, the 5-year aABMR$_h$-free, cg-free, and aABMR$_h$/cg-free survival were, respectively, 24.4%, 62.1%, and 20.8% in the HLA-DSA positive patients, versus 81.1%, 88.2%, and 73.4% in the HLA-DSA negative patients (censored for *de novo* DSA). In the multivariable logistic mixed models, patients with pretransplant HLA-DSA had higher probabilities of aABMR$_h$, cg, and aABMR$_h$/cg compared with pretransplant HLA-DSA negative patients (Table 2, upper part). We next evaluated the prevalence of aABMR$_h$, cg, and aABMR$_h$/cg according to the HLA-DSA status at the time of biopsy, hence taking into account both resolution and *de novo* occurrence of HLA-DSA (Figure 2). Patients with HLA-DSA had ABMR$_h$/cg in 42.5%, 43.2%, 37.9%, and 33.3% of the protocol biopsies at 3, 12, 24, and 60 months after transplantation, and in 56.9% of indication biopsies. In patients without HLA-DSA, aABMR$_h$/cg occurred in 6.4%, 7.7%, 10.4%, and 4.3% of protocol biopsies at 3, 12, 24, and 60 months after transplantation, and in 19.0% of indication biopsies. When considering HLA-DSA as a time-dependent covariate, its status not only differed between patients but also changed within patients. The between-patients effect compared the (logit of) probability of aABMR$_h$, cg, and aABMR$_h$/cg among patients who differed in their mean HLA-DSA status over posttransplant time, with significant odds ratios for aABMR$_h$, cg, and aABMR$_h$/cg (Table 2, lower part). The within-patients' effect constituted the change in the (logit of) probability when a patient develops HLA-DSA or vice versa, have resolution of HLA-DSA. These data could not confirm that patients with *de novo* HLA-DSA were more prone to cg development, or vice versa, that patients with resolution of HLA-DSA had a lower risk of cg development (Table 2, lower part). There were also between-patients effects for time-varying HLA-DSA on T-cell mediated rejection (TCMR) (OR 2.00, 95% CI, 1.22–3.27, P = 0.006), TCMR including borderline changes (OR 2.29, 95% CI, 1.49–3.51, P < 0.001) and mesangial matrix expansion (OR 1.87, 95% CI, 1.04–3.37, P = .04) and a significant within-patients effect on TCMR (OR 2.68, 95% CI, 1.04–6.91, P = .04) (Table S4). There was no association between HLA-DSA and isolated TCMR (excluding mixed rejections of aABMR$_h$ + TCMR), illustrating that the association with TCMR can be attributed to the mixed rejection cases.

Table 2. Between and within-patients effects of pre-transplant and time-varying HLA-DSA status on the occurrence of aABMRh, cg, and aABMRh/cg (n = 941).

Parameters	Outcome variable*	Between/Within		OR (95% CI)	*P*-value
Pre-transplant HLA-DSA	aABMRh	Between patients		22.7 (11.8–43.7)	<0.001
	cg	Between patients		5.76 (1.67–19.8)	0.006
	aABMRh/cg	Between patients		19.5 (10.6–35.9)	<0.001
Time-varying HLA-DSA	aABMRh	Between patients		28.3 (12.4–64.7)	<0.001
		Within a patient:	De novo	2.59 (1.04–6.51)	0.04
			Resolved	0.39 (0.15–0.97)	
	cg	Between patients		4.83 (1.04–22.4)	0.045
		Within a patient:	De novo	1.65 (0.17–16.1)	0.67
			Resolved	0.61 (0.06–5.89)	
	aABMRh/cg	Between patients		25.0 (11.6–54.1)	<0.001
		Within a patient:	De novo	3.06 (1.28–7.31)	0.01
			Resolved	0.33 (0.14–0.78)	

The estimates were based on multivariable logistic mixed models including a fixed effect of posttransplant time as a cubic b-spline with three inner knots, random intercepts, and linear random slopes for posttransplant time. Between- and within-effects were estimated via the mean-centering approach.

*Models were corrected for donor/recipient age, donor/recipient sex, race, cold ischemia time, BMI, donor type (living; DBD; DCD), repeat transplantation, and the number of HLA-ABDR mismatches.

$ABMR_h$ and transplant glomerulopathy as risk factors for graft failure

Next, we built a multivariable joint model to evaluate the effect of time-dependent $aABMR_h$, cg, and $aABMR_h$/cg on death-censored graft failure, initially excluding HLA-DSA as covariate (Figure 1a). $aABMR_h$ (Figure S4a), cg (Figure S4b) and $aABMR_h$/cg (Figure S4c) all significantly increased the hazard of death-censored graft failure (Table S5).

HLA-DSA causing graft failure mediated through $aABMR_h$ and transplant glomerulopathy

As HLA-DSA, $aABMR_h$, and cg were associated with the risk for graft failure, and HLA-DSA associated with the risk of $aABMR_h$ and cg, we next evaluated whether the association between HLA-DSA and graft failure was mediated through $aABMR_h$ and/or cg. In the joint models evaluating the association of $aABMR_h$, cg, and $aABMR_h$/cg with death-censored graft failure, pretransplant HLA-DSA was not retained as a significant determinant of graft failure (Figure 1b and Table 3). This suggested that the negative impact of pretransplant HLA-DSA was indeed mediated through the occurrence of $aABMR_h$ and cg (and $aABMR_h$/cg). However, when we added HLA-DSA as a time-varying variable to the model (Figure 1c), HLA-DSA significantly increased the risk for graft failure while conversely $aABMR_h$ lost significance (Table 3), indicating that the follow-up of antibodies is more important than the follow-up of $aABMR_h$ histology for determining graft failure. The same model applied to cg and $aABMR_h$/cg showed that, next to the impact of time-varying HLA-DSA, both phenotypes remained significantly associated with graft failure (Figure S4). The same conclusions were drawn when applying the MFI threshold of 1400 for HLA-DSA positivity (Table S6).

aABMRh and transplant glomerulopathy in the absence of HLA-DSA

The previous analyses suggested that patients with HLA-DSA had a higher risk for $aABMR_h$ and cg development, which in turn affected graft failure. However, patients without HLA-DSA also experience $aABMR_h$ and cg in their posttransplant histological follow-up. Given the higher prevalence of HLA-DSA negative patients, in absolute numbers, more $aABMR_h$ and cg were observed in this group than in the HLA-DSA positive patients (Figure 2). In the HLA-DSA negative group, a considerable absolute number of $aABMR_h$ was found in the indication biopsies before 14 days posttransplant (n = 54) and in the protocol biopsies up to 2 years (n = 45 at 3 months; n = 43 at 1 year; n = 46 at 2 years). The absolute number of cg was low overall,

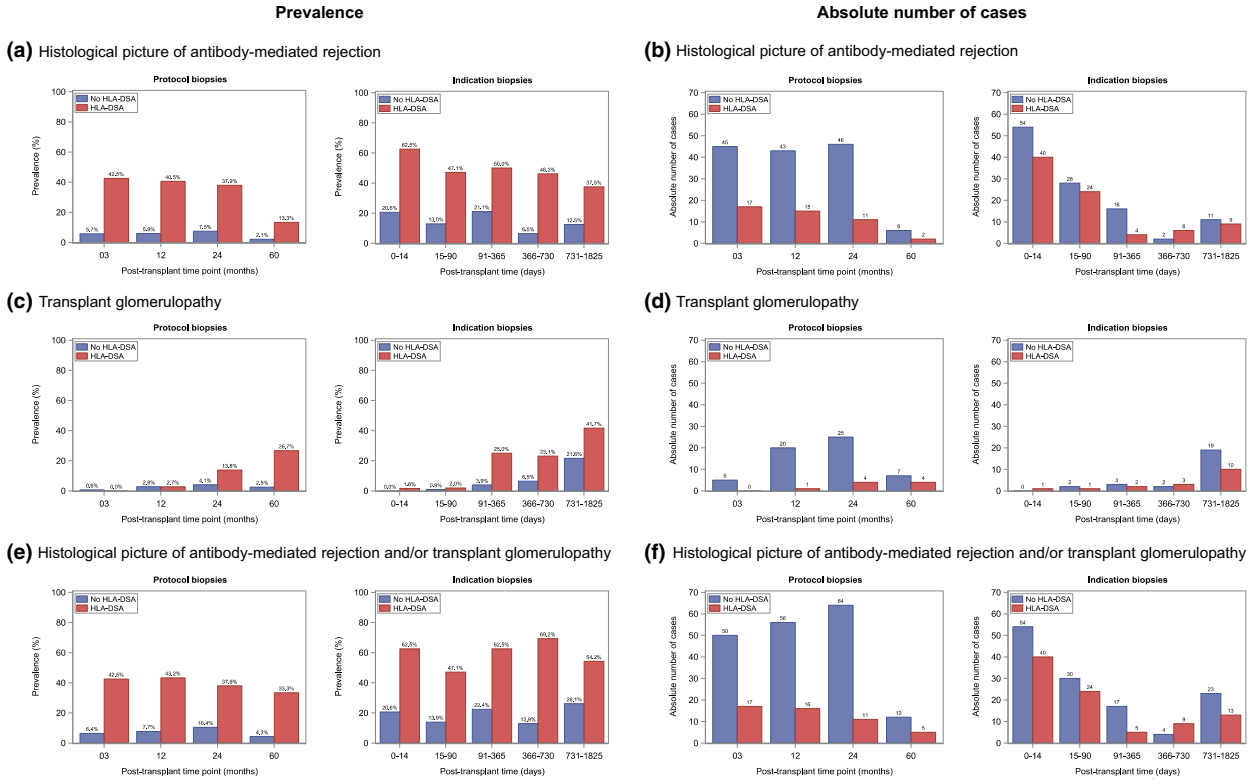

Figure 2 Association between DSA status and posttransplant histology (3594 biopsies in 941 transplantations). Prevalence according to DSA status at the time of biopsy for (a) the histological picture of antibody–mediated rejection (aABMR$_h$), (c) transplant glomerulopathy (cg), and (e) the histological picture of antibody–mediated rejection and/or transplant glomerulopathy (aABMR$_h$/cg) in protocol and indication biopsies, next to their absolute number of cases (b) for aABMR$_h$, (d) for cg and (f) for aABMR$_h$/cg).

yet in protocol biopsies in the HLA-DSA negative group, it increased to 25 at 2 years posttransplant (n = 5 at 3 months; n = 20 at 1 year) while in indication biopsies, most occurrences of cg were observed between 2 and 5 years posttransplant (n = 19). In a multivariable joint model applied to the pretransplant HLA-DSA negative patients only (Figure 1a), censored for *de novo* HLA-DSA occurrence, a negative effect on graft survival was found for cg and aABMR$_h$/cg, but not for aABMR$_h$ only (Table 4).

DISCUSSION

In this study, we evaluated the complex and time-dependent interplay between HLA-DSA, posttransplant aABMR$_h$, transplant glomerulopathy, and graft failure. We established that pretransplant HLA-DSA, as well as HLA-DSA in their posttransplant, time-varying nature, are important risk factors for graft failure. Furthermore, we demonstrated the cumulative impact of aABMR$_h$ on the occurrence of transplant glomerulopathy, and that the negative effect of pretransplant HLA-DSA is, at least partially, mediated through the occurrence of aABMR$_h$ and transplant glomerulopathy. Yet, when considering HLA-DSA as a time-dependent variable, only transplant glomerulopathy remained independently associated with graft failure; aABMR$_h$ on itself was not independently associated with impaired graft outcome in this respect. Finally, we demonstrated that these associations are also valid in patients without HLA-DSA, who can develop aABMR$_h$, associated with subsequent transplant glomerulopathy and decreased graft survival. However, if not evolving to transplant glomerulopathy, aABMR$_h$ was not identified as a risk factor for impaired graft outcome in HLA-DSA negative patients.

The finding that HLA-DSA associate with active lesions suggestive of ABMR is supported by extensive literature on this topic [6,31], including the association with its subclinical form on the short-term post-transplantation [32]. However, later after transplantation, we observed that the acute lesions of ABMR are replaced by transplant glomerulopathy and thereby confirmed this previously suggested association [33,34] using in-depth statistical evaluation of these time

Table 3. Association between time-dependent aABMRh, cg, and aABMRh/cg and death-censored graft failure, in multivariable joint longitudinal-survival modeling, including pretransplant and time-varying HLA-DSA as covariates (based on 3594 biopsies performed in 941 patients).

Model	Posttransplant phenotype	No phenotype occurrences	Parameters in the survival sub-model*	HR (95% CI)	*P*-value
Pretransplant HLA-DSA	aABMRh	401	Pretransplant DSA	1.34 (.64–2.69)	0.43
			aABMRh	1.20 (1.03–1.43)	0.02
	cg	121	Pretransplant DSA	1.46 (.74–2.88)	0.24
			Cg	1.33 (1.18–1.57)	<0.001
	aABMRh/cg	480	Pretransplant DSA	.92 (.42–1.91)	0.85
			aABMRh/cg	1.39 (1.18–1.66)	<0.001
Time-varying HLA-DSA	aABMRh	401	Time-varying DSA	4.03 (2.21–7.15)	0.002
			aABMRh	1.10 (.97–1.26)	0.13
	cg	121	Time-varying DSA	3.36 (1.97–5.85)	<0.001
			Cg	1.27 (1.14–1.47)	<0.001
	aABMRh/cg	480	Time-varying DSA	3.12 (1.82–5.93)	<0.001
			aABMRh/cg	1.24 (1.09–1.43)	<0.001

The estimates originated from separate joint models in which a longitudinal submodel (logistic mixed model) for the phenotypes was combined with a proportional hazards survival submodel for death-censored graft failure (events = 85).

*Survival and longitudinal sub-model were both corrected for donor/recipient age, donor/recipient sex, race, cold ischemia time, BMI, donor type (living; DBD; DCD), repeat transplantation, and the number of HLA-ABDR mismatches.

Table 4. Association between time-dependent aABMRh, cg, and aABMRh/cg and death-censored graft failure, in multivariable joint modeling in the group without pretransplant DSA (based on 3140 biopsies performed in 837 patients).

Parameters in the survival sub-model*	No. phenotype occurrences	HR (95% CI)	*P*-value
aABMRh	205	1.17 (0.97–1.43)	0.10
cg	79	1.25 (1.08–1.55)	0.004
aABMRh/cg	268	1.32 (1.07–1.61)	0.008

The estimates originate from separate joint models in which a longitudinal submodel for the phenotypes was combined with a proportional hazards survival submodel for graft failure. Censoring was applied at the time of *de novo* HLA-DSA occurrence (events = 56).

*Survival and longitudinal submodel were both corrected for donor/recipient age, donor/recipient sex, race, cold ischemia time, BMI, donor type (living; DBD; DCD) repeat transplantation, and the number of HLA-ABDR mismatches.

dependencies. This confirms the theory [6,30] and preclinical models [29,35] that transplant glomerulopathy arises through chronic persistent endothelial injury, evolving from acute lesions such as microvascular inflammation to chronic irreversible glomerular damage.

Although in patients with HLA-DSA, the majority of grafts experienced an episode of $aABMR_h$, when adjusted for the corresponding HLA-DSA evolution, graft failure was only significantly affected when transplant glomerulopathy was evident. Considering only pretransplant HLA-DSA, the effect on outcome seemed to be mediated through $aABMR_h$ episodes. The fact that this did not hold true when considering time-varying HLA-DSA instead of the stationary information on HLA-DSA only at time of transplantation is thought-provoking. Some transplants with pretransplant HLA-DSA may experience an early $aABMR_h$ episode, but later resolve their HLA-DSA spontaneously, preventing further injury and aborting the development of chronic irreversible damage. Our data indeed illustrated that the evolution of HLA-DSA (either resolving or *de novo* development) provides more information on graft outcome than a stationary measure of HLA-DSA at the time of transplantation. Only taking into account pretransplant HLA-DSA can be too simplistic, as HLA-DSA fluctuate over time. The significant effect of the evolution of HLA-DSA compared with the absence of effect when only considering pretransplant HLA-DSA in a model including biopsy follow-up could be related to two processes, probably both adding to this effect. On the one hand, the time-varying HLA-DSA variable takes into account *de novo* HLA-DSA, which have been stipulated to be more deleterious compared with pretransplant HLA-DSA in previous studies [13,16]. This also echoes a recent publication, which suggested that only *de novo* HLA-DSA predicted subsequent rejection in

patients without rejection [36]. On the other hand, more than 50% of the pretransplant HLA-DSA resolved during follow-up. Often, these resolving HLA-DSA are less harmful (more class I, lower MFI) compared with persistent HLA-DSA [11]. It therefore seems crucial to consider the kinetics of HLA-DSA over time, in order to understand their relevance.

Our study demonstrated that when HLA-DSA are included as time-dependent parameter, the diagnosis of aABMR$_h$ was not independently associated with graft failure. Although at first sight counterintuitive, this suggests that HLA-DSA exert their effects independent of the diagnosis of rejection, either by missed episodes of rejection (in between the staggered protocol biopsies) or through subtle lesions not fulfilling the complete Banff criteria for ABMR but nonetheless leading to chronic injury and graft failure. The fact that we showed that transplant glomerulopathy was retained as a predictor for graft failure independent of time-dependent HLA-DSA supports this last hypothesis. This is in apparent contrast to a recent paper, where neither HLA-DSA nor ABMR are associated with graft failure [36]. However, as this recent study was restricted to first biopsies that were primarily performed for graft dysfunction but that showed no rejection, the study design is not comparable to our strategy, and the interpretation of these data seems hampered by inherent selection bias [37]. Indeed, our study demonstrates that even in the presence of HLA-DSA, many of the episodes of ABMR and transplant glomerulopathy occur subclinically. Thus, limiting to indication biopsies only will inevitably lead to missing episodes of subclinical ABMR, which nonetheless will associate with transplant glomerulopathy and graft failure.

In addition to providing insights in the impact of HLA-DSA on rejection and graft failure, our study also highlights that the absolute number of aABMR$_h$ and transplant glomerulopathy episodes happening in HLA-DSA negative patients is not negligible. Although the probability of aABMR$_h$ and cg is lower in patients without HLA-DSA, the absolute number of cases of aABMR$_h$ and cg is greater in this much larger patient group than in the smaller HLA-DSA positive high-risk group. This is an illustration of the Rose paradox that describes the seemingly contradictory situation where the majority of cases of a disease come from a low-risk population of that disease while only a minority of cases come from the high-risk population of the same disease [38]. These absolute numbers will obviously differ in centers performing more high-risk transplantations, which explains center differences in the relative prevalence of HLA-DSA positive versus HLA-DSA negative aABMR$_h$/cg cases.

We also demonstrate that HLA-DSA negative aABMR$_h$ can progress to transplant glomerulopathy, which associates with graft failure. As aABMR$_h$ and cg are not uncommon in HLA-DSA negative patients, prevention of graft failure in this low-risk group should also be a focus of research, given the large size of this group. This again reminds us of the considerations put forward by Rose in his prevention paradox [38], which postulates that preventative strategies in the large low-risk group might have a larger effect on the outcome of the total population than preventative strategies focusing on a small subgroup of high-risk individuals. Applied to HLA-DSA negative cases with aABMR$_h$, research efforts could focus on the accuracy of HLA-DSA testing [23], on B-cell memory responses [39], on non-HLA alloimmunity [40], on direct NK cell activation through missing self [20], and on direct macrophage activation [41], and perhaps even on the nonspecificity of the histological lesions for underlying causes. Currently, it remains unclear which of these potential pathophysiologic processes are transient and do not progress to chronic irreversible injury, and which processes are deleterious, ultimately culminating in graft failure. In multiple studies on this same cohort, we showed that HLA-DSA negative ABMR/cg does not differ from ABMR/cg with HLA-DSA regarding histology or gene expression, but nonetheless has better outcome [11,19,42,43]. There is evidence supporting that the HLA-DSA negative cases represent a distinct entity, with a more transient histology of ABMR and less C4d deposition [19]. Concerning pathophysiology, recent studies indicate an important role for non-HLA DSA [44–48] and/or histological injury caused by the direct activation of natural killer cells because of missing-self recognition [20]. Nonetheless, since this etiology is not yet well established, we currently lack effective treatment for HLA-DSA negative ABMR/cg. Given that transplant glomerulopathy is an important determinant of outcome that often occurs subclinically, protocol biopsy studies that are not restricted to the short term after transplantation and that include HLA-DSA negative patients will convey important information in this regard.

In our cohort, we frequently observe HLA-DSA negative ABMR$_h$. An explanation for this high frequency could be sought in the high number of protocol biopsies, as well as within the methodological issues surrounding DSA detection. In all patients with ABMR$_h$, we carefully reevaluated all anti-HLA antibodies using

the same sensitive test, complemented with a retyping of the donors where necessary. We used a low MFI threshold of 500 for HLA-DSA positivity. Although some DSAs could have been missed, lower MFI cut-offs are rarely used, and could lead, because of the measurement fluctuation, to false positive DSA annotation. In addition, as shown previously [22], because of the high-resolution typing of donors in our cohort, we reclassified 32 former positive pretransplant DSAs to negative, thereby deflating the DSA frequency. Given the absence of second field HLA typing in many historical cohorts, we speculate that certain reported HLA-DSA were in fact not donor-specific, which would increase the proportion of HLA-DSA negative $ABMR_h$. Some HLA-DSA could also have remained undetected in the intravascular compartment because of homing. Yet, as recently shown [49], noncirculating HLA-DSA, solely detectable in the allograft, appear to be not so prevalent. Furthermore, non-HLA DSA and HLA-specific memory B cells, potentially present in the absence of circulating HLA-DSA [50–53], could explain some of our HLA-DSA negative $ABMR_h$ cases.

Despite the large number of biopsies, the inclusion of a large number of protocol biopsies, data on the evolution of HLA-DSA, and the use of advanced statistical modeling of the complex time-dependent associations, our study also has some limitations. This is a single-center cohort study in a Caucasian population with generally low risk transplantations, indicating that our results might not be generalizable to centers taking higher risks and using HLA-DSA desensitization strategies. We did not evaluate the evolution of the MFI over time but approached DSA as a dichotomous variable in the statistical analyses. Similarly, we considered $aABMR_h$ and cg only by light microscopy without electronic microscopy and as dichotomous variables, inherently losing information on the initial stages and severity of these lesions. Also, this compelled us to analyze these histological lesions on the logit scale. Consequently, by jointly modeling their longitudinal evolutions with a survival model for graft failure, the obtained hazard ratios have to be interpreted on this logit scale. Although an increase in the probability of the occurrence of the lesions translates in an increase of their logits, the point estimates of these associations remain difficult to interpret. Nevertheless, they do point out that, at any given time point after transplantation, an increased probability of $aABMR_h$, cg, or $aABMR_h$/cg leads to an increased hazard of kidney allograft failure.

We conclude that the evaluation of the kinetics of HLA-DSA is crucial to estimate the risk for graft failure, and that histological follow-up is necessary to discover, often subclinical, ABMR. Even without HLA-DSA, patients experience similar histological lesions, primarily in the early phase after transplantation. More research is necessary to decipher the causal processes underlying the heterogeneity in the evolution of histological changes suggestive of ABMR rejection after kidney transplantation, in order to take adapted preventative measures or provide better targeted therapies.

Authorship

Maarten Coemans: designed study, collected data, performed statistical analyses, interpreted results and wrote manuscript; Aleksandar Senev: collected data, produced and analyzed the HLA genotyping and anti-HLA antibody data; Elisabet Van Loon: collected data, interpreted results and wrote manuscript; Evelyne Lerut: performed biopsy readings; Ben Sprangers: collected data; Dirk Kuypers: collected data; Marie-Paule Emonds: assisted with producing the HLA genotyping and anti-HLA antibody data; Geert Verbeke: assisted with statistical analyses; Maarten Naesens: designed study, collected data, assisted with statistical analyses, interpreted results and wrote manuscript.

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Conflict of interest

All the authors declared no competing interests.

SUPPORTING INFORMATION

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

Table S1. Hazard ratios for death-censored graft failure, according to the univariable and multivariable Cox

models, including DSA as a time-dependent covariate (n=1000).

Table S2. Number and prevalence of aABMRh, transplant glomeruloptahy > 0, TCMR, TCMR including borderline changes, mixed rejections, arteriolar hyalinosis > 1, IFTA > 1, mesangial matrix expansion > 0 and vascular intimal thickening > 1 in protocol and indication biopsies (in 941 transplantations).

Table S3. Between and within-patients effects of pre-transplant and time-varying HLA-DSA status on the occurrence of TCMR, TCMR including borderline changes, arteriolar hyalinosis (> 1), IFTA (> 1), mesangial matrix expansion (> 0) and vascular intimal thickening (> 1) (3594 biopsies in 941 transplantations).

Table S4. Association between time-dependent aABMRh, cg and aABMRh/cg and death-censored graft failure, in multivariable joint modelling, excluding DSA as covariate (based on 3594 biopsies performed in 941 patients).

Table S5. Association between time-dependent aABMRh, cg and aABMRh/cg and death-censored graft failure, in multivariable joint modelling, including pre-transplant and time-varying DSA as covariate, based on a MFI threshold for DSA positivity threshold of 1400 (3594 biopsies performed in 941 patients).

Table S6. STROBE checklist.

Figure S1. Enrollment and renal allograft biopsies.

Figure S2. Kaplan-Meier curves for death-censored graft survival in the pre-transplant DSA positive versus pre-transplant DSA negative group, based on (a) an MFI value of 500 and (b) an MFI value of 1400 (n = 1000).

Figure S3. Association between aABMR$_h$ and the occurrence of transplant glomerulopathy.

Figure S4. Hazard ratios for graft failure of the median patient, according to the joint models excluding HLA-DSA, including pre-transplant HLA-DSA and including time-varying HLA-DSA, for (a) aABMR$_h$, (b) cg and (c) aABMRh/cg.

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