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

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Abbreviations

aABMR _h	histology of active antibody-mediated rejection
aABMR _h /cg	histology of active antibody-mediated rejection and/or transplant glomerulopathy
ABMR	antibody-mediated rejection
ADCC	antibody-dependent cellular cytotoxicity

cg	transplant glomerulopathy
HLA-DSA	donor-specific anti-HLA antibodies
HR	hazard ratio
MFI	median fluorescence intensity
OR	odds ratio
TCMR	T-cell mediated rejection

Conflict of interest statement

All the authors declared no competing interests.

Abstract

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 pre-transplant HLA-DSA were more prone to develop aABMR_h (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 aABMR_h/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 aABMR_h 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.

INTRODUCTION

Antibody-mediated rejection (ABMR) is a major threat for long-term kidney allograft survival [1,2,3,4]. ABMR is characterized by histological evidence of acute or chronic tissue injury, together with 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 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, due to a former transplantation, pregnancy or blood transfusion, or arise *de novo* after transplantation due to non-adherence 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 vs. resolving pre-transplant HLA-DSA, and pre-transplant vs. *de novo* HLA-DSA [11,13,14,15,16,17,18]. 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 [19,20,21], 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 post-transplant 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 XXXX 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 post-transplant renal allograft biopsies (protocol + indication biopsies), performed up to five 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 semi-quantitatively 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 semi quantitatively 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 semi-quantitatively 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], due to 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 XXXX (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 hazards models quantified the effect of pre-transplant HLA-DSA and of HLA-DSA as a time-varying covariate on graft survival. Graft failure was defined as 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 not developed *de novo* HLA-DSA were censored at two years after their last HLA-DSA measurement. The post-transplant 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 modelling 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 pre-transplant HLA-DSA on graft failure via the occurrence of these post-transplant 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 JMbays 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 Supplemental Figure 1). In 108 transplantations (10.8 %), pre-transplant 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 pre-transplant HLA-DSA group, there were significantly more females and repeat transplantations. In 59 cases, pre-transplant 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 were reported in Supplemental Table 1. In total, 3594 biopsies were performed in 941 patients, with 833 indication biopsies and 2761 protocol biopsies (Supplemental Figure 1).

HLA-DSA as risk factor for graft failure

Patients with pre-transplant 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 (Supplemental Figure 2A). 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 (Supplemental Table 2). When applying the 1400 MFI threshold for defining HLA-DSA positivity, comparable results were obtained. The univariable HR of pre-transplant HLA-DSA for graft failure (Supplemental Figure 2B) 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 (Supplemental Table 3). 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 (Supplemental Figure 3). On the basis of joint longitudinal-survival modelling, there was 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 pre-transplant HLA-DSA. In a sensitivity analysis restricted to the group of patients without pre-transplant 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 pre-transplant 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 aABMR_h and transplant glomerulopathy

Based on the evaluation of all 3594 post-transplant biopsies, the five-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, vs. 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 pre-transplant HLA-DSA had higher probabilities of aABMR_h, cg and aABMR_h/cg compared to pre-transplant 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 aABMR_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 post-transplant 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, $p=0.04$) and a significant within-patients effect on TCMR (OR 2.68, 95% CI, 1.04 – 6.91, $p=0.04$) (Supplemental Table 4). 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.

aABMR_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 (Supplemental Figure 4A), cg (Supplemental Figure 4B) and aABMR_h/cg (Supplemental Figure 4C) all significantly increased the hazard of death-censored graft failure (Supplemental Table 5).

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, pre-transplant HLA-DSA was not retained as a significant determinant of graft failure (Figure 1B and Table 3). This suggested that the negative impact of pre-transplant 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 (Supplemental Figure 4). The same conclusions were drawn when applying the MFI threshold of 1400 for HLA-DSA positivity (Supplemental Table 6).

aABMR_h 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 post-transplant histological follow-up. Given the higher prevalence of HLA-DSA negative patients, in absolute numbers, more aABMR_h and cg was 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 post-transplant (n=54) and in the protocol biopsies up to two years (n=45 at 3 months; n=43 at 1 year; n=46 at 2 years). The absolute number of cg was low overall, yet in protocol biopsies in the HLA-DSA negative group, it increased to 25 at two years

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post-transplant (n=5 at 3 months; n=20 at 1 year), while in indication biopsies most occurrences of cg were observed between two and five years post-transplant (n=19). In a multivariable joint model applied to the pre-transplant 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, post-transplant aABMR_h, transplant glomerulopathy and graft failure. We established that pre-transplant HLA-DSA, as well as HLA-DSA in their post-transplant, 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 pre-transplant 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 antibody-mediated rejection 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-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 pre-transplant 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 pre-transplant 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 pre-transplant HLA-DSA can be too simplistic, as HLA-DSA fluctuate over time. The significant effect of the evolution of HLA-DSA compared to the absence of effect when only considering pre-transplant 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 to pre-transplant 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 pre-transplant HLA-DSA resolved during follow-up. Often, these resolving HLA-DSA are less harmful (more class I, lower MFI) compared to 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_n 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 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 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 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_n and transplant glomerulopathy episodes happening in HLA-DSA negative patients is not negligible. Although the probability of aABMR_n and cg is lower in patients without HLA-DSA, the absolute number of cases of aABMR_n 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 vs. HLA-DSA negative aABMR_n/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 non-specificity 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 [44,45,46,47,48] and/or histological injury caused by the direct activation of natural killer cells due to 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 re-evaluated 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, due to measurement fluctuation, to false positive DSA annotation. In addition, as shown previously [22], due to the high-resolution typing of donors in our cohort, we reclassified 32 former positive pre-transplant 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 due to homing. Yet, as recently shown [49], non-circulating 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 [50,51,52,53], 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 modelling 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 modelling 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, antibody-mediated rejection. 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 antibody-mediated rejection after kidney transplantation, in order to take adapted preventative measures or provide better targeted therapies.

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TABLES

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

	Total (n=1000)	No pre- transplant HLA- DSA (n=892)	Pre-transplant HLA-DSA (n=108)	P value ^e
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 ^a	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	0.17
MFI ^a 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 ^a				
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 ^a , 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
Post-transplant data^b				
Overall graft survival ^c				
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 ^d				
at 1 year – %	95.2	95.3	94.4	0.70
at 2 years – %	93.9	94.5	89.7	0.06
at 5 years – %	89.0	90.9	72.9	<0.001

^aHLA = Human Leukocyte Antigen, MFI = Median Fluorescence Intensity,

TAC-MPA-CS = Tacrolimus-mycophenolic acid-corticosteroids

^b Based on complete follow-up

^c Overall graft survival = composite of graft failure and recipient death

^d Death-censored graft survival = graft failure censored at time of recipient death with a functioning graft

^e 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.

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

Parameters	Outcome variable ^a	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 Resolved	2.59 (1.04 – 6.51) 0.39 (0.15 – 0.97)	0.04
	cg	Between patients	4.83 (1.04 – 22.4)	0.045
		Within a patient: De novo Resolved	1.65 (0.17 – 16.1) 0.61 (0.06 – 5.89)	0.67
	aABMRh/cg	Between patients	25.0 (11.6 – 54.1)	<0.001
		Within a patient: De novo Resolved	3.06 (1.28 – 7.31) 0.33 (0.14 – 0.78)	0.01

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

Table 3. Association between time-dependent aABMRh, cg and aABMRh/cg and death-censored graft failure, in multivariable joint longitudinal-survival modelling, including pre-transplant and time-varying HLA-DSA as covariates (based on 3594 biopsies performed in 941 patients). The estimates originated from separate joint models in which a longitudinal sub-model (logistic mixed model) for the phenotypes was combined with a proportional hazards survival sub-model for death-censored graft failure (events = 85).

Model	Post-transplant phenotype	# phenotype occurrences	Parameters in the survival sub-model ^a	HR (95% CI)	P-value
Pre-transplant HLA-DSA	aABMRh	401	Pre-transplant DSA	1.34 (0.64 – 2.69)	0.43
			aABMRh	1.20 (1.03 – 1.43)	0.02
	cg	121	Pre-transplant DSA	1.46 (0.74 – 2.88)	0.24
			cg	1.33 (1.18 – 1.57)	<0.001
	aABMRh/cg	480	Pre-transplant DSA	0.92 (0.42 – 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 (0.97 – 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

^a 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 modelling in the group without pre-transplant DSA (based on 3140 biopsies performed in 837 patients). The estimates originate from separate joint models in which a longitudinal sub-model for the phenotypes was combined with a proportional hazards survival sub-model for graft failure. Censoring was applied at the time of *de novo* HLA-DSA occurrence (events = 56).

Parameters in the survival sub-model ^a	# 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

^a 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.

FIGURE LEGENDS

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 pre-transplant HLA-DSA and (C) including time-varying HLA-DSA as a covariate.

BMI = body mass index, aABMR_h = histological picture of active antibody-mediated rejection, cg = transplant glomerulopathy, aABMR_h/cg = histological picture of active antibody-mediated rejection and/or transplant glomerulopathy.

Figure 2. Association between DSA status and post-transplant 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).

FIGURES

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 pre-transplant HLA-DSA and (C) including time-varying HLA-DSA as a covariate.

A

B

C

Figure 2. Association between DSA status and post-transplant histology (3594 biopsies in 941 transplantations).

Prevalence

A. Histological picture of antibody-mediated rejection

Absolute number of cases

B. Histological picture of antibody-mediated rejection

C. Transplant glomerulopathy

D. Transplant glomerulopathy

E. Histological picture of antibody-mediated rejection and/or transplant glomerulopathy

F. Histological picture of antibody-mediated rejection and/or transplant glomerulopathy