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Clinical significance of alloantibodies in hand transplantation – a multicenter study

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Abbreviations: Acute rejection, AR; Antibody mediated rejection, AMR; Antibody negative, AB-; Antibody positive, AB+; Disabilities of the Arm, Shoulder and Hand, DASH; Donor-specific antibodies, DSA; Hand Transplant Scoring System, HTSS; Human Leukocyte Antigen, HLA; T-cell mediated rejection, TCMR; Upper extremity transplantation, UET; Vascularized Composite Allotransplantation, VCA

Abstract

Background: Donor-specific antibodies (DSA) have a strong negative correlation with long-term survival in solid organ transplantation. Although the clinical significance of DSA and antibody-mediated rejection (AMR) in upper extremity transplantation (UET) remains to be established, a growing number of single-center reports indicate their presence and potential clinical impact.

Methods: We present a multicenter study assessing the occurrence and significance of alloantibodies in UET in reference to immunological parameters and functional outcome.

Results: Our study revealed a high prevalence and early development of de novo DSA and non-DSA (43%, the majority detected within the first three post operative years). HLA class II mismatch correlated with antibody development, which in turn significantly correlated with the incidence of acute cellular rejection. Cellular rejections preceded antibody development in almost all cases. A strong correlation between DSA and graft survival or function cannot be statistically established at this early stage but a correlation with a lesser outcome seems to emerge.

Conclusions: While the phenotype and true clinical effect of AMR remain to be better defined, the high prevalence of DSA and the correlation with acute rejection highlight the need for optimizing immunosuppression, close monitoring and the relevance of an HLA class II match in UET recipients.

Introduction

Fueled by advances in prophylaxis and treatment of allograft rejection, organ transplantation and, more recently, vascular composite allotransplantation (VCA) have evolved rapidly.[1,2] Rejection episodes are however common, and most VCA recipients have experienced one or multiple episodes of acute skin rejection (AR).[3,4] In kidney,[5,6] pancreas,[7] heart,[8] lung,[9] and liver[10] transplantation, the existence of two mechanistically separate, although overlapping presentations of T cell-mediated (TCMR), and antibody-mediated (AMR) forms of rejection have been acknowledged. The diagnosis of rejection in hand transplantation is currently based on a five-graded histological classification system. Features indicating rejection include lymphocyte infiltration (including neutrophils) in the skin with epidermal and/or adnexal structures, dyskeratosis, epithelial apoptosis, and necrosis.[11,12] Although the clinical relevance of donor-specific antibodies (DSA) and AMR in VCA has not been established, there is an increasing number of single-center reports on the occurrence of DSA and B-cell aggregates in upper extremity allografts[13-15] indicating a need for a comprehensive appraisal of AMR in this novel field. Extrapolating from solid organ transplantation, a long-term negative effect of DSA on graft survival and function remains a threat. We report the results from the first multicenter study evaluating the prevalence of DSA in upper extremity transplantation (UET) recipients, and an association of DSA development with rejections and with the outcome.

Materials and Methods

Patient selection

This retrospective multicenter cohort study was based on a study specific collection of data from patients included in the International Registry on Hand and Composite Tissue Transplantation (IRHCTT), and from two additional cooperating single centers. All hand transplant centers known in the public domain as of July 12 2016, were invited to participate in this study. Immunological data including the assessment of DSA on 45 hand transplanted

patients from 15 centers were available for analysis. Patients with combined transplantations, such as hand and face or leg transplants, and patients with graft survival of less than three months were excluded. Eventually, data from 44 patients were considered suitable for statistical analysis. For functional outcome assessment, eight patients were excluded since they did not meet the twelve months follow-up criteria as assessed by the HTSS (Hand Transplant Scoring System) and/or DASH (Disabilities of the Arm, Shoulder and Hand) scores,[16] Functional data were available for 36 patients (Figure 1). The Regional Ethical Review Board in Stockholm, Sweden, approved the study (dnr: 2016/233-31/1).

HLA-matching, antibody detection, and acute rejection episodes

Class-I (HLA-A, HLA-B) and -II (HLA-DR) typing and HLA-mismatches were collected from all donor-recipient pairs. Antibody analyses were performed at local accredited laboratories. A test was considered positive as per local cut-off values (Table S1, SDC, http://links.lww.com/TP/B693). Patients with at least one positive test, either before or after transplantation were classified as antibody positive (AB+), regardless of antibody number and subtypes. The antibodies detected were hierarchically organized based on the presence of donor specificity. Donor specific antibodies (DSA) were subsequently divided into DSA class I, class II, and class I+II groups. All other antibodies, including non-DSA and non-HLA antibodies, were collectively referred to as "Other". Patients were classified as antibody negative (AB-) when all samples tested negative. Acute rejection (AR) episodes were registered as per the postoperative day and severity determined according to the five grades of the Banff CTA-07 classification.[11]

Immunosuppression treatment

Calcineurin inhibitor (CNI) use either throughout or conversion to other immunosuppressants than CNI, Tacrolimus target trough levels (>5ng/mL or <5ng/mL), were collected from all patients. Steroid treatment throughout, and temporary or complete discontinuation was also recorded.

Correlation Analysis

The cumulative incidence of ARs was compared using Kaplan-Meier estimates for multiple groups. Rejection episodes were grouped as grade I or higher, grade II or higher, and grade III or higher, respectively, while patients were divided into the following subgroups according to their antibody status: DSA-positive patients (DSA+), non-DSA and non-HLA-antibody positive patients ("Other"), and antibody negative patients (AB-). The DSA-positive patients were subdivided into groups of class I, class II and class I+II. The time distribution of de novo antibody formation was established by plotting the post operative day of the first antibody detection for each patient, using Kaplan-Meier estimates. Subsequently, the time association between ARs and the occurrence of de novo antibodies was also assessed with Kaplan-Meier estimate, correlating the time points against each other. No distinction was made regarding uni- or bilateral transplants for antibody analyses, but taken into account for the functional assessment.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 24 was used in all statistical analyses. Independent Samples T-test was used to compare AR grade I-IV means between AB+ and AB- patients. The ANCOVA model was used to adjust for confounders including the following as fixed factors: antibody status (AB+/AB-), recipient age (divided into age groups 21-35, 36-50, and 51-65), recipient gender and transplant type (unilateral/bilateral).

Incidence of (time to) graft rejection and antibody detection was evaluated by Kaplan-Meier estimates, and statistical significance determined using log rank testing. The survival data was further analyzed using the Cox proportional hazards regression, estimating the hazard ratio for DSA+ vs. AB- and "Other" vs. AB- using a multivariate model adjusting for the same age and gender factors as in the ANCOVA model. A hazard ratio (HR)=1 indicates no difference between groups. A HR>1 indicates a shorter time to rejection and thus a higher risk for rejection.

Chi-square test was used to evaluate the association of HLA-mismatches, antibody development and ARs. AB development was first analyzed per a univariate model followed by a multivariate logistic regression model adjusting for the same age and gender factors as in the ANCOVA model.

For functional outcome assessment, mean HTSS and DASH scores were calculated both per year and per patient. Delta HTSS and DASH scores were calculated by comparing the first and the last registered measurement. A higher HTSS score represents better outcome while a higher DASH score represents greater patient disability.[17] The minimum detectable DASH change at the 95% confidence interval (MDC_{95}) to discriminate if a patient's condition has worsened or improved, is 15 points.[18] All values were compared between AB- and AB+ patients using ANCOVA covariate analysis with adjustment for age, gender and transplant level. Uni- and bilateral transplants were analyzed separately. For AB+ patients, HTSS/DASH delta scores before and after AB-positivity were calculated and compared using paired T-test.

P-values less than 0.05 were considered significant (*$p<0.05$; **$p<0.01$; ***$p<0.001$), where *n.s.* denotes not significant. Data were expressed as means ± standard deviation (SD), unless stated otherwise. Descriptive variables were summarized as min-max and frequencies.

Results

Recipient and donor characteristics

Out of the 45 eligible patient datasets, one patient was excluded due to graft loss from technical failure within one week post transplant. The mean follow-up was 6.3 years (range 160 days to 17.5 years). Bilateral transplants were more common than unilateral transplants, and recipients were predominantly male (Table 1). Two patients (5%) were pre sensitized with DSA class II (n=1), and DSA class I+II (n=1) antibodies, respectively. Twenty patients developed de novo antibodies. In this AB+ cohort, 64% of patients were DSA positive (n=14), constituting two DSA class I, six DSA class II, and six DSA class I+II double positive patients, respectively. The remaining AB+ patients (n=8, referred to as "Other") had developed non-DSA (n=7) and non-HLA (n=1, anti-angiotensin II type 1 receptor) antibodies (Figure 1, left flow diagram). No major differences in recipient and donor age were identified between AB^+ and AB^- patients. No candidates were transplanted with a positive perioperative crossmatch.

Acute rejection episodes and antibody development

Development of ABs correlated significantly with the mean number of grade II rejection episodes per follow-up year (Table 2, top section). Adjusting for recipient age, gender and transplant type, the correlation between AB development and grade II rejections was still significant (p=0.019). The one grade IV rejection was observed in an AB+ patient. Four patients suffered graft losses, all in the AB+ group (on POD 278, 500, 771 and at 11 years due to severe confluent vasculopathy, rejection upon suspected non compliance, rejection, and unknown cause, respectively) (Table 2, bottom section). Their antibodies developed POD 297 (DSA class I/II, identified after amputation), 380 (DSA class II), 16 (non-DSA class I), and 6.5 years (DSA class II), respectively. The two pre sensitized patients developed rejection at day 6, grade II (preformed DSA class II) and at day 18, grade II (preformed DSA class I+II).

Despite the different histological findings, AMR and/or vasculopathy has to be considered the most likely cause for graft loss in all cases.

Among AB- patients, 59% experienced grade I and II rejections and 36% grade III rejections. Among AB+ patients 59% experienced grade I rejection, 82% grade II rejection, and 45% grade III rejection (Table 2, middle section). The most common antibodies were de novo DSA class II (n=12), followed by de novo DSA class I (n=8), which together constituted 51% of all formed antibodies. The remainder was made up by non-DSA (30.8%), pre transplant DSA (7.7%), pre transplant non-HLA antibodies (2.6%) and de novo non-HLA antibodies (5.1%) (Table 3).

Patients with DSA showed a higher number of rejections and more severe rejection episodes compared to the corresponding AB- group. The presence of any alloantibodies (DSA positive or "Other") showed significant differences in the cumulative incidence of grade I rejection episodes or higher (Figure 2A). Adjusting for recipient age, gender and transplant type, the hazard ratio between the group "Other" and development of grade I rejection was HR 2.9 (p=0.025), indicating that the risk of developing grade I rejection tends to be almost three times higher for the group "Other" compared to AB- patients. Patients with DSA showed a trend towards more grade II rejection episodes, while no apparent correlation was observed for grade III rejection episodes containing fewer events (Figure 2B-C). All AB+ patients experienced at least one grade I rejection (Figure 2A), while two of the AB- patients were rejection-free. None of the individual DSA classes was significantly correlated with the incidence and severity of rejection (Figure 3). Patients with DSA class I, however, showed a trend (*n.s.*) to develop earlier and higher-grade rejections (Figure 3C).

Although de novo antibodies were found to appear at any point during the post transplant period, about 50% were detected within the first three years. No correlation between time point and type of antibody was found (Figure 4). To address the sequence of events, the day

of de novo alloantibody detection was plotted against the occurrence of AR grade I-III (Figure 5). The majority of rejection episodes were diagnosed prior to alloantibody development indicating that a repetitive and/or ongoing immune response triggers the development of DSA rather than DSA preceding additional and or more severe cellular rejections.

Antibody expression changes were identified in at least two de novo AB+ patients converting back to AB-, and remained AB-. Two additional patients seroconverted twice. Five patients presented with multiple AB+ positive tests. Among the AB- patients, three presented with multiple negative tests up to 6 years postoperatively.

Effect of HLA matching on antibody development and acute rejections

The highest risk for de novo AB development was seen in patients with a two loci HLA-DR mismatch, while mismatches in the HLA-A and HLA-B loci did not correlate (Table 4). A univariate model analysis showed a higher risk for postoperative AB development among subjects with two HLR-DR mismatches compared to those with one mismatch (OR=4.7, p=0.041). Further analysis using multivariate logistic regression model adjusting for recipient age, gender and transplant type, revealed an OR similar for subjects with two mismatches (OR=4.5, p=0.07). The slightly larger p-value can be explained by the lower power when including all four factors in the same model, but also indicates the stability in the OR estimates.

Functional outcome

The overall number of patients and the follow-up time differed widely between patients. Although most patients were evaluated with HTSS/DASH scores, this was not done consistently. Some transplants were done before the introduction of HTSS, leading to lack of early HTSS scores in some patients. The total mean follow-up period for patients evaluated for functional outcome (Figure 1, right flow diagram) was 6.3 years (range 2-17). Per patient HTSS/DASH mean scores for uni- and bilateral transplants, showed no significant differences

between AB+ and AB- patients. HTSS/DASH means scores per follow-up year for all patients combined showed a significant difference between DASH scores for AB- and AB+ bilateral recipients. Mean DASH scores for all follow-up years combined: 26.84_{AB-} (±22.77) vs 36.02_{AB+} (±18.7) (p=0.006). This result was adjusted for age, gender and transplant level, and does not seem to indicate a clinically meaningful change based on the MDC_{95} requirement. Patients with ≥2 follow-up years (n=14) were included for per patient delta value analysis between first and last HTSS/DASH score. No significant differences were detected for uni- or bilateral recipients.

Next, delta mean HTSS/DASH values before and after change in AB status were compared. Several patients were excluded due to insufficient number of follow-up assessments, and both bilateral and unilateral transplants were therefore combined in the same group. HTSS scores (n=8) were 2.37_{AB-} (±3.42) vs $\Delta\text{-}12.87_{AB+}$ (±10.78) (p=0.018), and DASH scores (n=7) were $\Delta\text{-}6.34_{AB-}$ (SD ±14.42) vs $\Delta 1.37_{AB+}$ (±5.20) (p=0.4). Mean time until AB status change was 4.6 years (range 1-13). Even though HTSS showed a significant functional reduction following AB status change, it is difficult to exclude the impact of natural variation on post operative functional improvement.

Immunosuppression

All patients were treated with CNI. Tacrolimus target levels were >5ng/mL throughout in all centers except two: one targeted Tacrolimus levels <5 ng/mL 18 months post transplant (n=1), and one targeted between 4-8ng/mL after month three (n=1). Two patients had a temporary CNI cessation due to toxicity not linked to DSA development. One patient was switched to Belatacept two years post transplant, with preserved antibody status. One patient was temporarily switched to Rapamycin for ten months, one was converted from CNI to rapamycin, and two patients received rapamycin in addition to CNI, Cellcept, and prednisolone following repeated rejections. In the latter two patients Tacrolimus >5ng/mL at

the time of rejection. All patients except five received prednisolone from the start, of which six patients were gradually tapered and eventually discontinued. Prednisolone was temporarily discontinued in two patients. Out of five recipients treated with steroid-sparing regimens, three received Campath induction.

Discussion

Since the introduction of UET almost two decades ago, more than 100 hand transplants have been performed. It is well established that DSA can be detrimental to outcomes of several other types of solid organ allografts.[19] While patients can be pre sensitized by alloantigens from events such as previous transplantations, blood transfusions, and pregnancy, DSA more commonly emerge after transplantation. Experimental animal models have suggested a tentative role of pre existing DSA also in VCA.[20,21] An increasing number of single centers report the occurrence of DSA in human clinical UET. This is the first study comprehensively addressing the prevalence of DSA and a correlation with AR and the functional outcome in VCA worldwide.

Our data indicate that an immunogenic organ such as the hand,[22] with tissues of several different embryological origins is prone to develop alloantibodies (Figure 1). One potential confounder to the variation in antibody occurrence is center-dependent sampling differences. Data availability currently limits the resolution of describing VCA antibody dynamics. If anything, it is likely that the antibody prevalence in our study is underestimated since no standardized follow-up program exist. We identified an association between HLA class II mismatches and antibody development (Table 4). However, this study only takes HLA-DR class II typing into account. More detailed HLA studies, including also HLA-C, -DQ, and -DP mismatches, and molecular HLA typing, may add to our understanding of class II mismatch effect on antibody development. It is thus conceivable that some antibodies classified as non-DSA herein may be re-classified as actual DSA. The majority of antibodies were detected

within the first three post operative years (Figure 4). While the total number of HLA mismatched may impact on the occurrence of rejection,[4] the correlation with DSA development was only significant for HLA-DR in our assessment.

Importantly, we found that AB+ patients had a significantly higher incidence of rejection episodes grade I or higher when compared to AB- patients (Figure 2A). In other solid organ transplants, de novo DSA are mainly class II and lead to worse clinical outcome when compared to DSA class I antibodies.[23] While class II DSA are also the more common de novo antibodies in this series (Figure 1, Table 3), DSA class II was not more often linked to acute rejection than DSA class I (Figure 3 A-C). AB- negative patients did not invariably present with rejections, while all patients with DSA, non-DSA, and non-HLA antibodies experienced grade I rejection or higher within the first year post transplant (Figure 2A).

Comparisons of the time of antibody detection and cumulative rejection incidences (Figure 5A-C) suggests that almost all cellular rejection episodes occur before antibodies develop and/or are detected. There are however potential caveats to this conclusion. Not all centers have routinely run the diagnostics for alloantibodies. Hence, there is a chance that antibodies may have developed before the day of first detection. It is also known that cell-mediated responses commonly precede the antibody-mediated response.[24-26] It is not unlikely, that AMR also overlap with T cell–mediated rejection.[27] As no accepted AMR consensus criteria exist, we are humble to the fact that it remains to be validated to what extent diagnosed cellular changes constitute a causality with antibody development, AMR, and how often they embody separate events.

Although beyond the scope of this study, it will be important to understand contributing factors for alloantibody development in VCA. One such factor is non adherence, which can increase the risk of antibody development in solid organ transplantation. Non adherence has been described as relevant in VCA and may play a role.[14,28] Under-immunosuppression is a

significant immunological risk factor in kidney transplantation.[29,30] In this study, Tacrolimus levels >5ng/mL were not entirely sufficient to mitigate antibody development in all cases. Prednisolone management was quite different among the centers, without any obvious correlation to antibody status. The optimal immunosuppression regimen and levels to limit the risk for DSA development in VCA remain unknown at this point. In a cohort of 32 combined intestinal and vascularized composite allotransplantations (abdominal wall grafts), a clear relationship between development of late de novo DSA (>12 months post transplant) and changes either in immunosuppression and/or the adherence of the recipient was recently established.[31] Other factors possibly contributing to the DSA development are infections and the intensity of immunosuppression.[29,32]

Despite the use of C4d staining as a marker for AMR in kidney transplantation, the value of this method remains debatable. C4d staining has been applied in VCA, but the outcomes in the few reported cases were inconsistent. C4d staining was positive in areas showing rejection but also in areas showing no rejection, as well as normal host skin.[12,33] Firm conclusions about its usefulness in VCA can therefore not yet be made. In a case report of a patient with skin rejection and high proportions of CD20+ B-cells in the biopsy plus high levels of DSA, rituximab treatment resulted in remission of clinical symptoms, and disappearance of B-cell aggregates and DSA.[13]

With increased follow-up times in VCA patients, sufficient data will eventually be available to ascribe chronic rejection (CR) specific characteristics and develop consensus criteria. This will be the basis to evaluate the potential link between alloantibodies and CR. Results from preclinical models and clinical case reports already indicate that chronic rejection can be detrimental to graft function.[20,34-37] According to the fourth update of the IRHCTT reported that there was a slight trend towards functional deterioration with time, especially on the 7th to 9th year post transplantation.[3] This is related to the development of possible features of CR

in some cases. Other conditions associated with the transplantation procedure, namely cold preservation as compared to normothermic preservation, favored CR in experimental hind-limb transplants with sub-optimal immunosuppression.[38]

Although not the main scope of this study, we evaluated the presence of antibodies in relation to the functional outcome. The results indicate trends towards a difference in functional outcome over time. However, no statistical significant differences could be seen when comparing mean HTSS/DASH scores per patient (Figure S1, SDC, http://links.lww.com/TP/B693). This is not unexpected due to the small number of patients and disparate number of follow up years. Using delta scores, analysis of improvement in functional outcome identified the largest score difference in bilateral patients followed with HTSS. We also found a significant decrease in delta HTSS scores in AB+ patients following the change in AB status. However, our knowledge of the HTSS scoring system's consistency and reproducibility in detecting functional differences over time is limited, and these data should therefore be interpreted cautiously. Furthermore, due to the few collected data points before and after AB status change we could only analyze a small subset of patients. The trend towards a potential poorer functional outcome in AB+ recipients therefore requires further evaluation in larger patient cohorts with longer follow-up times, and an assessment if observed differences are clinically meaningful.

Taken together, our data supports the need for a structured and standardized follow-up of all VCA patients including the assessment of alloantibodies, DSA and AMR. We propose advancing the criteria for definition of AMR in VCA and add this to the existing Banff classification for VCA. Considering that almost all AB+ patients in our cohort had a previous TCMR, it is likely that TCMR indicates a higher risk for antibody development. Possibly, the pro-inflammatory milieu created by the TCMR trigger a clonal expansion of memory B cells,[26] followed by the development of de novo antibodies.

For accurate monitoring, we suggest monthly clinical visits for six months after a DSA positive rejection. A VCA patient has the advantage that the organ is accessible to close clinical examination, early rejection detection, and course assessment. Clinical evaluation of the allograft should include a skin biopsy in addition to the determination of the presence of swelling and/or edema, pain, or changes in sensation. If AMR is suspected, a deep tissue biopsy should be taken, as standard punch biopsies carry the risk of missing areas affected by CR in the deeper tissues.[39] The biopsy is then examined in H&E for TCMR criteria according to the current AR Banff classification, but augmented with immunohistochemistry for CD3, CD4, CD8, CD19, CD20, C4d, IgM, and IgG antibodies. To understand more comprehensively a potential link between alloantibodies and the impact on CR, the clinical examination and deep tissue punch biopsy should be complemented by Doppler-ultrasound, high resolution ultrasound imaging and/or MRI to investigate arteries for intimal thickening and luminal narrowing. In case DSA appear in a routine follow-up after VCA without any clinical and histological signs of AR and/or CR rejection, further antibody monitoring in the subsequent years seems advisable. Irrespective of whether the detected alloantibodies are incidental findings, all DSA should be investigated further for antibody specificity and mean fluorescence intensity by accredited laboratories.

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Figure Captions

Figure 1. Patient selection. (Left flow diagram) 45 patients met inclusion criteria for antibody

analysis, of which 50% were positive for alloantibodies. The group "Other" included seven non-DSA, and one non-HLA antibody positive patient, respectively. (Right flow diagram) 36 patients met inclusion criteria for functional analysis, using HTSS and DASH scores. Subgrouping was based on unilateral/bilateral transplant procedures, and antibody status (AB+/AB-). Abbreviations: Antibody positive (AB+), antibody negative (AB-), DSA (donor specific antibody), Hand Transplantation Scoring System (HTSS), Disability of Arm Shoulder and Hand (DASH).

Figure 2. Cumulative incidences of acute rejection grade I, II, and III. Kaplan Meier comparisons between the AB+ patients divided into DSA positive patients (n=14), non-HLA and non-DSA positive patients (n=8, collectively referred to as "Other"), and AB negative patients (n=22). (A) 14/14, 8/8, 20/22, of DSA positive, "Other", and AB negative patients, developed grade I rejection or higher, respectively. (B) 14/14, 6/8, 15/22, of DSA positive, "Other", and AB negative patients, developed grade II rejection or higher, respectively. (C) 8/14, 3/8, 8/22, of DSA positive, "Other", and AB negative patients, developed grade III rejection or higher, respectively. Statistics: significant, p-value < 0.05* (log rank test). Abbreviations: antibody (AB), donor specific antibody (DSA).

Figure 3. Cumulative incidence of acute rejections among DSA subtypes. Kaplan Meier comparisons between DSA positive patients divided into three subgroups: DSA class I (n=2), DSA class II (n=6), and DSA class I+II (n=6). There was no statistically significant difference on the impact of the DSA subclasses on rejections of grade I - III (A-C). Statistics: significant, p-value < 0.05* (log rank test). Abbreviations: donor specific antibody (DSA).

Figure 4. Timely distribution of de novo antibody development. The graph shows Kaplan Meier comparisons for the 20 patients with de novo alloantibodies: DSA class I (n=2), DSA class II (n=6), DSA class I+II (n=4), non-HLA and non-DSA (n=8, collectively referred to as "Other"). Two patients were pre-sensitized with DSA, and were therefore omitted in the analysis. Statistics: significant, p-value < 0.05* (log rank test). Abbreviations: donor specific antibody (DSA).

Figure 5. Cumulative incidences of acute rejection grade I, II, III, in relation to the day of

de novo antibody detection. Rejections plotted against the day of antibody confirmation, i.e. the day patients tested positive for *de novo* alloantibodies for the first time (marked by vertical line at day 0). Kaplan Meier comparisons were performed between patients positive for DSA class I (n=2), DSA class II (n=6), DSA class I+II (n=4), and non-HLA and non-DSA (n=8, termed "Other"). (A) Grade I rejection or higher, (B) grade II rejection or higher, and (C) grade III rejection or higher. Abbreviations: antibody (AB), donor specific antibody (DSA).

Table 1. Recipient and donor demographics included for antibody analyses.

		Mean (SD)	Min	Max
Duration of follow-up (years)		6.3 (4.5)	0.44	17.5
Age	Recipient	39 (12)	21	65
	Donor	34 (13)	14	59
		N		
Gender	Recipient	4 (F), 40 (M)		
	Donor	6 (F), 38 (M)		
Type of transplantation	Unilateral	18		
	Bilateral	26		

Abbreviations: Female (F), Male (M)

Table 2. Acute rejection episodes and graft losses. (Top section) Mean number of grade I-IV rejections (±SD) among AB+ and AB- patients per year of follow-up. (Middle section) Number of patients experiencing grade I-IV rejections, and the total number of grade I-IV ARs. (Bottom section) Patients free from AR, and number of patients having their grafts removed.

	Antibody positive (n=22)		**Antibody negative (n=22)**		**P-value**
	Mean (SD)		**Mean (SD)**		
AR grade I	0.73 (1.30)		0.56 (1.00)		0.636
AR grade II	0.73 (0.89)		0.29 (0.49)		0.030*
AR grade III	0.29 (0.57)		0.17 (0.28)		0.373
AR grade IV	0.0045 (0.0210)		0.0		0.329
	No. of patients	**No. of ARs**	**No. of patients**	**No. of ARs**	
AR grade I	13	61	13	55	
AR grade II	18	62	13	32	
AR grade III	10	20	8	21	
AR grade IV	1	1	0	0	
	N		**N**		
No ARs	0		2		
Graft losses	4		0		

Abbreviations: Antibody (AB); Acute rejection (AR). *Statistical significance, P-value < 0.05 (Independent Samples T-test). In one antibody positive patient the distal phalanges was amputated on POD 15, not included in the numbers above.

Table 3. Distribution of antibody types. Alloantibodies were detected in 22 patients. Two were pre-sensitized with DSA, one had a pre-transplant non-HLA antibody, and the remaining had *de novo* antibodies.

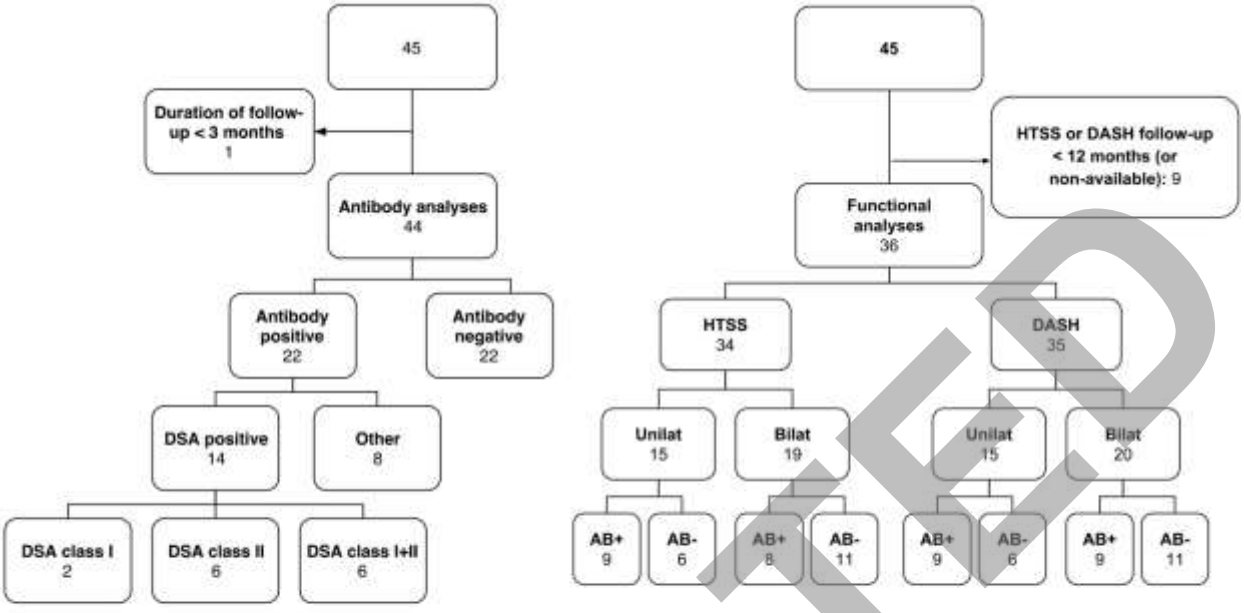
Pre-transplant antibodies	**N**
DSA (class I)	1
DSA (class II)	2
Other (Non-HLA antibody)	1
Post-transplant antibodies	**N**
DSA (class I)	8
DSA (class II)	12
Non-DSA (class I)	7
Non-DSA (class II)	5
Non-HLA	2

Abbreviations: Donor specific antibody (DSA), Human leukocyte antigen (HLA). N, the number of patients with positive antibody analyses (note: one patient can be positive for several antibody types).

Table 4. Association of HLA-mismatch at antigen class I loci HLA-A and HLA-B, and class II locus HLA-DR and acute rejection and antibody development. The two pre-sensitized patients (DSA) were excluded in this analysis.

	Proportion of AR in the first year[X] of transplantation (%)	P-value	Postoperative antibody development (%)	P-value
HLA-A				
0 mismatch (n=4)	75% (n=3)		50% (n=2)	
1 mismatch (n=16)	94% (n=15)	0.518	50% (n=8)	0.958
2 mismatch (n=22)	91% (n=20)		45% (n=10)	
HLA-B				
0 mismatch (n=1)	100% (n=1)		100% (n=1)	
1 mismatch (n=10)	100% (n=10)	0.456	50% (n=5)	0.549
2 mismatch (n=31)	87% (n=27)		45% (n=14)	
HLA-DR				
0 mismatch (n=0)	-		-	
1 mismatch (n=13)	92% (n=12)	1.000	23% (n=3)	0.033*
2 mismatch (n=29)	90% (n=26)		59% (n=17)	

[X]Three out of the 42 patients had a total duration of follow-up of less than one year (160, 278, and 330 days). Abbreviations: Acute rejection (AR), Human leukocyte antigen (HLA). *Statistical significance, p-value <0.05 (evaluated with Chi-square test).



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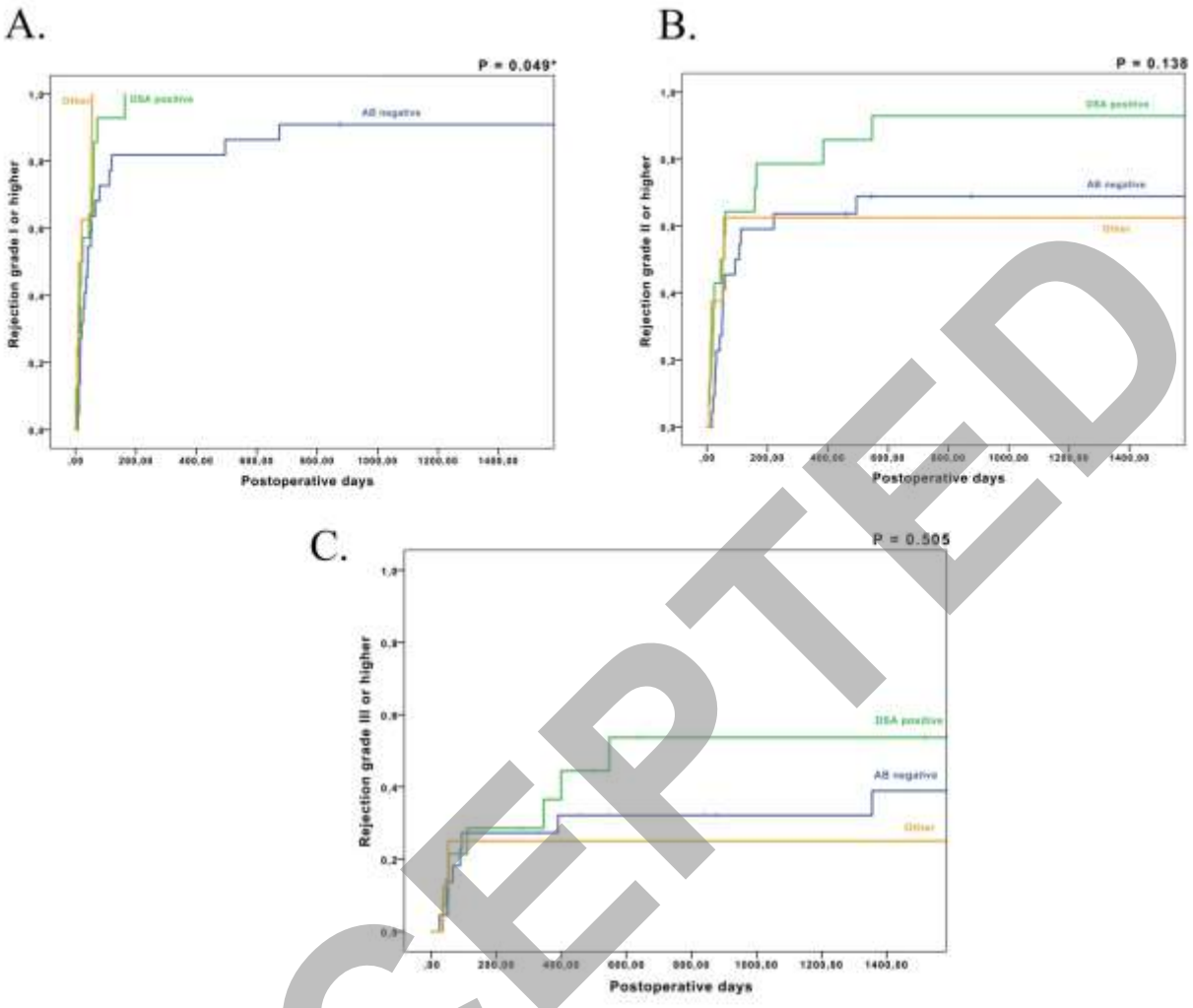

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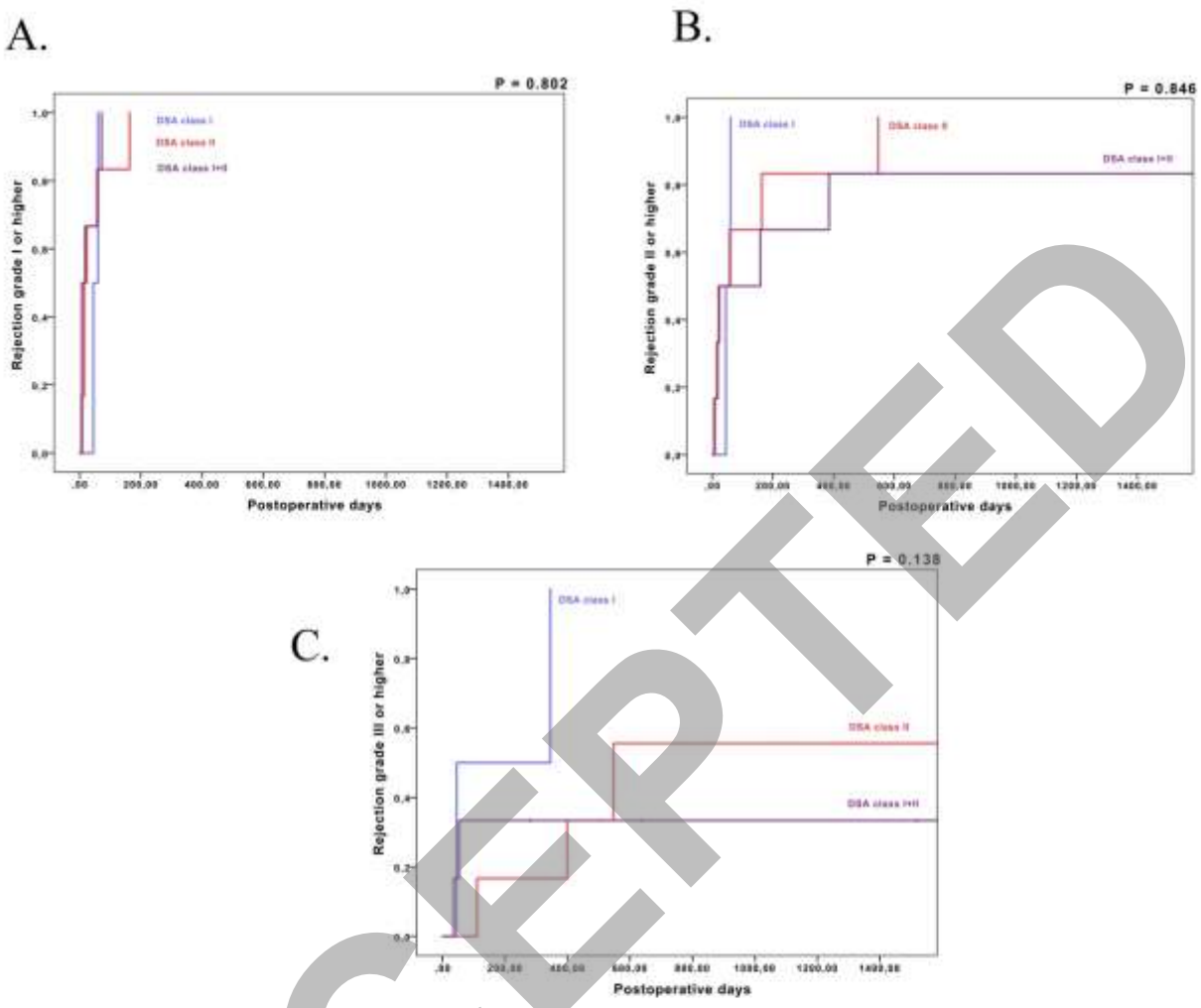

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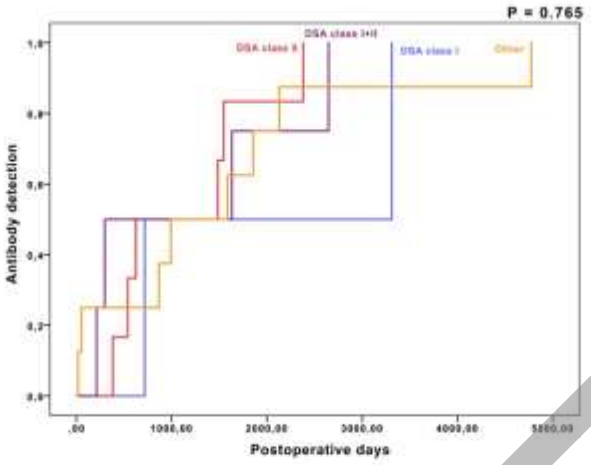

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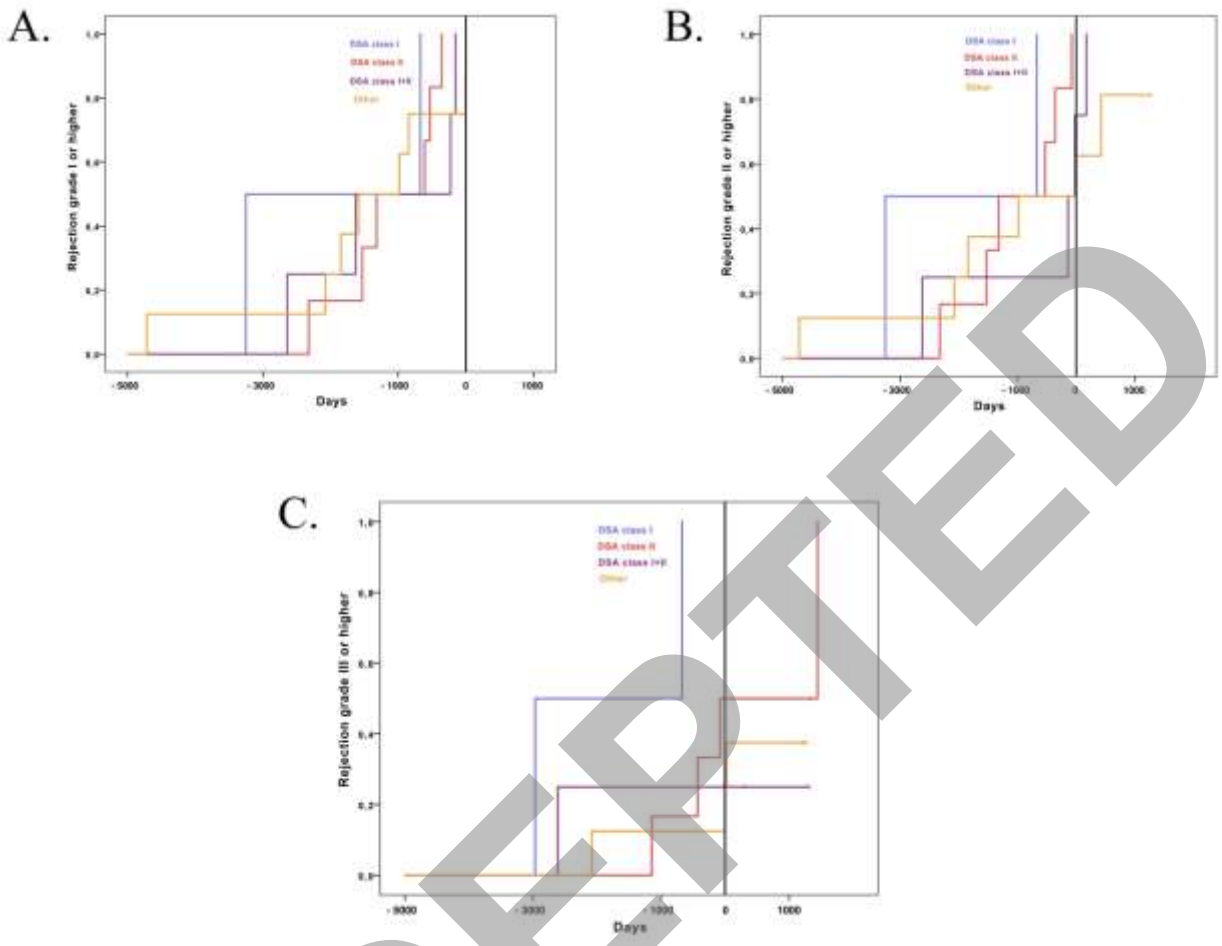

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