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# Long-term follow up for anti-HLA donor specific antibodies postrenal transplantation: high immunogenicity of HLA class II graft molecules

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*de novo* donor specific antibodies, graft failure, human leukocyte antigens class II antibodies, renal transplantation.

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## Summary

The clinical significance of *de novo* post-transplant anti-HLA donor-specific antibodies (DSA) was evaluated using 4241 serum samples collected between 2000 and 2007 from 597 renal transplant recipients. Patients transplanted before December 1996 ($n = 77$) were included in the historic group and those transplanted thereafter ($n = 520$) were included in the study group. All recipients were negative for DSA before transplantation (Tx). Post-Tx, *de novo* DSA were detected in 92/597 (15.4%) patients, while 196 had third party anti-HLA antibodies (DSA-negative). DSA were more frequent in the historic group (33.8%) compared with the study group (12.7%) ($P < 0.001$). Anti-HLA class-II DSA predominated in both groups (84.6% vs. 69.7%). Recipients of HLA class II-incompatible grafts developed DSA more frequently than those receiving HLA class II-compatible grafts (17.9% vs.7.9%, $P = 0.003$), directed mainly against HLA-DQ graft molecules (64/446, 14.4%). DSA production was not different between presensitized and nonsensitized patients ($P = 0.842$). Graft survival was higher in patients without antibodies compared with DSA-positive (log-rank test, $P = 0.002$) and DSA-negative patients (log-rank test, $P = 0.002$). Univariate and multivariate analysis showed independent association for DSA class I (HR = 31.78), DSA class II (HR = 20.92) and non-DSA (HR = 5.94) and graft failure. We conclude that HLA class II incompatible graft transplantations need careful monitoring and should be avoided in high immunological risk cases.

## Introduction

Renal transplantation (Tx) has become the preferred treatment for patients with end-stage renal disease, with the resolution of surgical problems, and developments in immunology and immunosuppression, over the last two decades. Clinical episodes of acute rejection after renal Tx have been significantly reduced. However, we have not achieved desired levels in prevention of chronic renal allograft failure, which over the long term, leads to kidney loss [1,2].

No efficient immunosuppression after renal Tx can gradually lead to graft failure. A number of studies have explored the effect of donor-specific antibodies (DSA) against human leukocyte antigens (HLA) on graft survival. DSA are involved in acute and chronic rejection, as well as decreased graft survival [3–8].

Moreover, the presence of preformed DSA is associated with increased graft loss, related to an increased risk of antibody-mediated rejection (AMR). Thus, the absence of preformed DSA tested by sensitive and specific techniques might identify patients with low immunological risk.

Here, we present the results of a prospective study, initiated in 2000, with the aim of investigating the significance of *de novo* DSA produced after renal transplantation. In this study, we followed up renal transplant recipients with no defined presensitization against donor HLA antigens. Post-Tx anti-HLA antibody screening for the development of *de novo* DSA was performed by highly sensitive Luminex technology. The risk of post-Tx DSA appearance, antibody specificity, and the influence of these antibodies on transplant outcome were analyzed.

## Materials and methods

### Study design

This study monitored the development of anti-HLA donor specific antibodies in 597 recipients of a kidney transplant between 1991 and 2007 at the Transplantation Unit of Laikon Hospital, Athens. The inclusion criteria were the absence of preformed DSA and early failures (less than 6 months post-Tx), availability of donor DNA for extended typing and consistent follow up. Retained clinical information, serum creatinine (sCr) levels and the immunosuppressive regimen were always available. From January 2000 until graft loss, patient death, or June 2010 (closing date of the study), 4241 sera samples were tested for anti-HLA antibodies by Luminex technique. The follow-up period ranged from 14 months to 10 years. The mean number of tests per patient was 7.1 (range: 4–15). Patients were divided into three groups based on the results of the anti-HLA antibody (HLAab) screening: (i) patients with DSA (DSA-positive), (ii) patients with antibodies against third party HLA (DSA-negative), and (iii) patients without anti-HLA antibodies (HLAab-negative). During the study, 17 patients died with a functioning graft.

### Patients

Seventy seven patients who were transplanted between January 1991 and November 1996 with a negative current T/B anti-human globulin augmented complement – dependent lymphocytotoxic crossmatch (AHG-CDC) – included in the historic group in this study. The remaining 520 patients who were transplanted from December 1996 to December 2007 with negative both T/B AHG-CDC and Flow-cytometry crossmatch were included in the study group. All sera tested negative for preformed anti-HLAab on the day of Tx and for historical sensitization. Patients in historic group were tested using ELISA and CDC in both historic sera and on the day of Tx. Patients in the study group were tested using CDC and Luminex methodology on the day of Tx and using CDC and ELISA ($n = 157$) or Luminex ($n = 363$) in historic sera.

Post-Tx screening for the detection of anti-HLA antibodies was performed using Luminex in both groups. Sera collected between 2000 and 2004 were tested retrospectively and thereafter prospectively until the end of the study. Both donors and recipients were phenotyped for HLA-A, B, Cw, DR, and DQ antigens using both serology and low resolution molecular methodology. A total of 446 patients were transplanted with incompatibility in HLA class I (A, B, Cw) and II (DR, DQ), whereas 151 patients were transplanted with compatible HLA-DR and HLA-DQ grafts. When HLAab recognized a graft antigen, we proceeded to additional donor typing to increase the resolution (high resolution typing), for confirmation of antibody specificity. Patient characteristics are shown in Table 1.

### Immunosuppression protocols

From 1991 to 1996, the immunosuppression regimen consisted of corticosteroids, azathioprin, and cyclosporine A (CsA). Patients treated after 1996, received corticosteroids, micophenolate acid (MPA), and a calcineurin inhibitor, either CsA or tacrolimus (Tac). A few cases received a mammalian target of rapamycin inhibitor (mTORi) instead of MPA. An anti-interleukin-2R inhibitor was administered routinely as induction therapy. However, in cases where prolonged delayed graft function was expected, sequential treatment with anti-thymocyte globulin (ATG) was given.

### Graft function and renal biopsies

The effect of anti-HLAab on allograft outcome was analyzed by separating the patients into three groups according to serum creatinine (sCr) levels: good and stable graft function (sCr < 2.0 mg/dl), graft dysfunction (sCr = 2.0–4.0 mg/dl), and graft failure (sCr > 4.0 mg/dl or return to hemodialysis).

Renal biopsies were performed following specific clinical indications such as deterioration of renal function estimated by serum creatinine clearance using Cockcroft-Gault formula (decrease by 20%), proteinuria >0.5 g/day or the presence of glomerular erythrocytes in urine. The biopsy findings graded according to the Banff classification update 2005. All patients were informed about the details of the various procedures and asked for consent. The protocol conformed to the ethical guidelines of the Declaration of Helsinki.

### Screening for anti-HLA antibody

A Luminex technique was used to detect IgG HLAab. Serum samples were analysed using a panel of micro-

**Table 1.** Patient characteristics.

| | Historic group ($n$ = 77) | Study group ($n$ = 520) | $P$-value |
|---|---|---|---|
| Recipient age (mean ± SD) | 49.2 ± 12.2 | 49.3 ± 12.8 | NS* |
| Recipient gender | | | |
| male | 44 (57.1%)¶ | 336 (64.6%) | NS* |
| female | 33 (42.9%) | 184 (35.4%) | |
| Donor organ source | | | |
| LD | 47 (61.0%) | 252 (48.5%) | 0.039* |
| DD | 30 (39.0%) | 268 (51.5%) | |
| Follow-up time in years (mean ± SD) | 16.9 ± 2.9 | 6.7 ± 3.1 | <0.001† |
| Prior transplants | | | |
| First transplants | 74 (96.1%) | 492 (94.6%) | NS‡ |
| Retransplants | 3 (3.9%) | 28 (5.4%) | |
| Number of HLA mismatches | | | |
| Class I (A, B, Cw locus), median (IQR) | 2 (1–3) | 2 (2–3) | 0.004§ |
| Class II (DR, DQ locus), median (IQR) | 1 (1–2) | 1 (0–2) | NS§ |
| Sensitization before Tx | | | |
| %PRAs < 5 | 50 (64.9%) | 341 (65.6%) | NS* |
| %PRAs ≥ 5 | 27 (35.1%) | 179 (34.4%) | |
| Baseline Immunosuppression | | | |
| CSA – based therapy | 70 (90.9%) | 276 (53, 1%) | <0.001* |
| TAC – based therapy | 2 (2.6%) | 169 (32.5%) | <0.001* |
| with AZA | 77 (100.0%) | 12 (2.3%) | <0.001* |
| with MMF | 0 (0.0%) | 409 (78.7%) | <0.001* |

*$\chi^2$ test.
†Student's $t$-test.
‡Fisher's exact test.
§Mann–Whitney test.
¶patient number (percent) unless otherwise indicated.
SD, standard deviation; NS, nonsignificant; LD, living donor kidney transplant; DD, deceased donor kidney transplant; PRA, panel reactive antibody; CsA, cyclosporine A; TAC, tacrolimus; AZA, azathioprin; MMF, mycophenolate mofetil.

spheres coated with purified HLA class I and II molecules (LABScreen PRA class I and II; One Lambda-Inc, Canoga Park, CA, USA).

All samples positive for LABScreen PRA were further analyzed for HLAab specificity using a LABScreen Single Antigen test (One Lambda-Inc). Sera from patients with negative %PRA but elevated sCr (>2.0 mg/dl) were also analysed for HLAab specificity, to define sub-threshold levels of circulating DSA. LABScreen assays, data analysis, and calculations were performed according to the manufacturer's instructions. Results were expressed as mean fluorescence intensity (MFI). HLAab specificity was considered positive if the normalized mean fluorescence intensity was higher than 1000.

### Statistical analysis

Continuous variables are presented as mean and standard deviation or as median (interquartile range-IQR), while quantitative variables are presented as absolute or relative frequencies. For proportion comparisons, chi-square and Fisher's exact tests were used. Student's $t$-test was computed to compare mean values. The Mann–Whitney test was used for the comparison of not normally distributes variables between the two groups. For repeated measurements, analysis of variance (ANOVA) was used to compare changes in sCr levels between the two groups over the follow-up period. Kaplan–Meier survival estimates were graphed for grafts over the follow-up period. Log-rank tests were used to compare survival curves. Recipient gender, age at Tx, donor type, number of previous transplants, peak intensity of antibody, time of antibody appearance, the presence of anti-HLA antibodies, either donor-specific or nondonor-specific, alloantibody reactivity, and HLA mismatches were first tested for their ability to predict graft survival using univariate Cox proportional-hazard models. Multiple Cox proportional-hazard analysis in a stepwise method was used to determine the independent predictors for graft failure. The assumption of proportional hazards was evaluated by testing for interaction with a continuous time variable. The covariates used in the multiple analysis concerned full data. Hazard ratios (HR) with 95% confidence intervals are referred from the results of multiple analysis. All reported $P$ values

are two-tailed. Statistical significance was set at 0.05 and analysis was conducted using SPSS statistical software (version 17.0, Chicago, IL, USA).

## Results

### *De novo* anti-HLA-DSA development and presensitization

Among the 597 recipients who were screened periodically on post-Tx sera for anti-HLA-specific antibodies, 92 patients (15.4%) had DSA class I or class II, 196 patients (32.8%) had third party anti-HLA antibodies (DSA-negative), and 309 patients (51.8%) were HLAab-negative.

The presence of DSA in the historic group and the study group is shown in Table 2. In the historic group, the incidence of DSA development was 33.8% ($n = 26$) compared with 12.7% ($n = 66$) of the study group ($P < 0.001$). Interestingly, anti-HLA class II DSA developed more frequently in the historic group (22/77) compared with the study group (46/520) ($P < 0.001$), although no significant difference in HLA class II compatibility were found between the two groups.

The effect of presensitization on *de novo* DSA development post-Tx was estimated in the study group patients as shown in Table 3. The incidence of *de novo* DSA development was not different between presensitized against third party HLA (%PRA ≥ 5) and nonsensitized (%PRA < 5) before transplantation patients ($P = 0.842$).

### Analysis of anti-HLA-DSA specificity

Anti-HLA class II DSA predominated in both historic and study group: 84.6% (22/26) DSA-positive patients in the historic group and 69.7% (46/66) DSA-positive patients in the study group developed anti-HLA class II DSA ($P = 0.142$).

**Table 2.** Anti-HLA antibody detection in the historic group and the study group.

| Anti-HLA antibody status | Historic group, *N* (%) | Study group, *N* (%) | *P*-value |
|---|---|---|---|
| Total number of patients | 77 | 520 | – |
| HLAab-negative | 31 (40.2) | 278 (53.5) | 0.030* |
| DSA-negative | 20 (26.0) | 176 (33.8) | NS* |
| DSA-positive | 26 (33.8) | 66 (12.7) | <0.001* |
| DSA-class I | 3 (3.9) | 16 (3.1) | NS† |
| DSA-class II | 22 (28.6) | 46 (8.8) | <0.001* |
| DSA-class I and II | 1 (1.3) | 4 (0.8) | NS† |

*$\chi^2$ test.
†Fisher's exact test.
HLA, human leukocyte antigen; HLAab, HLA antibody; DSA, donor-specific antibody; NS, nonsignificant.

**Table 3.** Anti-HLA sensitization before transplantation and anti-HLA antibody status post-transplantation in the 520 patients of the study group.

| Pre-transplant sensitization | | Post-transplant HLAab status | | |
|---|---|---|---|---|
| | Number of patients | DSA-positive *N* (%) | DSA-negative *N* (%) | HLAab-negative *N* (%) |
| %PRAs < 5 | 341 | 44 (12.9) | 44 (12.9) | 253 (74.2) |
| %PRAs ≥ 5 | 179 | 22 (12.3) | 132 (73.7) | 25 (14.0) |
| *P*-value* | – | 0.842 | <0.001 | <0.001 |

*$\chi^2$ test.
HLA, human leukocyte antigen; HLAab, HLA antibody; DSA, donor-specific antibody; PRA, panel reactive antibody.

The degree of HLA incompatibility affected DSA development post-transplantation: 80/446 (17.9%) recipients of an HLA class II incompatible graft and 12/151 (7.9%) recipients of an HLA class II compatible graft developed DSA post-transplantation ($P = 0.003$). Among the 80 DSA-positive patients who received an HLA class II incompatible graft, 85% ($n = 68$) developed anti-HLA class II, 8.8% ($n = 7$) developed anti-HLA class I, and 6.3% ($n = 5$) developed anti-HLA class I and II antibodies. Twelve DSA-positive patients who received an HLA class II compatible graft developed anti-HLA class I DSA.

The main HLA specificity of the DSA was against HLA-DQ graft molecules: 64 patients had anti-HLA-DQ antibodies, 17 patients had anti-HLA-DR antibodies, 20 patients had anti-HLA-A antibodies, six patients had anti-HLA-B antibodies, and four patients had anti-HLA-Cw antibodies.

### Time point of DSA appearance and its relationship to graft failure

The time point of DSA appearance was estimated in 52 patients who developed DSA during the study. The remaining 40 DSA-positive patients had antibodies by the first test (11 ± 5.0 years post-Tx).

There was no difference between DSA class I and DSA class II concerning the time point of antibody appearance post-transplantation: DSA class I appeared 3.3 ± 2.7 years and DSA class II appeared 2.3 ± 2.1 years post-transplantation ($P = 0.084$). DSA were detected more frequently in patients tested earlier in the post-Tx period: 51.9% (27/52) patients tested positive in the first 2 years, 26.9% (14/52) patients in the interval 2–4 years, 17.3% (9/52) patients in the interval 4–8 years, and 3.8% (2/52) patients at more than 8 years post-Tx.

During the follow-up period, 14/52 patients lost the graft. The mean time from DSA appearance to graft failure was 2.8 ± 3.0 years. Patients with DSA class I lost

their graft 3.9 ± 4.0 years after antibody appearance and patients with DSA class II lost their graft 2.6 ± 2.7 years after DSA development ($P = 0.096$).

DSA that developed early post-Tx were not found to be more deleterious compared with those that appeared later: Among 16 patients who developed DSA the first year post-Tx, 18.7% patients ($n = 3$) lost their graft and among 36 patients who developed DSA later, 30.5% patients ($n = 11$) lost their graft during the study ($P = 0.506$).

### Clinical outcome of the patients with antibodies

Over the entire follow-up period, 48/597 (8.0%) patients lost their graft: 28 DSA-positive patients (58.3%), 17 DSA-negative patients (35.4%) and three HLAab-negative patients (6.3%). Kaplan–Meier graft survival analysis in the study group revealed that the mean graft survival time was 6.8 years (SD = 3.0) for DSA-negative and 6.5 years (SD = 3.6) for DSA-positive cases (Fig. 1). Graft survival was significantly higher in HLAab-negative cases compared with DSA-negative (log-rank test, $P = 0.002$) and DSA-positive cases (log-rank test, $P = 0.002$) and in DSA-negative cases compared with DSA-positive cases (log-rank test, $P < 0.001$).

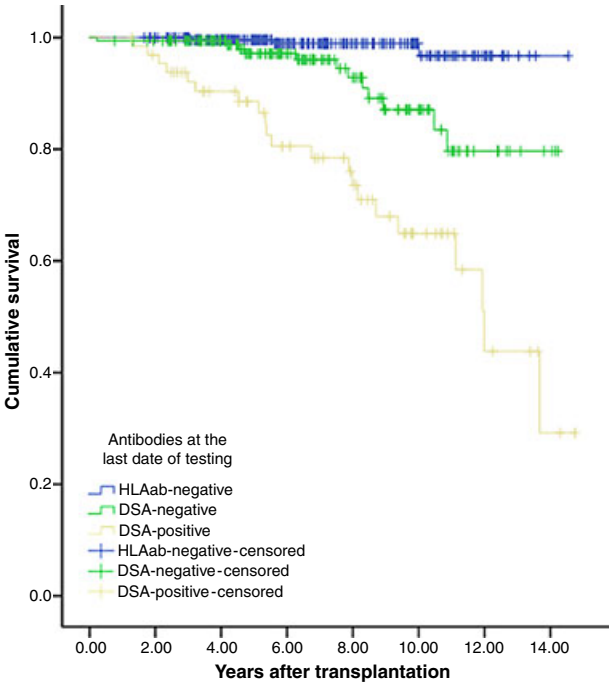


**Figure 1** Graft loss-censored allograft survival stratified by the presence of antibody. The curves were constructed according to the method of Kaplan–Meier and compared using the log-rank test. Graft survival was significantly higher in HLAab-negative cases compared with DSA-negative (log-rank test, $P = 0.002$) and DSA-positive patients (log-rank test, $P = 0.002$). Graft survival was also significantly higher in DSA-negative cases compared with DSA-positive cases (log-rank test, $P < 0.001$). Similar results were found in the 597 patients (data not shown).

**Table 4.** Variables predictive of graft failure by univariate Cox proportional analysis.

| | Hazard ratio | 95% CI | *P*-value |
|---|---|---|---|
| Anti-HLAab | | | |
| HLAab-negative | 1.0* | – | – |
| DSA-negative | 5.94 | 1.67–21.06 | 0.006 |
| DSA-positive | 22.54 | 6.69–75.89 | <0.001 |
| Alloantibody reactivity | | | |
| HLAab-negative | 1.0 | – | – |
| DSA-negative | 5.94 | 1.67–21.04 | 0.006 |
| DSA class I | 31.78 | 7.58–133.22 | <0.001 |
| DSA class II | 20.92 | 6.02–72.74 | <0.001 |
| DSA class I and II | 15.98 | 1.64–155.27 | 0.017 |

HLA, human leukocyte antigen; HLAab, HLA antibody; *indicates reference category; DSA, donor-specific antibody.

According to the univariate and multivariate Cox regression analysis in the study group, the presence of detectable anti-HLA antibodies, either donor-specific or nondonor-specific, was the only independent predictor for graft loss (Table 4). More precisely, DSA-positive (HR = 22.54, 95% CI: 6.69–75.89; $P < 0.001$) and DSA-negative patients (HR = 5.94, 95% CI: 1.67–21.06; $P = 0.006$) had greater hazard for graft failure compared with HLAab-negative patients. Moreover, DSA-positive patients had greater hazard for graft failure compared with DSA-negative patients (HR = 3.79, 95% CI: 1.75–7.74; $P < 0.001$). A sub-analysis concerning anti-HLAab specificity showed that patients with DSA class I (HR = 31.78, 95% CI: 7.58–133.22; $P < 0.001$) and those with DSA class II (HR = 20.92, 95% CI: 6.02–72.74; $P < 0.001$) had greater hazard for graft failure compared with HLAab-negative patients. Patients with DSA class I (HR = 5.35 95% CI: 1.88–15.25; $P = 0.002$) and DSA class II (HR = 3.52, 95% CI: 1.63–7.58; $P = 0.001$) had also greater hazard for graft failure compared with DSA-negative patients.

Recipient age at Tx, recipient gender, donor type, prior transplants, peak intensity of antibody, and time of DSA appearance had no significant effect on graft survival. In addition, no significant difference in graft survival time was found between recipients of an HLA class II incompatible graft and those transplanted with an HLA class II compatible graft.

The effect of anti-HLA antibodies on renal graft outcome in both historic and the study group was analysed by separating patients into three groups according to sCr, as shown in Table 5. In both groups, the incidence of

**Table 5.** Relationship between anti-HLA antibody production and graft function in patients of the historic and the study groups. In the historic group, the incidence of graft failure was significantly higher in patients with DSA (26.9%) compared with HLAab-negative patients ($P = 0.007$). In the study group, the incidence of graft failure among patients with DSA (31.8%) and DSA class II (32.6%) was significantly higher compared with DSA-negative patients (6.8%) ($P < 0.001$) and HLAab-negative patients (1.1%) ($P < 0.001$).

| | | Average serum creatinine | | |
|---|---|---|---|---|
| | N | >4 mg/dl *N* (%) | 2–4 mg/dl *N* (%) | <2 mg/dl *N* (%) |
| Historic group | | | | |
| All | 77 | 12 (15.6) | 16 (20.8) | 49 (63.6) |
| HLAab-negative | 31 | 0 (0.0)[a] | 6 (19.4) | 25 (80.6) |
| DSA-negative | 20 | 5 (25.0)[b] | 4 (20.0) | 11 (55.0) |
| DSA-positve | 26 | 7 (26.9)[c] | 6 (23.1) | 13 (50.0) |
| DSA-class I | 3 | 0 (0.0) | 0 (0.0) | 3 (100) |
| DSA-class II | 22 | 7 (31.8) | 5 (22.7) | 10 (45.5) |
| DSA-class I and II | 1 | 0 (0.0) | 1 (100) | 0 (0.0) |
| Study group | | | | |
| All | 520 | 36 (6.9) | 70 (13.5) | 414 (79.6) |
| HLAab-negative | 278 | 3 (1.1)[d] | 29 (10.4)[h] | 246 (88.5) |
| DSA-negative | 176 | 12 (6.8)[e] | 26 (14.8) | 138 (78.4) |
| DSA-positve | 66 | 21 (31.8)[f] | 15 (22.7)[i] | 30 (45.5) |
| DSA-class I | 16 | 5 (31.2) | 2 (12.5) | 9 (56.3) |
| DSA-class II | 46 | 15 (32.6)[g] | 12 (26.1)[j] | 19 (41.3) |
| DSA-class I and II | 4 | 1 (25.0) | 1 (25.0) | 2 (50.0) |

DSA, donor specific antibody; HLA, human leukocyte antigen; HLAab, HLA antibody.
[a] vs. [b] $P = 0.01$; [a] vs. [c] $P = 0.007$; [d] vs. [e] $P = 0.002$; [d] vs. f; d vs. g; e vs. f; [e] vs. [g] $P < 0.001$; [h] vs. [i] $P = 0.01$; [h] vs. [j] $P = 0.007$.

graft failure was significantly higher in patients with DSA compared with HLAab-negative patients ($P = 0.007$ and $P < 0.001$ respectively). In the study group, the incidence of graft dysfunction among patients with DSA and DSA class II was significantly higher compared with HLAab-negative patients ($P = 0.01$ and $P = 0.007$ respectively).

At the end of the study, 46.7% of the patients with DSA, 76.0% of the DSA-negative patients, and 87.7% of the HLAab-negative patients had good and stable graft function. Changes in sCr levels over the follow-up period were not significantly different between DSA-positive patients with good stable graft function (sCr <2 mg/dl) and those with graft function deterioration (sCr ≥2 mg/dl) ($P = 0.885$) (Fig. 2).

No significant difference in DSA HLA specificity was observed in patients with graft failure, graft dysfunction, or those with good long-term function. DSA were predominantly directed against mismatched HLA class II antigens. The Incidence of HLA class II DSA in DSA-positive patients was 23/28 (82.1%) in patients with graft failure, 19/21 (90.5%) in patients with graft dysfunction, and 31/43 (72.1%) in patients with good long-term graft function.

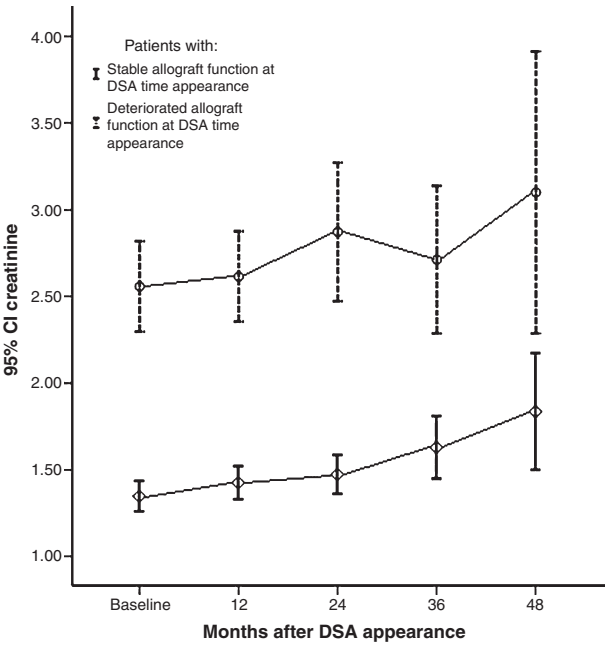


**Figure 2** Change in serum creatinine levels of recipients with donor-specific antibodies (DSA) at four different time points after DSA appearance. Changes in creatinine levels over the follow-up period were not significantly different between DSA-positive patients with good stable graft function (Cr <2 mg/dl) and those with deterioration of graft function (Cr ≥2 mg/dl) ($P = 0.885$).

### Association of antibodies with histopathology lesions in biopsies

We evaluated a total of 134 clinically indicated biopsies from 107 patients (Table 6). At the end of the study, 30/107 patients had returned to hemodialysis: 63.3% DSA-positive, 33.3% DSA-negative, and 3.3% HLAab-negative patients. Acute alloimmune injury was diagnosed in 38 cases, chronic alloimmune injury (CAI) in 69 cases, and nonimmune injury in 28 cases. C4d staining coincided with HLA antibodies in 31 biopsies, 25 with circulating DSA, (80.6%) and six with HLA antibodies against third party (19.4%).

Among DSA-positive patients, acute rejection episodes were more frequent in patients with DSA – class I (6/8, 75.0%) compared with patients with DSA – class II (8/40, 20.0%) ($P = 0.007$). This difference disappeared in the cases diagnosed with CAI ($P$ = NS). The risk of CAI development was significantly higher in DSA-positive patients (67.9%) compared with DSA-negative patients (37.2%) ($P = 0.005$) and HLAab-negative patients (44.2%) ($P = 0.04$).

## Discussion

Immunological memory if activated remains a substantial risk factor for graft deterioration and loss. The introduc-

**Table 6.** Biopsy results from 107 DSA-positive, DSA-negative and HLAab-negative patients. Alloimmune injury was diagnosed in 107/134 biopsies (79.9%). Acute antibody-mediated rejection (AAMR) was found in 5/53 (9.4%) biopsies from patients with circulating DSA and 3/43 (6.9%) biopsies from DSA-negative patients. All AAMR were C4d-positive. In 13/53(24.5%) biopsies from DSA-positive patients, acute cellular rejection (ACR) was diagnosed. Among patients with chronic alloimmune injury, C4d deposition was more frequent in DSA-positive cases (37.8%) compared with DSA-negative (7.0%) ($P = 0.001$) and HLAab-negative cases (10.5%) ($P = 0.007$). In 34 biopsies, nonalloimmune injury was found: CNI toxicity, recurrent disease, BK nephropathy or unspecified chronic changes.

| | | Acute alloimmune injury | | | Chronic alloimmune injury/rejection | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total no. biopsies ($N = 134$) | AAMR ($N = 8$) | ACR ($N = 30$) | All ($N = 38$) | C4d-pos ($N = 27$) | C4d-neg/ND ($N = 42$) | All ($N = 69$) | Nonimmune injury ($N = 28$) | Patients with graft failure ($N = 30$) |
| | | *N* (%) | *N* (%) | *N* (%) | *N* (%) | *N* (%) | *N* (%) | *N* (%) | *N* (%) |
| HLAab-negative | 38 | 0 (0.0) | 7 (18.4) | 7 (18.4) | 4 (10.5)[c] | 13 (34.2) | 17 (44.7)[f] | 12 (31.6) | 1 (2.6) |
| DSA-negative | 43 | 3 (6.9) | 10 (23.3) | 13 (30.2) | 3 (7.0)[d] | 13 (30.2) | 16 (37.2)[g] | 10 (23.2) | 10 (23.2) |
| DSA-positive | 53 | 5 (9.4) | 13 (24.5) | 18 (33.9) | 20 (37.8)[e] | 16 (30.1) | 36 (67.9)[h] | 6 (11.3) | 19 (35.8) |
| DSA-I | 8 | 3 (37.5) | 3 (37.5) | 6 (75.0)[a] | 3 (37.5) | 1 (12.5) | 4 (50.0) | 0 (0.0) | 2 (25.0) |
| DSA-II | 40 | 1 (2.5) | 7 (17.5) | 8 (20.0)[b] | 16 (40.0) | 15 (37.5) | 31 (77.5) | 6 (15.0) | 17 (42.5) |
| DSA-I and II | 5 | 1 (20.0) | 3 (60.0) | 4 (80.0) | 1 (20.0) | 0 (0.0) | 1 (20.0) | 0 (0.0) | 0 (0.0) |

AAMR, acute antibody-mediated rejection; ACR, acute cellular rejection; ND, not defined; DSA, donor-specific antibody; HLA, human leukocyte antigen; HLAab, HLA antibody.
[a vs. b] $P = 0.007$; [c vs. e] $P = 0.007$; [d vs. e] $P = 0.001$; [f vs. h] $P = 0.04$; [g vs. h] $P = 0.005$.

tion of more sensitive and specific techniques as flow cytometry crossmatch and Luminex assays for the detection of preformed anti-HLA – DSA [9] remains controversial with respect to the graft outcome. In our center, we introduced flow cytometry crossmatch and Luminex methodology for DSA detection pre-Tx many years ago, to identify the possible immunological risk for transplantation. As the goal in transplantation is to succeed long-term good graft survival, transplantation with low immunological risk may contribute to that point. Subsequently, we decided to screen for *de novo* anti-HLA DSA production a cohort of 520 renal transplant recipients who received a kidney transplant with low immunological risk. A control group was selected from patients transplanted until 1996 with a negative AHG-CDC crossmatch. The prevalence of *de novo* DSA was higher in the historic group (33.8%) compared with the study group (12.7%). This finding confirms that of recent reports which emphasize the importance of the use of sensitive and specific techniques to define DSA existence pre-Tx [10]. Furthermore, the shift in immunosuppression regimen from azathioprine to MPA after 1996 might have contributed to a limitation of *de novo* DSA production post-Tx [11].

Previous studies report a varying percentage of patients with anti-HLAab after renal Tx. Differences are attributed to differences in techniques for antibody detection, immunosuppression type, size and type of patient population, follow-up time, and the presence of preformed anti-HLA DSA before Tx [12–15]. In this study, the incidence of *de novo* DSA development post-Tx was not different between presensitized against third party HLA and non-sensitized pre-Tx patients ($P = 0.842$). In contrast, previous studies show that pre-Tx sensitization is related to poor transplant survival and require more attention. No earlier reports used highly sensitive methods to exclude patients with preformed antibodies against donor antigens [16–18].

The importance of HLA class II antigens in Tx has been widely discussed [15,19–22]. Technical limitations had made the identification and interpretation of the importance of antibodies against HLA class II confusing. The current study revealed that recipients of HLA class II incompatible renal grafts are at higher risk for DSA development compared with recipients of HLA class II compatible grafts. Interestingly, in that case the antibodies were predominantly directed against incompatible HLA class II (73/80 patients), particularly against HLA-DQ, and despite the presence of incompatible HLA class I. Similarly, in a multivariate analysis, it has been shown that HLA – DR matching not only improved graft survival, but it was also significantly associated with the development of DSA and non-DSA post-Tx [23,24].

Moreover, it was also clear from the results that when there was HLA-DR and -DQ incompatibility, the antibodies that appeared first in the circulation were HLA-DQ graft specific. These results support the theory about hierarchy in alloantigen recognition post-Tx as it has been described previously [25–27]. Our observation that the majority of anti-HLA class II DSA recognized HLA-DQ graft molecules (64/68 patients) confirms the results of previous studies [20,24,26]. We estimate that the high incidence of HLA-DQ antibodies is related to the high

number of polymorphic epitopes that are expressed on both α and β chains of the HLA-DQ molecule [28].

We found no difference between HLA-class I and HLA-class II DSA in the time point of antibody appearance or the time from antibody appearance until graft failure. Concerning the time of antibody appearance post-Tx, it was found that in approximately half the DSA-positive cases, antibodies appeared within the first 2 years post-Tx. This observation indicates a higher incidence of activated alloimmune mechanisms in the first year post-Tx. Previously, we showed that allo-antibodies and specific T-cell alloreactive clones against incompatible HLA class II are also detected in the first year post-renal Tx [29].

Antibodies formed soon after Tx were not found to be more detrimental to the graft than antibodies formed later, as it has been reported by others [30]. Probably, the careful selection of patients, excluding those with immunological memory to donor antigens, reduced the probability of rapid post-Tx production of high affinity harmful DSA. Moreover, the frequency of DSA appearance reduced over time post-Tx. This could be explained by the development of regulatory-adaptation mechanisms that may inhibit antibody production.

In the past years, evidence supported a significant role of anti-HLAab in a slow progressive tissue injury and graft dysfunction [31–33]. Here, we clearly show that the slow change in sCr levels over time did not differ between DSA-positive patients with good and stable graft and those with graft dysfunction. DSA were present for months to years in the circulation before graft dysfunction or graft failure, as it has already been reported by others [7,8].

This study, like other studies, show a highly significant association between graft dysfunction or graft failure and the presence of DSA and DSA class II [21,22]. In the past years, there has been an increasing interest in antibody-mediated acute and chronic renal allograft injury. One split complement C4d is now widely accepted as a marker for antibody-mediated rejection in renal and cardiac transplantation. Our results confirm previous observations about the importance of antibodies on either acute or chronic graft injury [21,22,34,35]. Clinically indicated biopsies from 107 patients showed that circulating DSA, particularly anti-HLA class I, were associated with acute antibody-mediated or acute cellular rejection. Moreover, higher incidence of chronic antibody-mediated injury in DSA-positive patients was found irrespectively of antibody specificity in comparison with DSA-negative patients and HLAab-negative patients. Although only 55.5% (20/36) of the DSA-positive patients with histopathological lesions of chronic alloimmune injury had C4d deposition in peritubular capillaries, the absence of C4d staining does not rule out the participation of antibodies in graft injury. Non-complement binding antibodies may also cause injury to the endothelial cells [36].

In our study, nondonor-specific anti-HLA antibodies were also associated with graft dysfunction and graft failure compared with the absence of anti-HLAab. In that case, the presence of anti-HLA antibodies indicates an active immune system that may support non-HLA donor-specific immunity. Indeed, five DSA-negative patients who experienced graft loss had MICA antibodies (data not shown). Furthermore, in certain cases non DSA may actually be the same specificities as DSA. Cai *et al.* showed that some nondonor-specific antibodies may react with nondonor antigens that share epitopes with donor mismatched HLA antigens [37].

Only 8.0% of the patients lost their graft during the study. The great majority of the patients (77.5%, 463/597) experienced good and stable graft function. Graft survival was significantly higher in HLAab-negative cases compared with DSA-negative and DSA-positive cases as has been reported previously [34].

Parameters that predict clinical outcome were studied in a Cox analysis. This analysis revealed that the presence of anti-HLA antibodies either DSA class I, II or both or non-DSA was the only independent factor for graft loss. On the other hand, neither time of DSA appearance nor anti-HLAab strength had a strong effect on graft outcome, in discordance with a recent study which had a different study group and used a different approach [31].

In conclusion, this study demonstrates the importance of HLA class II incompatibility in renal transplantation. Recipients of an HLA class II incompatible graft are at higher risk for antibody development compared with recipients of an HLA class II compatible graft and need careful monitoring post-transplantation. When anti-HLA antibodies, either class II or class I develop after transplantation, they both seem to be associated with graft dysfunction and graft failure. Transplantation on full HLA – DR and – DQ compatibility might benefit high immunological risk transplantation such as re-transplantation or transplantation with incompatible blood group donor.

## Authorship

I-SAN: designed and performed the study, collected and analyzed the data, and wrote the paper. AG: designed the study, conducted data analysis and wrote the paper. ENK: analyzed the data. MND: collected data. MDA: analyzed the data. AGK: participated in the study design. JNB: participated in the study design, clinical evaluation and in writing the paper.

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