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The Clinical Spectrum of *De Novo* Donor-Specific Antibodies in Pediatric Renal Transplant Recipients

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The development of donor-specific HLA antibodies (DSA) is associated with worse renal allograft survival in adult patients. This study assessed the natural history of *de novo* DSA, and its impact on renal function in pediatric renal transplant recipients (RTR). HLA antibodies were measured prospectively using single-antigen-bead assays at 1, 3, 6 and 12 months posttransplant followed by 12-monthly intervals and during episodes of allograft dysfunction. Of 215 patients with HLA antibody monitoring, 75 (35%) developed DSA at median of 0.25 years posttransplant with a high prevalence of Class II (70%) and HLA-DQ (45%) DSA. DSA resolved in 35 (47%) patients and was associated with earlier detection (median, inter-quartile range 0.14, 0.09–0.33 vs. 0.84, 0.15–2.37 years) and lower mean fluorescence intensity (MFI) (2658, 1573–3819 vs. 7820, 5166–11 990). Overall, DSA positive patients had more rapid GFR decline with a 50% reduction in GFR at mean 5.3 (CI: 4.7–5.8) years versus 6.1 (5.7–6.4) years in DSA negative patients ($p = 0.02$). GFR decreased by a magnitude of 1 mL/min/1.73 m^2 per log10 increase in Class II DSA MFI ($p < 0.01$). Using Cox regression, independent factors predicting poorer renal allograft outcome were older age at transplant (hazard ratio 1.1, CI: 1.0–1.2 per year), tubulitis (1.5, 1.3–1.8) and microvasculature injury (2.9, 1.4–5.7). In conclusion, pediatric RTR with *de novo* DSA and microvasculature injury were at risk of allograft failure.

Abbreviations: AMR, antibody-mediated rejection; CI, confidence interval; DSA, donor-specific HLA antibodies; eGFR, estimated GFR; IQR, inter-quartile range; MFI, mean fluorescence intensity; NDS-HLA, nondonor-specific HLA antibodies; RTR, renal transplant recipients

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Introduction

Renal transplantation is the gold-standard treatment for patients with end-stage kidney disease. Increased renal allograft survival over recent years has been due to improvements in the early postoperative period and in the long term, rates of renal allograft loss have not changed (1). It is now being increasingly recognized that B cells play a role in mediating this long-term renal allograft attrition through the development of HLA-specific antibodies detected using high throughput single-antigen screening with the Luminex platform (2–4). Nevertheless, there are technical limitations to this method, and it has to be recognized that the HLA configuration on the Luminex beads might not correspond to the *in vivo* conformation on cell surfaces (4).

Clinically, there is accumulating evidence in the adult literature for the association of donor-specific HLA antibodies (DSA) with poorer renal allograft outcomes (2,3). The current paradigm is that chronic antibody-mediated rejection (AMR) can run an indolent course with an inexorable progression from microvascular endothelial injury in the early phase to graft arteriosclerosis and transplant glomerulopathy, which only then manifests clinically with a reduced GFR and increased proteinuria (2). Nevertheless, there are DSA positive renal transplant recipients (RTR) with a clinically stable course and studies to date have not fully addressed the fluctuating nature of DSA levels over time (5–7). Adult studies also cannot automatically be applied to the pediatric population and specifically in this case, B cell subpopulations in children mature as the child gets older (8). We address these concerns in our prospective cohort study of pediatric RTR using multivariable analysis and multilevel linear modeling to assess longitudinal data on effects of DSA on GFR and graft outcome (9).

Patients and Methods

Study design

Posttransplant HLA-specific antibody testing was standardized in all pediatric RTR in our center from January 1, 2006 (Figure 1). All new transplants had serial HLA testing at 1, 3, 6 and 12 months and annually thereafter with existing patients having annual testing. In addition, HLA-specific antibody tests were performed during episodes of renal allograft dysfunction (defined as a 10% decrease in estimated GFR [eGFR] from baseline).

Clinical data were collected at 3 monthly intervals. eGFR was calculated using modified Schwartz formula using the internally validated k-value of 33 (10,11). Baseline eGFR used in survival analysis was taken at 1 month posttransplant for patients transplanted after January 1, 2006. For patients transplanted before this, baseline eGFR was taken on this date.

Ethics approval for this program was obtained from the University College London Institute of Child Health and Great Ormond Street Hospital for Children NHS Trust Research Ethics Committee.

Patients

All patients were cross match negative using flow cytometric cross-matching for T and B-cells pretransplant. In addition, multiple pretransplant sera were screened for the presence of HLA-specific antibody. Where patients screened positive for the presence of antibody, samples were analyzed to ensure no DSA was present pretransplant using the methods outlined below. Immunosuppression protocol for standard risk patients consisted of corticosteroids (intravenous methylprednisolone 600 mg/m^2 presurgery following by oral prednisolone weaning course over 3 months), azathioprine (60 mg/m^2) and tacrolimus (trough levels 8–12 mg/L). Basilix-imab was used for subsequent retransplants. Azathioprine was substituted for mycophenolate mofetil in high-risk transplants with immunosuppression thereafter tailored to individual patients' requirements. No changes were made to the immunosuppression based on antibody results alone.

Patients were grouped into three groups, according to the cause of renal failure; ''structural defects,'' ''immune-mediated'' and ''others'' as we hypothesized that patients with structural defects were at increased risk of urinary tract infections which might impact on long-term renal allograft function and also immune-mediated diseases might have a different effect on DSA. Of note, glomerular diseases, which were not immune-mediated, for example, congenital nephrotic syndrome were classed in the ''others'' group. Full list of diagnosis are provided in Table S1.

Biopsies were only performed in the presence of renal allograft dysfunction (''for cause'') and no protocol biopsies were performed. Histological classification was based on Banff 2009 criteria (12). Microvascular injury was defined in this study by any presence of peritubular capillaritis, glomerulitis or glomerular double contouring. C4d and CD20 staining were performed using immunohistochemistry. T cell–mediated rejection was treated with pulsed intravenous methylprednisolone and/or high dose oral corticosteroids. Two patients had acute AMR and responded to treatment with pulsed intravenous methylprednisolone, rituximab with or without plasma exchange. Chronic AMR was treated with intravenous rituximab (750 mg/m^2) and/or intravenous immunoglobulin (2 g/kg).

HLA antibody screening

Both pre- and posttransplant patient serum samples were tested for the presence of HLA-specific antibodies using the LABScreen Mixed Class I and II antibody screening kit (OneLambda, Canoga Park, CA). Samples testing positive using locally derived, lot specific, cut-off ratios, were then further analyzed using the appropriate HLA Class I and/or Class II LABScreen single-antigen kits, also from OneLambda. The beads were analyzed using the Luminex 100 platform (Austin, TX), and data assessed using the kit specific HLA Fusion software (One Lambda). Antibodies to all loci including HLA-A, -B, -Cw, DR, DQB, DQA and DPB were included. Calculated mean fluorescence intensity (MFI) values were normalized against the internal negative control bead and the negative serum control using the HLA Fusion software. All donors and recipients were DNA typed to the one field level. Where allele specific antibodies were identified on single-antigen screening that could be potential DSA based on donor typing information, the donor was typed at the two field level for the alleles in question. If these alleles were not represented on the screening panel, sequence data presented on the International Immunogenetics database (http://www.imgt.org/) was used to assign or eliminate the presence of DSA based on shared sequences. In the rare instance where multiple beads representing a single antibody specificity were present and the four digit donor type was not known, the mean positive MFI value was calculated for that specificity. For the purpose of this study, MFIs of multiple DSA within Class I and Class II were added together to provide a total DSA MFI value. No cut-off for positive DSA MFI was applied due to the lack of consensus in the literature.

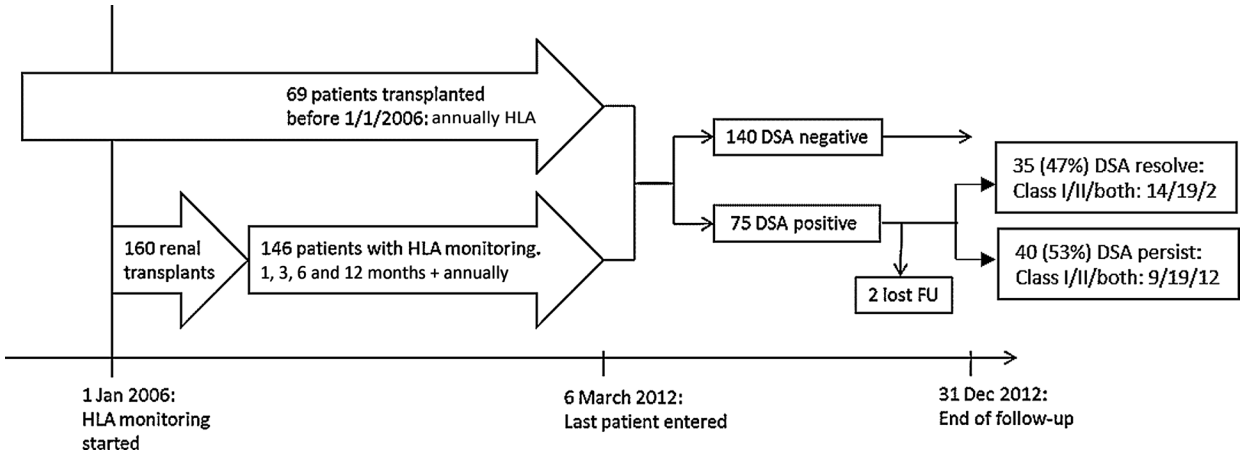

Figure 1: Study design. HLA monitoring was started on all patients on January 1, 2006. At that point, 69 active renal transplant recipients (RTR) had HLA monitoring in addition to 146 new RTR. FU = follow-up.

Statistics

Data were presented as mean (95% confidence interval [CI]) and median (inter-quartile range [IQR]) as appropriate. Comparisons between groups were performed with Mann–Whitney. Comparisons of proportions were performed using Fischer's test. Survival analysis was performed using Kaplan–Meier and log-rank test. Multivariable analysis was performed using Cox regression with forward stepwise (likelihood ratio) method. Statistical analysis was performed using GraphPad Prism 5.0 (LaJolla, CA) and SPSS 20 (Chicago, IL) with p-values < 0.05 considered significant. Multilevel linear modeling was performed using nlme on the R statistical platform (13,14).

Results

HLA antibody monitoring

One hundred and sixty children underwent renal transplantation during our study period from January 1, 2006 to December 31, 2012. Of these, 146 were prospectively monitored for posttransplant HLA-specific antibody production at 1, 3, 6 and 12 months and annually thereafter posttransplant and during episodes of renal allograft dysfunction (Figure 1). Cumulative frequency of antibody tests was 60%, 86% and 98% at 3, 6 and 12 months, respectively. In addition, there were 69 children transplanted before January 1, 2006 who had clinical follow-up and were therefore included in the study. A total of 888 HLA antibody tests were available for analysis with a median of four tests (IQR 3–4) per patient with 19 patients only having one HLA antibody test result available. Baseline characteristics of patients are shown in Table 1 with 11 patients having previously failed transplants and other patients having low sensitization risk pretransplant and no preformed DSA. During the course of follow-up, 85/215 (40%) patients were negative for HLA-specific antibodies. 55/215 (26%) patients developed HLA-specific antibody which were nondonor-specific (NDS-HLA) on at least two different time points. Seventy-five of 215 (35%) patients became DSA positive; of these, 23/75 (31%) were Class I DSA, 38/75 (51%) were Class II and 14/75 (19%) were positive to both Class I and II. Breakdown of HLA antibody groups are as follows: HLA-A (16 patients), HLA-B (21 patients), HLA-C (7 patients), HLA-DPB (1 patient), HLA-DQA/B (34 patients) and HLA-DR (14 patients). There were no differences in baseline characteristics of patients who developed DSA compared to DSA negative patients (Table 1).

DSA levels fluctuated during follow-up and 35/75 (47%) were negative on at least two consecutive occasions at last follow-up. Fifty-three of 75 DSA positive patients were transplanted after January 1, 2006 and had antibody monitoring from the start of transplantation. Overall, 27/53 (51%) were first detected within the first 3 months, 6/53 (11%) were detected between 3 and 6 months after transplantation and 6/53 (11%) were detected between 6 and 12 months posttransplant. Comparing patients who had persistent versus resolved DSA, the resolved group had DSA, which were detected earlier and had lower MFI (Table 2; Figure 2). The DSA persistent group also developed more concomitant Class I and II DSA.

Biopsy findings

A total of 182/213 (85%) patients had 437 biopsies (Table 3). There was no difference between the proportion of patients having biopsies in the DSA positive and DSA negative

Table 1: Baseline characteristics of patients

	DSA negative (N = 140)	DSA positive (N = 75)	p-Value[1]
Age at transplant (years)	10.1 (4.1–14.0)	9.7 (5.4–14.0)	0.92
Donor type, living donor	74 (53%)	40 (5%)	1.0
Male	85 (61%)	51 (68%)	0.30
Ethnicity			
White	91 (65%)	44 (59%)	0.44
Black	9 (6%)	8 (10%)	
Asian	40 (29%)	23 (31%)	
Retransplants	4 (3%)	7 (9%)	0.05
Pretransplant HLA sensitization (CRF%)			
0	120 (86%)	59 (79%)	0.15
1–10	7 (5%)	1 (1%)	
>10	12 (9%)	11 (15%)	
NA	1	4	
Diagnosis			
Structural defects	69 (49%)	36 (48%)	
Immune-mediated	19 (14%)	10 (13%)	0.98
Others	52 (37%)	29 (39%)	
Mismatch	2 (2–3)	2 (2–3)	0.06
Follow-up length (years)	3.1 (1.7–5.1)	2.8 (1.4–5.2)	0.71

CRF, calculated reaction frequency; DSA, donor-specific HLA antibodies; NA, not available.
Data are presented as median (inter-quartile range) or n (%).
[1]Fischer's test for comparison of proportions and Mann–Whitney test for comparison of continuous variables.

Table 2: Characteristics of DSA

	DSA positive (N = 75)	DSA resolve (N = 35)	DSA persist (N = 40)	p-Value[1]
Time first detected posttransplant (years)	0.25 (0.10–1.16)	0.14 (0.09–0.33)	0.84 (0.15–2.37)	<0.01
Age first detected (years)	12.7 (6.40–15.9)	11.1 (5.8–15.7)	14.8 (7.8–16.0)	0.27
MFI first detected (units)	3433 (1861–6588)	2409 (1399–3544)	5835 (2903–10 660)	<0.001
Peak MFI (units)	4981 (2246–9647)	2658 (1573–3819)	7820 (5166–11 990)	<0.0001
Class 1/Class 2/Both	23/38/14 (31%, 51%, 18%)	14/19/2 (40%, 54%, 6%)	9/19/12 (23%, 47%, 30%)	0.019

DSA, donor-specific HLA antibodies; MFI, mean fluorescence intensity.
Data are presented as median (inter-quartile range).
[1]Comparing DSA resolve and DSA persist, Mann–Whitney.

group. There was also no difference in the proportion of patients with tubulitis and vasculitis. The number of episodes of tubulitis and vasculitis were also similar between the two groups. Of note, there were also six patients with vasculitis and the absence of tubulitis on biopsy (''isolated v-lesions'') (15). Three of these patients were DSA positive and the remaining three were NDS-HLA-positive. As expected, biopsy findings of C4d, microvasculature injury and CD20 cells were more common in the DSA group. Four patients had BK virus associated nephropathy; one patient was DSA negative and three patients were DSA positive. There were no cases of posttransplant lymphoproliferative disorder. Comparing patients who had persistent versus resolved DSA, the persistent group was associated with more tubulitis (16/40 vs. 6/35, p = 0.042). There were lower rates of vasculitis, C4d and

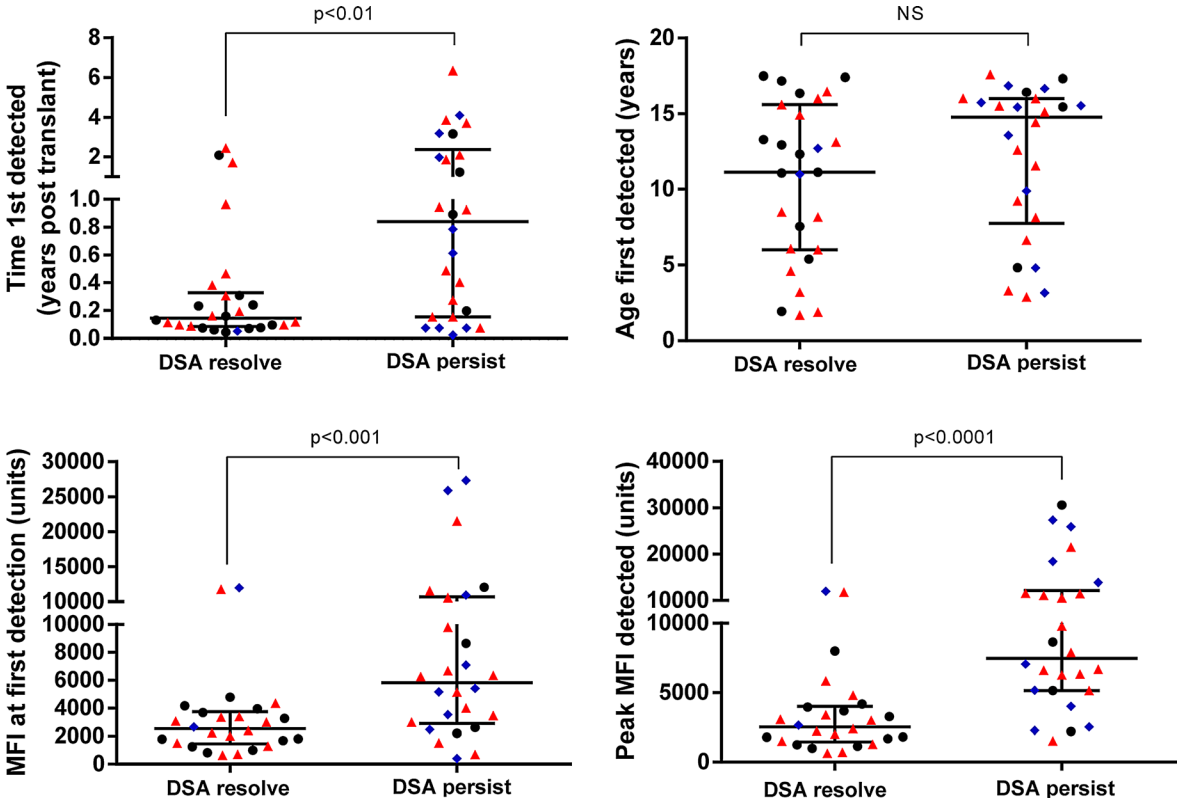

Figure 2: Comparison of factors associated with donor-specific HLA antibodies (DSA) resolve versus DSA persist. Bars represent median and inter-quartile range. ● Class 1 DSA ▲ Class 2 DSA ◆ Both Class 1 and 2.

Table 3: Biopsy findings in DSA negative and DSA positive patients

	DSA negative (N = 140)	DSA positive (N = 73)	p-Value[1]
Tubulitis	48 (34.3%)	24 (32.9%)	0.8796
Mean episodes per person	1.4 (69/48)	1.83 (44/24)	
Vasculitis	18 (12.9%)	11 (15.1%)	0.6771
Mean episodes per person	1.3 (23/18)	1.4 (16/11)	
C4d	9 (6.4%)	17 (23.3%)	0.0007
Microvasculature injury	7 (5.0%)	12 (16.4%)	0.0237
CD20	17 (12.1%)	18 (24.7%)	0.0308

DSA, donor-specific HLA antibodies.
Data presented as n (%).
[1]Fischer's test for comparison of proportions.

microvasculature injury though this did not reach statistical significance (Table S2).

Renal allograft function

Median GFR at baseline was 64.5 (IQR 51.1–79.0) mL/min/1.73 m^2. The overall rate of GFR decline was low with a median change of −2.6 (IQR −10.1 to 2.3) mL/min/1.73 m^2 per year at 2 years posttransplantation and −4.2 (−8.1 to 1.5) mL/min/1.73 m^2 per year at 3 years posttransplantation. Therefore, we utilized a primary end point of 50% reduction in GFR for survival analysis as previously established in a pediatric study (16). Records were analyzed to exclude causes of reversible GFR loss, for example, treated acute rejection, dehydration and infection. DSA positive patients had a higher risk of GFR decline and lost 50% of GFR at a mean of 5.3 (CI 4.7–5.8) years compared to DSA negative patients at 6.1 (CI 5.7–6.4) years ($p = 0.02$; Figure 3). There was no significant risk of GFR decline comparing patients who had NDS-HLA antibodies to HLA antibody negative patients. Interestingly, there was no difference between subgroups of patients with persisting DSA versus patients with DSA, which resolved, although there was a larger fall in GFR in the persisting DSA group in the early follow-up period.

Cox regression was used to analyze risk factors for GFR decline. Univariate Cox regression for risk factors analyzed are shown in Table 4. Among the clinical factors, only age at transplant was associated with GFR decline. Donor type and pretransplant HLA calculated reaction frequency of >10% approached statistical significance ($p = 0.06$). Biopsy findings were all significantly associated with GFR decline. There was positive agreement between patients with tubulitis and vasculitis (Spearman's correlation 0.4, $p < 0.001$). There was also positive agreement between biopsy findings of AMR (C4d, microvasculature injury and CD20). There is literature to suggest that C4d is not a good predictor of graft outcome and C4d negative AMR has recently been recognized in the Banff schema (17–19). Therefore, to avoid multi-co-linearity, C4d was not included in the final model. Microvasculature injury and CD20 were included as there is evidence supporting both these factors in predicting renal allograft outcome (20–23). In the multivariable Cox regression analysis, the three independent factors predicting GFR decline were age at transplant (hazard ratio 1.1, CI 1.0–1.2), number of tubulitis episodes (1.5, CI 1.3–1.8) and the presence of microvasculature injury (2.9, CI 1.4–5.7).

Multilevel linear modeling of GFR

To allow for analysis of the relationship between GFR and DSA over time, a multilevel linear model was employed (9). This enables analysis of fluctuating MFI levels and its effect on GFR rather than treating DSA as a positive or negative result. A two-level model was used; that is, longitudinal measurements of GFR and DSA within individual patients (level one) and differences between patients (level two). To evaluate if a multilevel approach was indeed necessary we

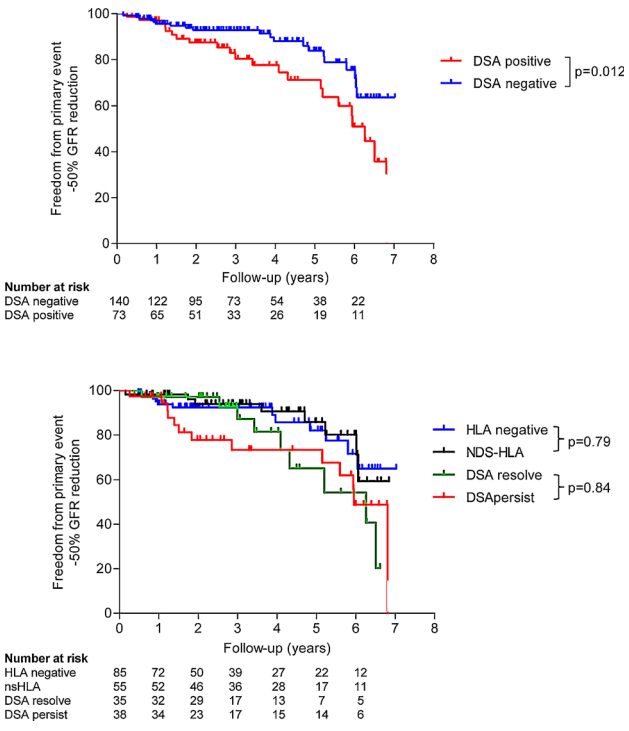

Figure 3: Kaplan–Meier survival curves for 50% GFR reduction according to HLA result.

Table 4: Cox regression analyses of factors associated with GFR decline

	Hazard ratio (CI)	p-Value
Univariate Cox regression		
Age at transplant (years)[1]	1.1 (1.0–1.2)	0.02
Donor type: LD/DD[1]	1.8 (1.0–3.3)	0.06
Male/female		0.67
Pretransplant HLA sensitization (CRF%)[1]		
1–10% versus 0	0	0.98
>10% versus 0	2.3 (0.9–5.6)	0.06
Diagnosis		
Structural defects versus others	1.6 (0.9–2.4)	0.16
Immune-mediated versus others	2.4 (1.4–3.4)	0.09
Mismatch	1.0 (0.7–1.4)	0.88
DSA[1]	2.1 (1.1–3.8)	0.019
Tubulitis[1]	1.5 (1.2–1.7)	<0.001
Vasculitis[1]	2.4 (1.6–3.5)	<0.001
C4d	2.5 (1.2–4.9)	0.011
Microvasculature injury[1]	2.9 (1.5–5.9)	<0.01
CD20[1]	1.7 (1.3–2.3)	<0.001
Multivariable Cox regression		
Age at transplant (years)	1.1 (1.0–1.2)	0.018
Tubulitis	1.5 (1.2–1.8)	<0.001
Microvasculature inflammation	2.9 (1.4–5.7)	<0.01

CRF, calculated reaction frequency; DD, deceased donor; LD, living donor.
[1]Univariate factors included in multivariable analyses.

compared a random intercept model to a single level null model fitted with restricted maximum likelihood. Indeed the comparison suggested that the multilevel model was a better candidate than the single level model ($\chi^2 = 411$, $p < 0.001$). This is because the multilevel model inherently accounts for individual differences in mean GFR. Serial correlation was addressed by using an autocorrelation structure of order 1 (AR1). An intercept only model with the AR1 correlation structure was significantly better that a model without ($\chi^2 = 119$, $p < 0.001$).

Considering the possibility that the effect of time on GFR could vary from person to person (i.e. GFR changes at different rates) we compared a model with a fixed time effect to a model with a random time effect. Allowing for a random slope for the effect of time did not show a significant improvement to the model ($\chi^2 = 4.026$, $p > 0.05$), and therefore, the simpler model with a fixed effect was used.

Variables included in the model were all patient characteristics; and concomitant values of GFR, Class I and II DSA MFI posttransplant. A logarithmic scale was used for DSA MFI and a value of one was added to all MFI as the logarithm of zero is not defined. The analysis was limited to patients with at least three HLA antibody results. Therefore, 170 patients were analyzed in the model with a total of 767 time points posttransplant. Results are presented in Table 5.

In concordance with the univariate analysis, the only significant patient characteristic was age at transplant with older patients having a lower GFR of 1.4 ($\pm$ 0.3) mL/min/1.73 m^2 per year. A diagnosis of structural defects just achieved statistical significance. The average baseline GFR was 85.7 mL/min/1.73 m^2. There was a significant overall rate of GFR decline at 2.8 ($\pm$ 0.3) mL/min/1.73 m^2 per year. The effect of Class II DSA was also significant with a decrease of 1.5 ($\pm$ 0.5) mL/min/1.73 m^2 per logarithmic increase in DSA MFI.

Discussion

This was a prospective cohort study of HLA-specific antibody monitoring which is the largest to date in pediatric RTR who were at low risk for sensitization. As no changes were made to immunosuppression based on results of HLA antibodies alone, we have observed the natural history of DSA as detected by single-antigen beads and correlated this to renal allograft function. Consistent with the literature, DSA positive patients were associated with a greater GFR decline though our study additionally highlighted subtle differences within the DSA positive group. First, DSA, which were detected early posttransplant and had lower MFI levels could resolve. Second, DSA was not an independent risk factor in multivariable Cox regression unless patients developed microvascular injury. Last, the magnitude of effect on GFR is proportional to the MFI level for Class II DSA.

In this study, DSA were detected early posttransplant at a median of 0.25 years. No threshold was applied for reporting positive results as there was no standardized cut-off value in the literature, and we wanted to capture all results (4). Recent adult studies with positive MFI thresholds of 300–500 have also corroborated this finding (3,21,24). In addition, donor-specific B cells were detected 2 months posttransplant using an ELISPOT method even in a group of nonsensitized first transplants (25). In our study, almost half of the DSA became undetectable on last follow-up. These were associated with lower MFIs and were detected early posttransplant. One possible explanation is that the Luminex method detects low-level cross-reactive epitopes that are produced by inflammation following transplantation surgery (26). In the short-term, the outcome of DSA resolved patients was similar to DSA negative patients which may suggest a degree of accommodation consistent with *in vitro* data showing that endothelial cells are able to increase Akt phosphorylation and increase Bcl expression in the presence of low levels of Class I DSA (27). Long-term outcomes though were similar to DSA positive patients, suggesting heterogeneity in these patients or alternative nonantibody-mediated mechanisms of rejection for example antigen presentation to T cells leading to increase cellular-mediated rejection.

Although there is an accumulating body of evidence for a causative effect of DSA on renal allograft loss in adults, the

Table 5: Two-level model of GFR with predictors of patient characteristics and DSA

Random effects			95% CI		
Random intercept (SD)	13.1				
			10.6–16.2		
Residual (SD)	14.1				
			12.0–16.2		
Φ (autocorrelation)	0.52				
			0.37–0.65		
Model fit					
Log likelihood	−3086				
BIC	6272				
Fixed effects	Regression coefficient	Standard error	DF	t-Value	p-Value
Intercept (baseline GFR)	85.68	5.062	594	16.925	<0.0001
Time posttransplant (years)	−2.82	0.322	594	−8.771	<0.0001
Class 1 DSA MFI (log [C1 + 1])	−0.15	0.272	594	−0.535	0.59
Class 2 DSA MFI (log [C2 + 1])	−1.46	0.485	594	−3.018	0.0027
Age at transplant (years)	−1.45	0.282	164	−8.771	<0.0001
Diagnosis					
Structural versus others	−5.63	2.793	164	−2.017	0.0454
Immune versus others	−6.02	4.248	164	−1.418	0.1581
Donor type (LD/DD)	4.49	2.544	164	1.766	0.0793
Mismatch	−1.99	1.363	164	−1.460	0.1463

BIC, Bayesian information criterion; DD, deceased donor; DF, degrees of freedom; DSA, donor-specific HLA antibodies; LD, living donor; MFI, mean fluorescence intensity.

literature in pediatric populations is sparse and is compounded by small patient numbers (3,28). There are three pediatric *de novo* DSA studies to-date (29–31). Miettinen et al (31) showed no effects on renal allograft outcome as measured by ^{51}Cr-EDTA GFR with a follow-up time of up to 10 years posttransplant. The remaining two studies showed increased plasma creatinine in DSA positive patients (29,30). All studies used antibody induction and triple-immunosuppression. The reasons for the different outcomes are not clear, hence, the need for larger studies. Our study is the first pediatric cohort to show poorer renal allograft function using Kaplan–Meier survival analysis. However, in the multivariable analysis, DSA did not remain a significant risk factor and was superseded by microvasculature injury. This could be explained by a lag bias in the detection of microvasculature injury as biopsies were only performed at the time of renal allograft dysfunction with elevated plasma creatinine, which is a late marker of allograft dysfunction (2). Patients with microvasculature injury are often resistant to current treatment modalities including anti-thymocyte globulin, rituximab and plasmapheresis (20,21). Episodes of tubulitis can potentially lead to development of DSA due to increased inflammation and B cell activation (5). It is also recognized that patients with combined cellular and antibody-mediated rejection have the least favorable outcome (32). Age at transplant was another independent risk factor that should be further investigated. One obvious explanation is the association of noncompliance with mixed cellular and antibody-mediated rejection leading to renal allograft loss although data were not available to answer this question (33). However, it would be interesting to postulate if there are any regulatory elements in younger patients, which can mitigate the effects of DSA.

The largest number of DSA in this study was targeted at the HLA-DQ locus. This could be because HLA-DQ is currently not being matched in organ allocation algorithms in the United Kingdom. Historically, this was due to the strong linkage disequilibrium between HLA-DR and HLA-DQ. Nonetheless, with the current high resolution HLA antibody testing, it is increasingly being recognized that HLA-DQ antibodies can be formed separate from HLA-DR and is associated with increased risk of transplant glomerulopathy and graft failure (34–36). The matching of HLA-DQ is complex and needs to take into account polymorphisms in both the alpha- and beta-chains (37,38). A large body of work remains to determine relative frequencies of HLA-DQ alleles in the population, the method of determining unacceptable HLA-DQ mismatches and the impact of increased waiting times due to increased matching criteria on organ allocation program (37–40). However, DQ mismatching is likely to be part of algorithms for matching in the future.

There was a large quantity of data accumulated in the study with multiple data time points for each patient. This presents a difficulty for standard statistical methods, which does not allow analysis of changes in DSA MFI over time. To our knowledge, we are the first study to use a multilevel linear model to achieve this. This hierarchical model allows data collected at different levels to be studied without

violating assumptions of independence in linear multiple regression. For example, the effect of DSA for each patient would be inter-dependent at each time point but the effects of DSA could be different for each patient. The model makes an assumption on linearity and therefore does not specifically analyze DSA MFI, which increase and then decrease. Changes in DSA early posttransplant might be overly represented compared to changes in GFR occurring late posttransplant. However, this limitation is minimized by the large number of patients in the model. There were also a large number of patients without DSA, which generates an average decline in GFR over time and therefore allows analysis of deviation from this trend in DSA positive patients. It would be interesting to build on this model with additional variables to see if it can be improved. One such variable could be longitudinal immunosuppression data.

We have observed findings of microvasculature injury in the DSA negative group. There were also three patients with ''isolated v-lesions'' and NDS-HLA antibodies (41). Therefore, one limitation of the study was that non-HLA antibodies specific for targets such as MICA and angiotension II Type 1 receptor were not measured (30,42,43). Additionally, the prognostic value of DSA can potentially be improved by adding C1q assays (44). However, it should be noted that DSA+ C1q negative patients have worse renal allograft survival compared to DSA negative patients (44). Another caveat is that the Luminex assay only provides a semi-quantitative result mainly due to inter-assay variability. This can be improved using a standard operating protocol which reduces MFI variability to 25% (45). In this study, MFI values were normalized against the patient's background value and the negative serum value to standardize results. A different computational normalization method using the LOESS curve fitting algorithm can be performed to improve variation (45). Overall, the calculated GFR change due to changes in MFI can only be taken as an approximation of magnitude of effect rather than a definite result and the statistical model requires further validation in different cohorts. In addition, respective Class I and Class II DSA MFI values were taken as a whole and added together. This method has not been validated and assumes equal importance of each HLA type.

In conclusion, our study provides evidence for the association of DSA with poor long-term allograft outcome in pediatric RTR. We propose that there is a graded response to DSA MFI levels with low levels possibly resolving and high levels quantitatively associated with lower GFR. Patients with microvasculature injury have the highest risk for renal allograft dysfunction. We propose that prospective HLA antibody monitoring is important in pediatric patients and high DSA MFI levels should be targeted with increased immunosuppression even in the absence of a renal allograft dysfunction with an aim to prevent the development of microvascular injury. This should be performed systematically in the setting of a clinical trial (46).

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1: Breakdown of diagnostic categories.

Table S2: Biopsy findings in DSA persistent and DSA resolved patients. Data presented as n (%). *Fischer's test for comparison of proportions.