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Persistent C4d and Antibody-Mediated Rejection in Pediatric Renal Transplant Patients

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Abstract

Background—Pediatric renal transplant recipient survival continues to improve, but antibody-mediated rejection (ABMR) remains a significant contributor to graft loss. ABMR prognostic factors to guide treatment are lacking. C4d staining on biopsies, diagnostic of ABMR, is associated with graft failure. Persistent C4d+ on follow-up biopsies has unknown significance, but could be associated with worse outcomes.

Methods—Retrospective cohort of 17 pediatric renal transplant patients diagnosed with ABMR. Primary outcome at 12 months was a composite of 50% reduction in estimated glomerular

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filtration rate, transplant glomerulopathy, or graft failure. Secondary outcome was the urine protein-to-creatinine ratio at 12 months. We used logistic and linear regression modeling to determine if persistent C4d+ on follow-up biopsy was associated with the outcomes.

Results—Forty-one percent reached the primary outcome at 12 months. Persistent C4d+ on follow-up biopsy occurred in 41%, was not significantly associated with the primary outcome, but was significantly associated with the secondary outcome (estimate 0.22, 95% CI 0.19 to 0.25, $p < 0.001$), controlling for confounding.

Conclusions—Persistent C4d+ on follow-up biopsies was associated with a higher urine protein-to-creatinine ratio at 12 months. Patients who remain C4d+ on follow-up biopsy may benefit from more aggressive or prolonged ABMR treatment.

Keywords

Donor-specific antibody; C1q; humoral rejection; proteinuria; transplant glomerulopathy; graft failure

Introduction

Pediatric renal transplantation has dramatically improved patient survival and quality of life for children with end-stage renal disease. The discovery of recipient alloantibodies to donor antigens led to improved renal transplant outcomes^{1, 2}. Despite the increased sensitivity and specificity of donor-specific antibody (DSA) testing, antibody-mediated rejection (ABMR) remains a significant cause of acute and chronic renal allograft injury³. ABMR is the major contributor to late allograft failure⁴. In ABMR, antibodies bind to allograft endothelial antigens expressed on the peritubular and glomerular capillaries, leading to necrosis, apoptosis, and ischemic injury⁵. In addition to the presence of circulating DSA, the diagnosis of acute ABMR requires histological evidence of recent endothelial injury, such as glomerulitis or peritubular capillaritis and linear peritubular capillary C4d staining^{6, 7}. A byproduct of complement fixation and activation, C4d remains bound to the vascular endothelium in ABMR⁸. Histologic staining of C4d therefore is a useful biomarker of ABMR and is strongly associated with graft failure^{9–13}.

Peritubular capillary C4d staining detected by immunohistochemistry is less sensitive than immunofluorescence, which is taken into account by the Banff classification in determining the threshold for positivity. Glomerular C4d staining is nonspecific on immunofluorescence microscopy, but when present on immunohistochemical staining may suggest ABMR^{14, 15}. Little is known, however, about the importance of persistent C4d staining on follow-up biopsies. There are a lack of prognostic indicators for ABMR treatment, which limits clinical trials¹⁶. Persistent C4d staining on follow-up biopsies may provide evidence for ongoing microvascular injury and could be a risk factor for worse outcomes. Our goal was to describe prognostic factors associated with acute/active ABMR in order to improve ABMR treatment. Our hypothesis was that persistent C4d staining on follow-up biopsy is associated with poor clinical outcomes.

Methods

Study Design

We performed a retrospective cohort study of 17 pediatric patients who underwent renal transplantation at Lucile Packard Children's Hospital at Stanford in Palo Alto, CA, between 2008 and 2014 (208 total patients) and were diagnosed with acute/active ABMR before October 1st, 2014 (1.2% yearly incidence of ABMR). Exclusion criteria included transplantation at another institution, C4d-negative ABMR, and lack of follow-up biopsy. Subjects were diagnosed with ABMR by indication biopsy or surveillance protocol biopsy per institutional protocol, and ABMR diagnosis required the presence of all three features of acute/active ABMR, based on the updated Banff 2015 criteria¹⁷: 1) histologic evidence of acute tissue injury (one or more of microvascular inflammation, intimal or transmural arteritis, acute thrombotic microangiopathy, and acute tubular injury in the absence of any other apparent cause); 2) evidence of current/recent antibody-vascular endothelium interaction [any C4d staining by immunohistochemistry on paraffin tissue (C4d >0, Figure 1a and Figure 1b) or at least moderate microvascular inflammation (Banff g+ptc 2)]; and 3) serologic evidence of DSA. One pathologist reviewed each biopsy to confirm the diagnosis (NK). The immunostaining protocol on the Leica Bond consisted of 1) heat-induced epitope retrieval with Leica's EDTA-based ER2; 2) incubation with primary polyclonal rabbit C4d antibody (cat. no. B1-RC4D, Biomedica, Austria, 1/80 dilution); and 3) antibody detection using the Leica Bond Refine polymer detection kit (cat. no. DS9800, Leica Biosystems Ltd, Newcastle, UK). After a diagnosis of rejection is made, our protocol is to perform routine follow-up biopsies at six to eight weeks after the initial biopsy to assess the response to treatment unless there is a contraindication such as active infection. The Institutional Review Board approved the study.

Data Collection

We noted demographic and pre-transplant clinical data, including sex, self-reported race (Caucasian, African American, Hispanic, or Asian), underlying renal disease, history of dialysis, prior transplant, and sensitization status (panel reactive antibody >40% or positive DSA). We recorded transplant data, including donor status, antigen match, induction immunosuppression medication and dosage, steroid status, and presence of delayed graft function (requiring dialysis within the first week post-transplant). Data relevant to the diagnosis of ABMR was documented, including subclinical rejection (diagnosed on surveillance protocol biopsy), time to rejection, and non-compliance as documented in the Electronic Medical Record. Biopsy data was recorded, including concurrent tubulointerstitial or vascular rejection, Banff T-cell-mediated rejection (TCMR) grade, peritubular capillary and glomerular C4d staining, and Banff scoring of all histological and immunohistochemical parameters including peritubular capillaritis and glomerulitis. Transplant glomerulopathy was diagnosed in the presence of one or more glomerular capillaries with basement membrane double contours by light microscopy (cg1b), as only two biopsies (from two different subjects) had electron microscopic evaluation; neither had evidence of glomerular or peritubular capillary basement membrane abnormalities (cg0)^{6, 17}. Immunodominant DSA characteristics were noted, including class I or II and C1q positivity. We categorized ABMR treatment [high-dose pulse intravenous methylprednisolone,

intravenous rituximab, intravenous immunoglobulin, intravenous thymoglobulin (ATG), plasmapheresis, intravenous bortezomib, and oral steroid status], time to follow-up biopsy, and C4d status on follow-up biopsy (persistent C4d+).

Serum creatinine and urine protein-to-creatinine ratio (UPCR, mg protein/mg creatinine) were recorded at baseline, at the time of ABMR diagnosis, and 12 months after ABMR diagnosis. Estimated glomerular filtration rate (eGFR) was calculated using the updated Schwartz equation¹⁸. Proteinuria was defined as UPCR >0.2^{19, 20}. We recorded any use of anti-proteinuric medications (angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers) during the study period.

Outcomes

The primary outcome 12 months after ABMR diagnosis was a composite of 1) 50% reduction in eGFR from baseline to 12 months after ABMR diagnosis, 2) transplant glomerulopathy on biopsy, or 3) graft failure. UPCR 12 months after ABMR diagnosis was the secondary outcome. Our primary predictor was persistent C4d positivity (C4d+) on follow-up biopsy.

Statistical Analyses

Categorical variables were summarized with frequency distributions and continuous variables were summarized by mean with standard deviation (*SD*) or median with interquartile range (*IQR*). Between-group differences were analyzed using Fisher's Exact test for categorical variables and t-test or Wilcoxon Rank-Sum test to analyze continuous variables. Spearman correlation coefficients were used to analyze the relationship between continuous covariates and the continuous outcome. To build the final models, we first used bivariate analyses to examine the relationships between each potential confounder and both the primary predictor and each outcome measure. Potential confounders were included in the final models if they were 1) associated with the predictor and the outcome at $p < 0.2$; or 2) associated with a >10% change in the significant predictor-outcome estimate. Creatinine at baseline and at ABMR diagnosis was *a priori* included in the primary outcome model, and the UPCR at baseline and at ABMR diagnosis was *a priori* included in the secondary outcome model. We used logistic regression modeling for the primary outcome and general linear regression modeling for the secondary outcome. Enterprise Guide software, Version 7.11 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA) was used for all analyses.

Results

Baseline and Transplant Characteristics

Subjects were racially diverse (47% Hispanic, 29% Caucasian, 12% African American, and 12% Asian) and 71% were male (Table 1). One subject (6%) had a prior renal transplant and 35% were sensitized prior to transplantation. Of the six subjects who were sensitized, three received pre-transplant (two) or perioperative (one) intravenous immunoglobulin for desensitization but no further desensitization was performed. The majority (76%) received less than a 2 out of 6 antigen-matched allograft. All subjects received induction

immunosuppression, predominantly with ATG (76%). The majority of subjects (65%) were on steroid-free maintenance immunosuppression, and all subjects received tacrolimus and mycophenolate. Three subjects (18%) had delayed graft function.

ABMR Characteristics

Table 2 lists subjects' ABMR characteristics. The mean time to rejection was 21.0 months after transplant and 53% had documented non-compliance. The majority of subjects had class II immunodominant DSA (76%), most commonly DQ (71%), and 94% were C1q-positive. Mixed rejection was common, with concurrent acute T-cell-mediated tubulointerstitial rejection (TCMR grade I, 65%) and/or vascular rejection (TCMR grade II, 6%). The majority of biopsies were graded Banff IB (35%) but 29% were negative for TCMR. Glomerulitis was present in 24% of cases. One subject had evidence of acute and chronic active ABMR on index biopsy (subject 15, Banff cg=1). Complete Banff scores for the histological and immunohistochemical components of each index biopsy are shown in Supplementary Table 1.

Overall, ABMR treatment was heterogeneous. All subjects received intravenous immunoglobulin, 76% received rituximab, 71% received high-dose pulse steroids, and 53% received ATG (the latter two agents were used to treat concomitant TCMR). Eighteen percent received plasmapheresis and 12% received bortezomib. Of the eight subjects who were on steroid-free maintenance immunosuppression, 50% were changed to a steroid-based protocol after ABMR diagnosis.

Persistent C4d Staining

Forty-one percent of subjects were persistently C4d+ (C4d >0) on their follow-up biopsy (Supplementary Tables 2 and 3). The median time to follow-up biopsy was 2.0 months (IQR 1.6, 6.3). The C4d+ and C4d- groups differed only in their UPCR at ABMR diagnosis [median (IQR) 2.17 (0.37, 4.15) vs 0.28 (0.11, 0.53), $p = 0.03$] and their total treatment dose of ATG [mean (*SD*) 6.3 mg/kg (1.2) vs 8.5 (1.3), $p = 0.04$]. There were no differences by persistent C4d staining in DSA status (class or C1q positivity) or other pathology characteristics.

Primary Outcome

Seven subjects (41%) reached the composite primary outcome at 12 months after ABMR diagnosis (Figure 2). Three subjects had a 50% reduction in their eGFR, two subjects had transplant glomerulopathy, and three subjects had graft failure. One of the subjects whose graft failed also had transplant glomerulopathy. There were no differences in baseline, transplant, or ABMR characteristics between groups, including creatinine at baseline and at ABMR diagnosis, except that subjects who reached the primary outcome were older at transplant (16.3 years vs 14.9, $p = 0.05$) and had an earlier follow-up biopsy [median (IQR) 1.6 months (1.3, 1.8) vs 3.8 (2.0, 8.6), $p = 0.02$]. There were no differences in ABMR treatment between the two groups. The two groups did not differ in rates of persistent C4d+ (43% vs 40%, $p = 1.00$). Table 3 shows the final regression model for the primary outcome at 12 months, controlling for creatinine at baseline and at ABMR diagnosis. Persistent C4d+

was not significantly associated with the primary outcome (OR 1.81, 95% CI 0.18 to 17.87, $p = 0.61$).

Secondary Outcome

Data on proteinuria at 12 months after ABMR diagnosis was available in 11 subjects (65%). The median (IQR) UPCR at 12 months was 0.23 (0.08, 0.66). One subject received the angiotensin-converting enzyme inhibitor lisinopril during the study period. On initial bivariate analyses, subjects with persistent C4d+ had higher UPCR at 12 months [median 0.66 (0.28, 0.71) vs 0.09 (0.01, 0.23), $p = 0.04$] (Figure 3). The UPCR at 12 months differed by presence of TCMR [median (IQR) 0.66 (0.23, 0.71) vs 0.14 (0.01, 0.23), $p = 0.1$]. UPCR at baseline (correlation coefficient 0.37, $p = 0.14$) and at ABMR diagnosis (correlation coefficient 0.5, $p = 0.04$) were correlated with UPCR at 12 months. Table 3 shows the final regression model for the secondary outcome. Data was available for eight subjects, and the model controlled for UPCR at baseline, UPCR at ABMR diagnosis, and concurrent TCMR. Use of lisinopril was not a significant confounder in the model. Persistent C4d+ was associated with a 0.22 increase in the UPCR at 12 months (0.19 to 0.25, $p < 0.001$).

Discussion

Our study identified a novel independent risk factor associated with adverse outcomes after acute/active ABMR in pediatric renal transplant recipients. Persistent C4d+ on follow-up biopsies was associated with a higher UPCR 12 months after ABMR diagnosis. Proteinuria is a known marker of chronic kidney disease in transplant recipients and predicts graft failure^{21–23}. The reason for this persistent C4d staining is unclear and as C4d staining is not specific for ABMR, could indicate dynamic humoral activity, accommodation, continued microvascular damage, chronic ABMR, or repeated episodes of acute ABMR^{11, 24–26}. Tissue-bound C4d generally indicates recent immunologic activity, usually within weeks²⁴. There was no difference in C4d persistence by time to follow-up biopsy (median 2.0 months). Our study provides evidence supporting the prognostic value in staining for C4d on follow-up biopsies when evaluating a patient's response to ABMR treatment.

The association between persistent C4d+ and UPCR at 12 months was independent of the UPCR at baseline and at ABMR diagnosis, both of which were also significantly associated with UPCR at 12 months. This is consistent with the literature, wherein worse proteinuria at the time of ABMR diagnosis is associated with decreased treatment response and worse ABMR outcomes²⁷. We also controlled for concomitant TCMR, and consistent with previous studies in adult transplant recipients, we found that TCMR was associated with worse outcomes^{14, 28, 29}.

To our knowledge, this study is the largest to date to investigate ABMR in pediatric renal transplant recipients. Thirty-five percent of our subjects were sensitized at the time of transplant but this had no effect on outcomes. Steroid-free immunosuppression had no effect on outcomes and was a common maintenance immunosuppression protocol in our population. Non-compliance was common but we could not detect a difference in outcomes given the small sample size. Our study did not find any differences according to immunodominant DSA but this is likely because the majority of our subjects had class II

DSA and all but one subject was C1q-positive. Class II DSA, and in particular C1q-positive DSA, are associated with a higher risk of C4d positivity, ABMR, and graft loss^{30, 31}. Interestingly our study found no difference in outcomes by treatment.

Our study's small sample size and single-center experience combined with a lack of a standard ABMR treatment protocol limited the study. We may have missed additional cases of transplant glomerulopathy that were normal on light microscopy due to the lack of electron microscopy except in two biopsies. Treatment in our population was consistent with the standard of care in pediatric renal transplant recipients^{32–35}. Due to the small sample size we were unable to subcategorize into early or late ABMR, which could also mask differences in the outcomes. Persistent C4d+ may be associated with worse clinical outcomes in patients with ABMR. Our study suggests that serial C4d staining on follow-up biopsies in patients with ABMR has prognostic utility and could guide ABMR treatment protocols in pediatric renal transplant recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1a.

Diffuse Peritubular Capillary Staining with C4d

The peritubular capillary in the center demonstrates >10 leukocytes (Banff ptc=3). C4d immunohistochemistry stain on paraffin tissue at x400 magnification

Figure 1b.
Diffuse Glomerular Endothelial Cell C4d Staining
C4d immunohistochemistry stain on paraffin tissue at x600 magnification

Figure 2.
Kaplan-Meier Plot of Outcome-Free Probability Over One Year
Number of Subjects at Risk

Figure 3.
UPCR at 12 Months by Persistent C4d+ Status
* $p = 0.04$ by Wilcoxon Rank-Sum test. C4d+ = 5, C4d- = 6. Bar denotes median, diamond denotes mean, box represents IQR, and whiskers include 1.5x IQR

Table 1

Patient Characteristics by Primary Outcome

		Yes	No
	N = 17	n = 7 (41%)	n = 10 (59%)
Male	12 (71%)	5 (71%)	7 (70%)
Age (yr)	15.7 [3.9, 16.3]	16.3 [15.8, 17.8] *	14.9 [1.8, 15.7]
Race			
Hispanic	8 (47%)	3 (43%)	5 (50%)
Caucasian	5 (29%)	3 (43%)	2 (20%)
African American	2 (12%)	1 (14%)	1 (10%)
Asian	2 (12%)	0 (0%)	2 (20%)
Diagnosis			
CAKUT	5 (29%)	1 (14%)	4 (40%)
Secondary GN	4 (24%)	2 (29%)	2 (20%)
Unknown	4 (24%)	1 (14%)	3 (30%)
Primary GN	3 (18%)	3 (43%)	0 (0%)
ARPKD	1 (6%)	0 (0%)	1 (10%)
Living Donor	2 (12%)	1 (14%)	1 (10%)
Antigen Match 1/6	13 (76%)	4 (57%)	9 (90%)
Delayed Graft Function	3 (18%)	1 (14%)	2 (20%)
Baseline Creatinine (mg/dL)	0.9 (0.4)	1.08 (0.37)	0.78 (0.4)
Baseline eGFR (mL/min/1.73 m ²)	109.2 (36.4)	91.7 (25.6)	121.4 (39.0)
Baseline UPCR [†]	0.18 (0.15)	0.25 (0.2)	0.14 (0.12)
Baseline Proteinuria [†]	5 (45%)	3 (75%)	2 (29%)

N(%), mean (SD), median [IQR].

* Denotes group difference with $p < 0.05$ by Wilcoxon Rank-Sum test;

[†] $n=11$. Congenital anomaly of the kidney and urinary tract (CAKUT), glomerulonephritis (GN), autosomal recessive polycystic kidney disease (ARPKD).

Table 2**Antibody-Mediated Rejection Characteristics by Primary Outcome**

		Yes	No
Clinical	N = 17	n = 7 (41%)	n = 10 (59%)
Subclinical Rejection	5 (29%)	2 (29%)	3 (30%)
Creatinine at Diagnosis (mg/dL)	1.2 [0.9, 3.6]	2.5 [0.9, 10.0]	1.1 [0.8, 2.2]
eGFR at Diagnosis (mL/min/1.73 m ²)	72.6 [16.8, 99.6]	36.9 [11.7, 74.5]	89.1 [17.0, 108.6]
UPCR at Diagnosis *	0.37 [0.19, 2.01]	1.48 [0.33, 4.15]	0.28 [0.11, 0.53]
Proteinuria at Diagnosis *	11 (73%)	5 (83%)	6 (67%)
Donor-Specific Antibodies			
Class II	13 (76%)	6 (86%)	7 (70%)
DQ	12 (71%)	6 (86%)	6 (60%)
C1q Positive	16 (94%)	7 (100%)	9 (90%)
Pathology			
Peritubular Capillary C4d Staining (%)	60 [20, 90]	60 [15, 90]	55 [20, 100]
Banff C4d Score (0–3)	3 [2, 3]	3 [2, 3]	2.5 [2.0, 3.0]
Glomerular C4d Staining Present	12 (71%)	4 (57%)	8 (80%)
Glomerular C4d Staining (%)	20 [0, 100]	10 [0, 30]	65 [10, 100]
Peritubular Capillaritis Present	8 (47%)	4 (57%)	4 (40%)
Banff ptc Score (0–3)	0 [0, 1]	1 [0, 2]	0 [0, 1]
Treatment			
Rituximab	13 (76%)	5 (71%)	8 (80%)
Steroid Pulse	12 (71%)	6 (86%)	6 (60%)
Treatment with ATG	9 (53%)	5 (71%)	4 (40%)
Plasmapheresis	3 (18%)	1 (14%)	2 (20%)
Bortezomib	2 (12%)	1 (14%)	1 (10%)

N(%), median [IQR].

*
n=15.

Table 3

Final Outcome Models

<u>Composite Outcome at 12 Months</u> *	OR	95% CI	P Value
Persistent C4d+	1.81	0.18 to 17.87	0.61
<u>UPCR at 12 Months</u> †	Estimate	95% CI	P Value
Persistent C4d+	0.22	0.19 to 0.25	<0.001

*
n=17, logistic regression, controlling for creatinine at baseline and ABMR diagnosis;

†
n=8, general linear regression, controlling for UPCR at baseline and ABMR diagnosis and ACR.