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ORIGINAL ARTICLE

IVIG and rituximab for treatment of chronic antibody-mediated rejection: a prospective study in paediatric renal transplantation with a 2-year follow-up

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Summary

Chronic antibody-mediated rejection (AMR) is the major cause of late renal allograft loss. There is, however, no established treatment for this condition. We report the results of a prospective pilot study on an antihumoral therapy (AHT) consisting of high-dose intravenous immunoglobulin G (IVIG) and rituximab in 20 paediatric renal transplant recipients. Donor-specific HLA antibodies (HLA DSA) were quantified by Luminex-based bead array technology. Loss of eGFR decreased significantly from 7.6 ml/min/1.73 m^2 during 6 months prior to AHT to 2.1 ml/min/1.73 m^2 ($P = 0.0013$) during 6 months after AHT. Fourteen patients (70%) responded: nine of nine patients (100%) without and five of 11 (45%) with transplant glomerulopathy ($P = 0.014$). C4d positivity in PTC decreased from $40 \pm 18.5\%$ in the index biopsy to $11.6 \pm 12.2\%$ ($P = 0.002$) in the follow-up biopsy. In four of nine biopsies (44%) C4d staining turned negative. During 2 years of follow-up, the median loss of eGFR in each of the four 6-month periods remained significantly lower compared with prior to AHT. Class I DSA declined in response to AHT by 61% ($p = 0.044$), class II DSA by 63% ($p = 0.033$) 12 months after intervention. AHT with IVIG and rituximab significantly reduces or stabilizes the progressive loss of transplant function in paediatric patients with chronic AMR over an observation period of 2 years, apparently by lowering circulating DSA and reducing intrarenal complement activation.

Introduction

The role of alloantibodies in chronic renal allograft deterioration and the corresponding morphological changes are increasingly recognized. In the Banff '05 meeting report, criteria for late or chronic antibody-mediated rejection (AMR) were defined to eliminate the unspecific term 'chronic rejection' [1]. The diagnostic criteria of chronic AMR include the following: (i) defined morphological features including transplant glomerulopathy; (ii) diffuse C4d deposition in PTC; and (iii) the presence of donor-specific antibody (DSA) [1].

The pivotal clinical relevance of chronic AMR is underscored by studies suggesting a primary role of antibody-mediated injury as a major cause of long-term kidney allograft loss [2–4]. In one study for example, 63% of late kidney graft failures after index biopsy were attributable to AMR and antibody-mediated microcirculation injury [3]. While a variety of strategies have been proven effective in treating acute AMR, chronic AMR still represents a major therapeutic challenge [5,6]. The development of chronic AMR, which may culminate in irreversible structural damage, is considered to be a continuous process associated with fluctuating levels of (*de novo*) DSA, with

or without detectable deposits of capillary C4d [7–13]. There is currently, no established treatment for chronic AMR and the development of strategies to reverse or at least halt chronic active rejection remains a big challenge.

We have previously published the results of a pilot study with an antihumoral regimen consisting of high-dose intravenous immunoglobulin G (IVIG) and rituximab in six paediatric renal transplant recipients with chronic AMR: Transplant function could be improved or stabilized in four patients during the 12 months after intervention [14]. Here we report an update of our experience with a larger group of patients ($n = 20$) and with longer follow-up over 24 months.

Patients and methods

Patients and study protocol

Patient characteristics are listed in Table 1. Twenty paediatric renal transplant recipients developed chronic AMR according to Banff '05 criteria [1] and were included in this prospective pilot study between July 2004 and May 2010. Nineteen of the 20 patients had received their first transplant. The initial immunosuppressive therapy post-transplant consisted of a triple regimen including a cyclosporine microemulsion (CsA) in 11 patients, tacrolimus (TAC) in nine patients, in conjunction with mycophenolate mofetil (MMF) and methylprednisolone. All patients had been subjected to graft biopsy before index biopsy; none of the patients showed positive C4d staining in these previous biopsies. Fourteen of 20 patients (70%) had experienced previously borderline or T-cell-mediated rejection and six patients had CNI-arteriolopathy (transmural hyalinosis of the arteriolar wall) with or without mild to moderate interstitial fibrosis and tubular atrophy. Eight patients underwent CNI minimization, six of them because of CNI-arteriolopathy and two because of infectious complications. All patients were screened in regular intervals for BK viremia and/or viruria according to international guidelines [15]. In case of positive results, the kidney allograft biopsy was investigated for BK nephropathy by SV40 staining. All 20 patients were tested negative for BK viremia and/or viruria at the time of index biopsy.

In the 6-month period of prior to initiation of antihumoral therapy, the immunosuppressive regimen remained unchanged. The type of immunosuppressive therapy given at the time of initiation of antihumoral therapy is shown in Table 1. During the subsequent 12-month period, the maintenance of immunosuppressive regimen remained unmodified in 11 patients (subgroup A). In nine patients CsA therapy was switched to TAC because of concomitant borderline cellular infiltrates. Because of pronounced interstitial inflammation in the index biopsy four of these nine patients were also subjected to a course of high-dose methylpednisolone before initiation of antihumoral therapy (subgroup B). The mean serum creatinine immediately before the course of high-dose steroids was 2.1 ± 0.3 mg/dl and 1 week thereafter 1.9 ± 0.2 mg/dl ($P = 0.76$). There was no difference between CNI target levels in the period of 6 months before versus 6 months after initiation of antihumoral therapy.

Presumed risk factors for chronic AMR were nonadherence (supposed and/or reported) to immunosuppressive medication in seven patients and reduction or discontinuation of CNI or MMF in 12 patients (Table 1).

Table 1. Patient characteristics at time of index biopsy.

Parameter	Patients ($n = 20$)
Age (years)	15 (10–19)
Gender, *n* (%)	7 (35) female, 13 (65) male
Donor type, *n* (%)	
Deceased donor	16 (81)
Living donor	4 (19)
Time post-transplant (months)	66 (40–117)
HLA mismatch	2.0 ± 0.2
Initial immunosuppressive regimen, *n* (%)	
TAC	9 (45)
CsA	11 (55)
MMF	19 (95)
AZA	1 (5)
MP	16 (80)
IL-2 receptor antagonist	0 (0)
Maintenance immunosuppressive regimen	
TAC dose (mg/m^2/day)	6.0 ± 0.7
TAC predose level (ng/ml)	5.6 ± 1.0
CSA dose (mg/m^2/day)	150 ± 0.9
CSA predose level (ng/ml)	51.0 ± 5.5
MMF dose (mg/m^2/day)	633 ± 73.6
MPA-AUC (μg*h/ml)	60.5 ± 7.6
MP dose (mg/m^2/day)	2.8 ± 0.2
Previous BPAR	
0	5 (25%)
1	5 (25%)
2	6 (30%)
≥3	4 (20%)
Presumed main risk factors for chronic AMR, *n* (%)	
Noncompliance	7 (35)
CNI minimization	8 (40)
MMF dose reduction or discontinuation	4 (20)
3rd transplantation	1 (5)
Proteinuria (g/mol creatinine)	
≥20–200 ($n = 13$)	36 (16–75)
≥200 ($n = 7$)	292 (223–417)

Data are *n* (%), normally distributed data are mean ± SD, non-normally distributed data are median (IQR). BPAR, biopsy-proven acute rejection including borderline changes; CsA, cyclosporin A; TAC, tacrolimus; MMF, mycophenolate mofetil; MPA, mycophenolic acid; MP, methylprednisolone.

Thirteen of 20 patients (65%) had mild proteinuria at the time of index biopsy; seven (35%) patients had heavy proteinuria (Table 1). Sixteen patients required antihypertensive therapy with at least one antihypertensive drug; angiotensin-converting enzyme (ACE) inhibitors were administered in 11 patients, angiotensin 2 receptor (ATR) antagonists in five patients. Neither ACE nor ATR antagonists were newly administered or stopped in the period of 6 months before or after initiation of antihumoral therapy.

Inclusion criteria were (i) clinical evidence of slowly deteriorating graft function; (ii) deposition of the complement split product C4d in PTC as an immunological footprint of complement activation and anti-donor humoral activity; (iii) serological evidence for donor-specific anti-HLA antibodies (anti-HLA DSA) at the time of index biopsy, and (iv) informed consent for treatment with IVIG and rituximab by the parents or guardians.

The treatment regimen for chronic AMR consisted of four weekly doses of IVIG (1 g/kg body weight per dose), followed by a single dose of rituximab (375 mg/m^2 body surface area) 1 week after the last IVIG infusion. Response to antihumoral therapy was defined as a reduction of the rate of loss of GFR by at least 30% in the period of 6 months after initiation of antihumoral therapy compared with the period 6 months prior to intervention. All patients received trimethoprim-sulfamethoxazole as prophylaxis for pneumocystis jirovecii for the first 6 months after intervention.

The length of follow-up was 24 months after initiation of antihumoral therapy in 15 patients and 18 months in two patients. Three patients lost their graft before completing the 24-month follow-up, two at 6 months and one at 18 months.

Laboratory evaluations

Estimated glomerular filtration rate (eGFR) of renal allografts was assessed by creatinine clearance calculated according to Schwartz *et al.* [16]. In patients with graft loss, eGFR was set to a value of 5 ml/min/1.73 m^2. Samples of venous blood in ethylenediaminetetraacetic acid were used for immunophenotyping by flow cytometry. CD20, expressed on all normal mature B cells, was used as a specific B-cell marker. CsA and TAC blood concentrations were measured by a monoclonal immunoassay (EMIT^{TM}; Dade Behring, Marburg, Germany) according to the manufacturer's instructions. Plasma mycophenolic acid (MPA) concentrations were measured by the EMIT^{TM} immunoassay (Dade Behring) on a Cobas-Mira analyser according to the manufacturer's instructions. MPA exposure was estimated by a validated algorithm based on a limited sampling strategy during the first 2 h after MMF dosing [17].

Histological review

Biopsies were evaluated using the Banff '05 and '07 classification respectively [1,18]. For detection of C4d in PTC we used a polyclonal anti-C4d antibody (C4dpAb; Biomedica, Vienna, Austria) as described elsewhere in detail [19]. Because a previous report had shown that focal C4d positivity (10–50% of PTC) in PTC had similar impact on graft survival as diffuse pattern (>50%) [20], we also included patients with focal C4d positivity in PTC. Specimens stained with haematoxylin and eosin, periodic acid Schiff stain, methenamine silver and the Masson trichrome stain were used to analyse the lesions in allograft biopsies according to the definitions provided by the Banff '97 working classification of renal allograft pathologic features [21].

Detection of donor-specific antibodies

Patients were tested prior to antihumoral therapy and 6 and 12 months thereafter for DSA using the LABScreen Luminex kit (One Lambda, Canoga Park, CA, USA), which uses single HLA-coated beads and enables identification of IgG alloantibody specificities against HLA-A, -B, -C, -DRB1/3/4/5, -DQA1, -DQB1, -DPA1 and -DPB1 antigens. Because no clinically validated cutoff for the Luminex assay is recommended by the provider company, a mean fluorescence intensity (MFI) of ≥500 was used to define the cutoff for antibody positivity. For high-resolution typing of recipient and donor HLA-A, -B, -C, -DRB1, -DQA1 and -DQB1 antigens, CTS-PCR-SSP Tray and CTS-Sequence kits (Heidelberg, Germany), and for HLA-DRB3/4/5, -DPA1 and -DPB1 antigens, Olerup SSP kits (Saltsjöbaden, Sweden) were used.

Statistical analysis

Normal distribution of the data was evaluated using the Shapiro-Wilks test. Normally distributed data are expressed as mean ± SD, non-normally distributed data as median (IQR). For the assessment of transplant function, all serum creatinine values measured in our institution were considered; at least one value per patient and month was available. For the evaluation of these high-frequency eGFR assessments over time, we applied an advanced smoothing algorithm to reduce bias because of outliers (LOESS procedure, SAS V9.2, SAS Institute, Cary, NC, USA). Thereby high-quality data on longitudinal eGFR evolutions were obtained. Longitudinal changes of eGFR were evaluated by one-way ANOVA on repeated measurements. Pair-wise comparison between baseline and subsequent time points was performed using the CONTRAST option of the GLM procedure. The day of

rituximab administration was defined as day 0 for the calculation of eGFR values before and after initiation of antihumoral therapy. The association of the presence of transplant glomerulopathy with the response to antihumoral therapy was explored with the Fisher's exact test. The association of the degree of histological lesions graded by Banff '05 criteria [1] with the response to therapy was analysed by logistic regression analysis. Differences between dependent data were assessed by the Student's *t*-test for paired samples. A *P*-value of less than 0.05 was considered as statistically significant. All statistical analyses were performed by using SAS V9.2.

Results

Histopathological features

The frequency of the histological lesions graded by Banff criteria [1] in the index biopsy of the renal allograft is depicted in Fig. 1. Ten (50%) patients had focal and 10 (50%) had diffuse C4d. All 20 patients had *de novo* DSA against HLA antigens of the organ. Transplant glomerulopathy score 2 to 3 was present in 11 of 20 patients (55%); none of these patients presented with glomerulitis. Eighteen of 20 patients (90%) showed considerable amounts of interstitial inflammation and/or tubulitis in their renal graft biopsy (Fig. 1). No patient fulfilled the criteria of acute T-cell-mediated rejection ≥ type IA or acute AMR according to the Banff '05 criteria [1]. There were variable CD20-positive infiltrates ranging from <10% to 50% in 12 of 20 (60%) patients. Six of 20 patients (30%) showed CNI-arteriolopathy.

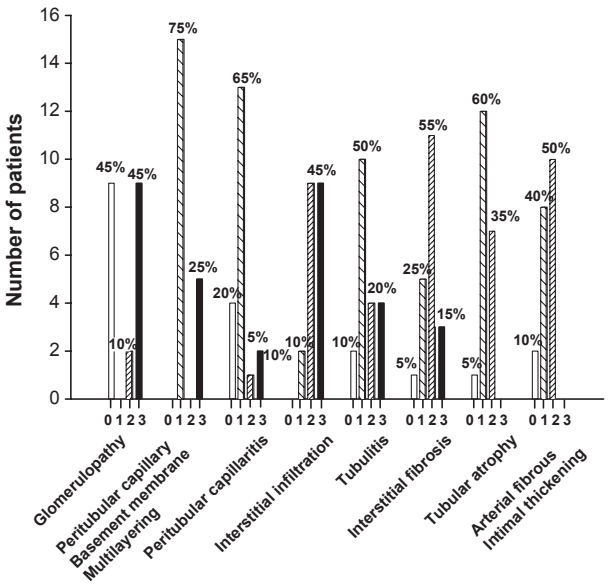

Figure 1 Frequency of histological lesions graded by Banff 05 criteria [1] in the index biopsy of the renal allograft.

Follow-up biopsies, performed as indication biopsies 3–17 months after initiation of antihumoral therapy, were available in nine of 20 patients (45%). In four of these nine biopsies, transplant glomerulopathy, which had not been present in the index biopsy, became detectable. The percentage of C4d positivity in PTC decreased from 40 ± 18.5% in the index biopsy to 11.6 ± 12.2% ($P = 0.002$) in the follow-up biopsy. In four of nine biopsies (44%) C4d staining turned completely negative.

Graft function and response to therapy

The response of graft function to antihumoral therapy is depicted in Fig. 2a and b. All patients showed progressive

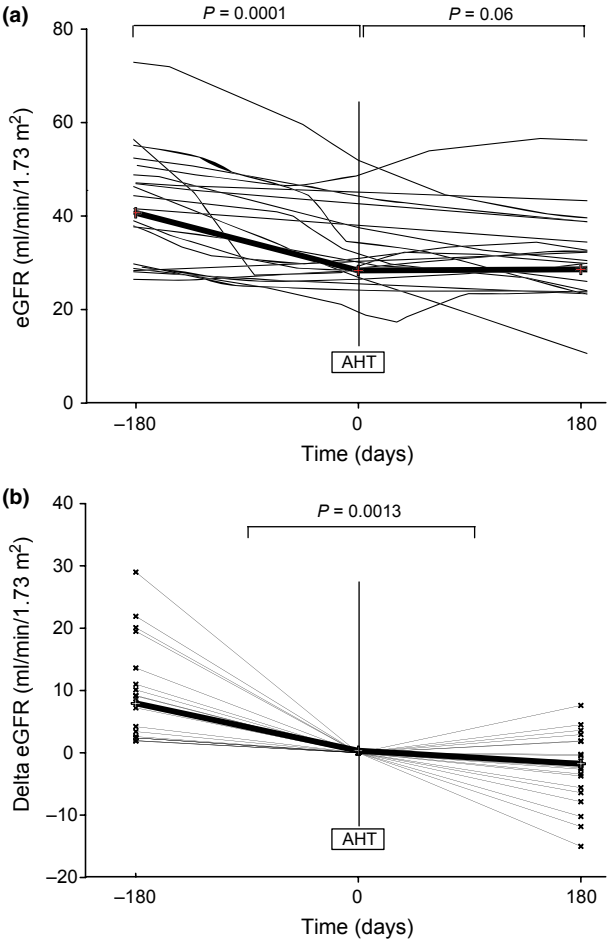

Figure 2 (a) Individual (standard line) and median eGFR values (bold line), estimated according to the formula of Schwartz *et al.* [14], in 20 paediatric renal transplant recipients with chronic AMR during the period 6 months prior to and after initiation of antihumoral therapy (AHT) with IVIG and rituximab, which is indicated by the vertical line. (b) Individual (standard line) and median values (bold line) for the change of eGFR during the period 6 months prior to and after initiation of antihumoral therapy.

deterioration of transplant function prior to intervention: The median eGFR declined from 43.0 (28.7–49.6) ml/min/1.73 m^2 at 6 months prior to antihumoral therapy to 30.6 ml/min/1.73 m^2 (27.6–40.3) at baseline. Antihumoral therapy was associated with a stabilization of eGFR 6 months (30.3 ml/min/1.73 m^2; 24.1–36.6; Fig. 2a) and 12 months (27.5 ml/min/1.73 m^2; 19.3–35.1) after intervention (not shown). The loss of eGFR declined from 7.6 ml/min/1.73 m^2 (−29.2 to 1.9) during the 6 months prior to antihumoral therapy to 2.1 ml/min/1.73 m^2 (−15.1 to 7.6; $P = 0.0013$) during the 6 months after intervention (Fig. 2b). We also analysed the loss in eGFR over time after eliminating the two patients from the analysis that returned to dialysis during the first 6 months after intervention. For the remaining 18 patients, the loss of eGFR declined from 3.8 ml/min/1.73 m^2 (−29.2 to 1.9) during the 6 months prior to antihumoral therapy to 1.5 ml/min/1.73 m^2 (−5.9 to 1.8; $P = 0.01$) during the 6 months after intervention. An improvement of eGFR was noted in eight patients, a stabilization in six patients. Altogether, 14 of 20 patients (70%) responded to antihumoral therapy. All patients without transplant glomerulopathy ($n = 9$, 100%) responded compared to only five of 11 patients (45%) with transplant glomerulopathy ($P = 0.014$). By logistic regression analysis, the degree of transplant glomerulopathy ('cg') graded by Banff '05 criteria [1] (odds ratio −0.22; $P = 0.032$) and the degree of arterial fibrous intimal thickening ('cv') (odds ratio −0.49; $P = 0.042$) were associated with the response to therapy, while the degree of peritubular capillaritis, interstitial inflammation, tubulitis or interstitial fibrosis/tubular atrophy was not. In responders, the degree of transplant glomerulopathy (median 1.5; range, 0–3; $P = 0.032$) and arterial fibrous intimal thickening (median 1, range, 0–2; $P = 0.026$) was significantly lower than in nonresponders (median 3; range, 3 to 3; and median 2; range, 1–2, respectively).

Figure 3 shows the response to antihumoral therapy in subgroup A ($n = 11$), in whom the concomitant immunosuppressive therapy remained unmodified during the period of 6 months before and after intervention, and in subgroup B ($n = 9$), in whom the concomitant immunosuppressive therapy was intensified (for details, see Patients and study protocol) because of concomitant borderline cellular infiltrates. In both subgroups, the loss of eGFR post therapy was significantly lower than that pre therapy. This difference remained significant, when the two patients that returned to dialysis during the first 6 months after intervention (one in each group) were eliminated from the analysis (subgroup A, $P = 0.04$; subgroup B, $P = 0.001$). Both the mean change of eGFR prior to intervention (subgroup A, −22.0 ± 15.4 ml/min/1.73 m^2; subgroup B, −11.2 ± 12.7 ml/min/1.73 m^2; −22.0 ± 15.4 ml/min/1.73 m^2; $P = 0.10$) and post intervention (subgroup A, −9.8 ± 11.2 ml/min/1.73 m^2; subgroup B, −0.18 ± 13.5 ml/min/1.73 m^2; −9.8 ± 11.2 ml/min/1.73 m^2; $P = 0.11$) were not significantly different between the two subgroups. This difference remained significant, when the two patients that returned to dialysis during the first 6 months after intervention (one in each group) were eliminated from the analysis (subgroup A, $P = 0.04$; subgroup B, $P = 0.001$).

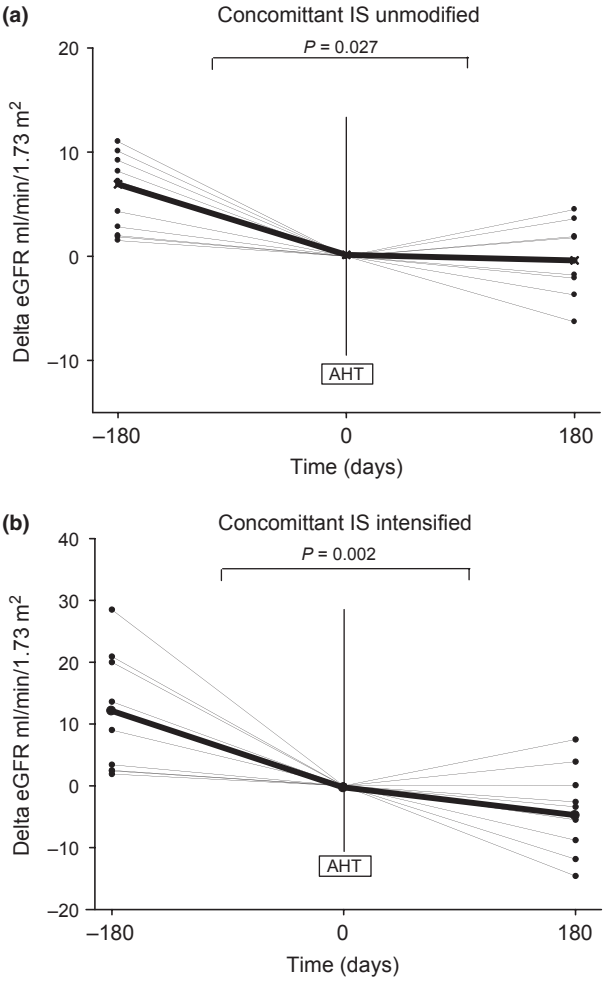

Figure 3 Individual (standard line) and mean values (bold line) for the change of eGFR during the period 6 months prior to and after initiation of antihumoral therapy (AHT). (a) Subgroup A ($n = 11$), in whom the concomitant immunosuppressive therapy remained unmodified. (b) Subgroup B ($n = 9$), in whom the concomitant immunosuppressive therapy was intensified (for details, see Patients and study protocol) because of concomitant borderline cellular infiltrates.

The course of eGFR values during the entire observation period of 24 months is depicted in Fig. 4a, the change of eGFR in Fig. 4b. In each of the four 6-month

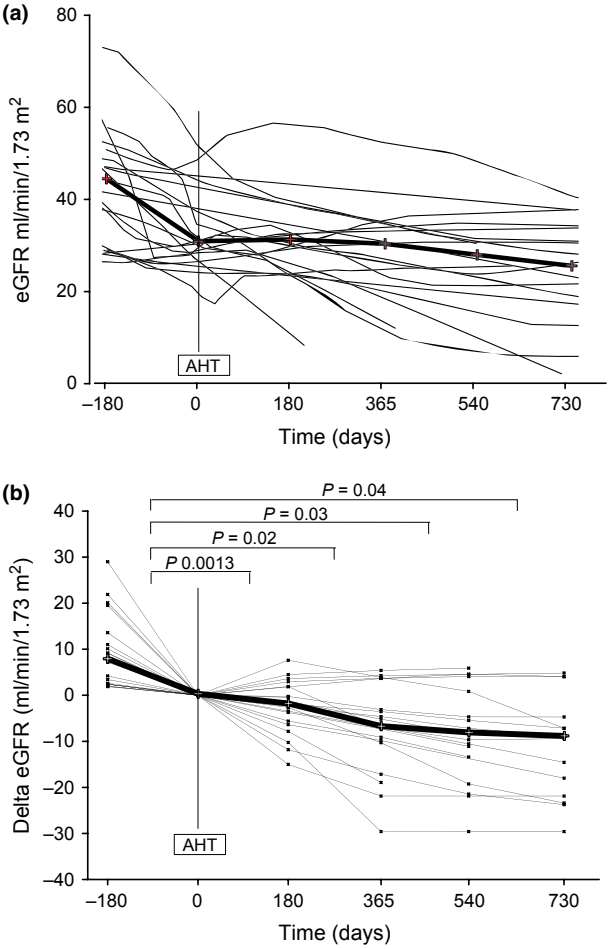

Figure 4 (a) Individual (standard line) and mean eGFR values (bold line) in 20 paediatric renal transplant recipients with chronic AMR during the 1st and 2nd year after initiation of antihumoral therapy. The slopes of the respective regression lines in the 1st compared with the 2nd year post intervention were not statistically different ($P = 0.876$). (b) Individual (standard line) and mean values (bold line) for the change of eGFR during the period 6 months prior to initiation of antihumoral therapy and the four 6-month periods during the 2 years of follow-up. The respective delta eGFR was determined in comparison to the previous time point.

periods during the 2 years of follow-up, the respective mean loss of eGFR remained significantly lower than that prior to intervention (Fig. 4b). Two patients lost their graft at 6 months, one patient at 18 months and one patient at 2 years after intervention because of progressive chronic rejection. Overall, the stabilization of graft function persisted in 15 of the remaining 16 patients in the second year after initiation of antihumoral therapy. We also analysed the loss in eGFR over time after eliminating patients from the analysis in the respective interval they returned to dialysis. In each of the four 6-month periods during the 2 years of follow-up, the respective mean loss of eGFR remained significantly lower than that prior to intervention (period 0–6 months after intervention versus 6 months prior to intervention, $N = 18$, $P < 0.001$; period 6–12 months after intervention versus 6 months prior to intervention, $N = 18$, $P = 0.001$; period 12–18 months after intervention versus 6 months prior to intervention, $N = 17$, $P = 0.013$; period 18–24 months after intervention versus 6 months prior to intervention, $N = 16$, $P = 0.037$.)

The degree of proteinuria at baseline was significantly ($P = 0.007$) higher in patients ($n = 7$) who did not respond to antihumoral therapy [292 g protein/mol creatinine (range, 72.9–418)] than in responders (31.1 g protein/mol creatinine (range, 16.9–151). Proteinuria did not significantly change in response to antihumoral therapy in both groups at 6 months, 12 months and 24 months ($P = 0.98$ and $P = 0.71$ respectively).

Treatment with IVIG and rituximab was well tolerated; specific side effects were not observed. One patient acquired pneumocystis jirovecii pneumonia 2 years after initiation of antihumoral therapy, which fortunately resolved.

Immunological findings

No patients had pre-existing anti-HLA DSA. At the time of index biopsy *de novo* anti-HLA DSA as detected by the Luminex assay were present in all 20 (100%) patients. Ten of 20 patients (50%) had more than one DSA (HLA class I and/or class II antibodies), but only the antibody with the highest MFI was considered as the immunodominant antibody. In 13 of 20 patients (65%) the immunodominant DSA were directed against HLA class II DR, DQ or DP DSA antigens. Four of these 13 patients (31%) had also class I DSA.

The mean percentage decline of MFI of the immunodominant HLA class I DSA in response to antihumoral therapy was 59.3% 6 months and 61% 12 months after intervention ($P = 0.044$) (Fig. 5). The respective mean decline of the immunodominant HLA class II DSA was 40% at 6 months and 63% at 12 months ($P = 0.033$). The number of patients that had a significant reduction in DSA, defined as reduction of >50% of baseline, was 2/7 (28%) for HLA class I DSA and 5/13 (38%) for HLA class I DSA. In seven patients (35%) HLA DSA turned negative (<500 MFI) during the 12-month period after intervention. These patients all responded to antihumoral therapy and experienced no graft loss during the observation period of 2 years. The response to antihumoral therapy was not related to the MFI of DSA at baseline or the degree of decline in DSA in response to antihumoral therapy. Nondonor-specific HLA antibodies were detected in 17 of 20 patients (85%) prior to antihumoral

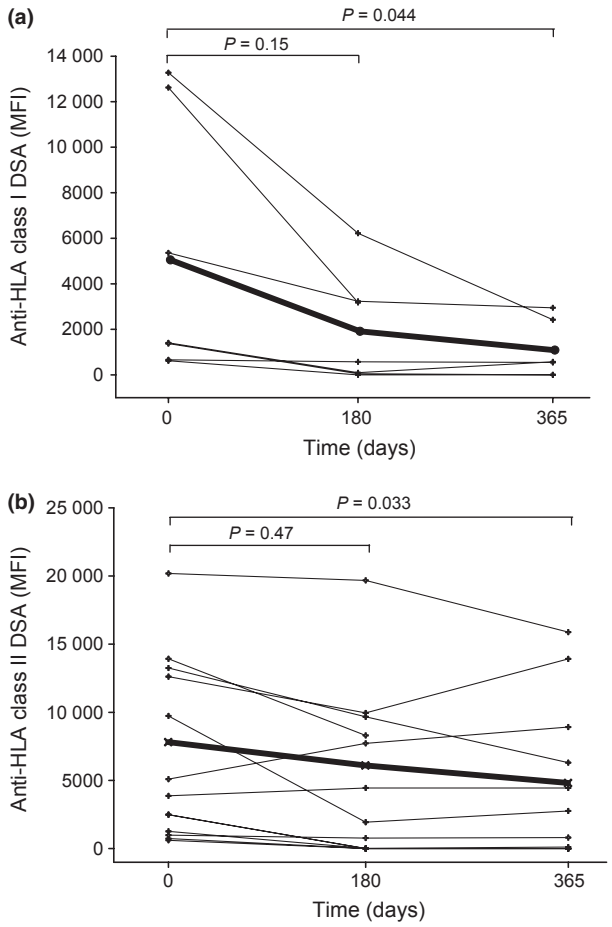

Figure 5 Individual (standard line) and mean values (bold line) of immunodominant donor-specific HLA antibodies (HLA DSA) against class I (panel A) or class II (panel B) antigens before (day 0) and in response to antihumoral therapy with IVIG and rituximab during the 1st year after intervention. HLA DSA was quantified using Luminex-based bead array technology. MFI, mean fluorescence intensity.

therapy and in 16 of 20 (80%) patients 6 months post therapy.

Complete depletion of circulating CD20+ cells, defined as <5 cells/μl, was observed in all patients at 6 months after initiation of antihumoral therapy ($n = 20$). At 12 months data on circulating CD20+ cells were available in eight patients: circulating CD20+ cells were partially depleted in seven patients and recovered to the normal range (>200 cells/μl) in one patient. Full recovery of CD20+ cells at 2 years was observed in four of 10 patients with available data, two additional patients showed partial depletion and in four patients the complete depletion of circulating CD20+ cells persisted. The prevalence of hypogammaglobulinemia, defined as a serum IgG concentration <7 g/l, was 4/20 (20%) prior to intervention, 6/20 (30%) 12 months after intervention and 6/20 (30%) 24 months after intervention.

Discussion

The main finding of this prospective pilot trial in paediatric patients with chronic AMR is that an antihumoral therapy consisting of four doses of IVIG and one dose of rituximab is associated with an improvement or stabilization of eGFR in the majority of patients: 14 of 20 patients (70%) responded to treatment as measured 6 months after intervention, and this response persisted over a 24-month observation period. Nevertheless, the decline of graft function went on, albeit to a lower degree and 4/20 grafts (20%) were lost within 2 years after intervention. The presence of transplant glomerulopathy in the index biopsy was associated with a lesser response to antihumoral therapy; nevertheless, five of 14 patients (35%) with transplant glomerulopathy in this high-risk group responded. This observation is important, because the natural course of transplant function in patients with already established transplant glomerulopathy is unfavourable [22]. We also found that the degree of arterial fibrous intimal thickening was inversely correlated with the response to therapy. This is the largest study to date on an exploratory treatment of chronic AMR using a well-defined course of IVIG + rituximab. In addition, there is good follow-up at 2 years examining the eGFR and DSA levels in these patients. These results confirm and extend our previous observation on the efficacy of IVIG and rituximab in six patients with chronic AMR, which was the first report on this topic [14].

The rationale for treating chronic AMR with the combination of IVIG and rituximab was the following: first, we sought to rapidly enhance the clearance of circulating DSA by IVIG, because rituximab on its own does not appear to reduce DSA titres [23]. There are numerous proposed mechanisms how IVIG exerts its immunomodulatory action. They include modification of circulating alloantibody concentration through induction of anti-idiotypic circuits, antigen binding through the Fab part of the immunoglobulin molecule, Fc receptor-mediated interaction with antigen-presenting cells to block T- and B-cell activation, and inhibition of complement activity [24]. Second, we attempted to prevent further antibody production by rituximab, which depletes B cells as antigen-presenting cells and precursors of mature plasma cells in the circulation and the lymphoid tissue, prevents B-cell proliferation, and induces apoptosis and lysis of B cells through complement-dependent and -independent mechanisms [25]. Consequently, it is expected that antibody formation will be suppressed. An additional potential mechanism of action of rituximab is the direct targeting of CD20-positive cells that infiltrate the allograft [26]; such graft infiltration was apparent in 60% of graft biopsies in our study.

All patients in this study were tested positive by Luminex SA testing for *de novo* HLA DSA; in 65% of patients these DSA were directed against HLA class II antigens. Antihumoral therapy significantly decreased circulating DSA against class I and class II HLA antigens over the observation period of 12 months. In seven patients, the respective DSA were not anymore detectable 6 and 12 months after intervention. This observation is promising because higher HLA class II antibody levels are related to an increased risk of developing transplant glomerulopathy and also to the presence of C4d in PTC [27]. Our observation, that peritubular C4d deposits decreased by 71% in follow-up biopsies suggests that the mechanism of action of antihumoral therapy may involve both the suppression of circulating DSA and the reduction of intrarenal complement activation.

In general, antihumoral therapy was well tolerated in our patients. IVIG therapy has rarely been associated with the development of acute renal failure, which was not observed in the present study. The monoclonal anti-CD20 antibody rituximab is being used widely in renal transplant patients for the depletion peripheral B cells [28] and very few side effects have been reported so far.

Fehr *et al.* has published their experience with the use of IVIG and rituximab in the setting of CAMR [29]. They reported four patients with CAMR who were treated with a combination of IVIG + rituximab. Rituximab/IVIG improved kidney allograft function in all four patients, whereas DSA were reduced in two of the four patients.

Another potential treatment modality for CAMR is the use of bortezomib. Bortezomib is a proteosome inhibitor that leads *in vitro* to apoptosis of alloantibody-producing plasma cells *in vitro* [30]. Two recent studies are to be mentioned, in which bortezomib was administered without additional antihumoral measures [31,32]. Wahrmann *et al.* [31] reported two sensitized dialysis patients, in whom two subsequent bortezomib cycles, the second combined with steroids, did not or only modestly affect alloantibody levels. Sberro-Soussan *et al.* [32] reported that bortezomib applied as a sole treatment did not affect post-transplant alloantibody levels in kidney transplant recipients with subclinical AMR. On the other hand, there are two case reports [33,34] and one experimental study [35] suggesting some interesting effects of this compound in the context of chronic AMR. Nevertheless, the impact of bortezomib on the clinical course of chronic AMR remains uncertain.

An obvious limitation of the current prospective study is the lack of an untreated control group for comparison. However, in view of the unfavourable natural course of chronic AMR, it was felt to be unethical to perform a controlled trial with an untreated control group in a paediatric patient population. Instead, each patient served as his own control. We sought to eliminate potential sources of bias by including all patients with newly diagnosed chronic AMR in our centre into this protocol. Nevertheless, we recognize that a prospective, randomized trial will be needed to validate these initial findings. Since a control group was not included, we also cannot exclude that a significant component of the beneficial effect observed in the whole cohort is related to the immunosuppression intensification in the subgroup A.

We conclude that an antihumoral treatment regimen consisting of IVIG and rituximab significantly improved or stabilized the progressive loss of transplant function in paediatric patients with chronic AMR over an observation period of 2 years. Mechanistically, this regimen appears to be operative by lowering circulating HLA DSA and reducing intrarenal complement activation. This treatment protocol may represent a significant advance in the management of this common and often difficult-to-treat post-transplant complication.

Authorship

HB, CS, RW and BT: designed and performed the study. HB and SR: collected the data. HB, CS, RW, EW and BT analysed the data. HB, CS and BT: wrote the paper. All: critically reviewed and improved the manuscript.

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