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The influence of non-HLA antibodies directed against angiotensin II type 1 receptor (AT1R) on early renal transplant outcomes

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Keywords
angiotensin II type 1 receptor antibodies, antibody-mediated rejection, humoral rejection, non-HLA antibodies, renal transplant injury, renal transplantation.

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Summary

Non-HLA antibodies (Abs) targeting vascular receptors are thought to have an impact on renal transplant injury. Anti-angiotensin II type 1-receptor-activating antibodies (anti-AT1R) have been mentioned to stimulate a severe vascular rejection, but the pretransplant screening has not been introduced yet. The aim of our study was to assess the incidence and importance of anti-AT1R antibodies and their influence on renal transplant in the 1st year of observation. We prospectively evaluated the presence of anti-AT1R antibodies in 117 consecutive renal transplant recipients in pre- and post-transplant screening. Anti-AT1R antibodies were observed in 27/117 (23%) of the analyzed recipients already before transplantation. The function of renal transplant was considerably worse in anti-AT1R(+) group. The patients with anti-AT1R Abs >9 U/ml lost their graft more often. Biopsy-proven AR was described in 4/27 (15%) pts in the anti-AT1R(+) group and 13/90 (14.4%) in the anti-AT1R(−) group, but more severe cases of Banff IIB or antibody-mediated rejection (AMR) were more often observed in anti-AT1R (+) 4/27 (15%) vs. 1/90 (1.1%) in anti-AT1R(+) ($P = 0.009$). Patients with anti-AT1R Abs level >9 U/ml run a higher risk of graft failure independently of classical immunological risk factors. The recipients with anti-AT1R Abs developed more severe acute rejections described as IIB or AMR in Banff classification. More recipients among the anti-AT1R-positive ones lost the graft. Our study suggests monitoring of anti-AT1R Abs before renal transplantation for assessment of immunologic risk profiles and the identification of patients highly susceptible to immunologic events, graft failure, and graft loss.

Introduction

Non-HLA antibodies (Abs) targeting vascular receptors are thought to have an impact on renal transplant injury. Anti-angiotensin II type 1-receptor-activating antibodies (anti-AT1R) have been mentioned to stimulate a severe vascular rejection, but the pretransplant screening has not been introduced yet. Dragun *et al.* [1,2] noticed the role of anti-angiotensin II type 1 receptor antibodies (anti-AT1R Abs) in renal transplant patients with steroid refractory acute vascular rejection. Our observations showed the importance of non-HLA antibodies early but also long time after transplantation [3,4]. Elevated levels of anti-AT1R Abs and anti-endothelin A receptor antibodies (anti-ETAR

Abs) were observed as associated with cellular and humoral rejection and the early onset of microvasculopathy after heart transplantation [5]. Recently, the pretransplant presence of anti-AT1R Abs has been described as an independent risk factor for long-term graft loss in association with a higher risk of early AR episodes [6]. Another analysis of anti-AT1R Abs and DSA (anti-HLA) in pre- and post-transplant sera from 351 consecutive kidney recipients showed that patients with both anti-AT1R and DSA had lower graft survival than those with DSA alone [7].

Angiotensin type 1 receptor is a G protein-coupled receptor that mediates most physiologic and pathophysiologic actions of the angiotensin II [8]. The activity mainly includes arterial blood pressure and water–salt balance. The human gene for angiotensin type 1 receptor is located on chromosome 3 and contains four exons. The human gene for angiotensin type 1 receptor is located on chromosome 3 and contains four exons. Four major transcripts are produced with very different rates of translation [9]. The mRNA processing leads to different levels of AT1R expression with several polymorphisms of AT1R [10].

Autoreactive and alloreactive responses may lead to anti-AT1R antibodies development in multiple ways. Presentation of target antigens may induce an immunological response in various conditions of cellular stress. Inflammatory events stimulated by ischemia, infection injury, and the transplant process itself might lead to *de novo* expression of autoantigens and loss of tolerance [9–11]. Anti-AT1R Abs may also develop through transfusions, pregnancies, or prior transplant and arise after tapering of immunosuppressive drugs or as a result of noncompliance [12].

Pre- or post-transplant screening of anti-AT1R antibodies has not been introduced yet, because there are still many doubts and the outcomes do not seem to be convincing enough. An easy to use ELISA test for the detection of AT1R antibodies appears to be an interesting tool with the specificity of 100% and the sensitivity of 88% [13]. It seems obvious that a thorough characterization of immunological risk at the time of transplantation would improve individualization of therapy and help avoid acute or chronic rejection without excessive immunosuppression [14].

We decided to verify the activity and incidence of anti-AT1R Abs in renal transplant recipients early after transplantation.

Methods

We prospectively evaluated the presence of anti-AT1R antibodies in 117 consecutive renal transplant recipients in pre- and post-transplant screening (before and in 1st, 3rd, 6th, and 12th months after transplantation). The transplantation was performed in 2011 and 2012. Serum samples were collected and assessed prospectively. Anti-AT1R antibodies were assayed by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (CellTrend, Luckenwalde, Germany).

The patients' sera for the determination of antibody concentrations were obtained along routine examinations. Venous blood was drawn into sterile 10-ml serum separator tubes. Samples were centrifuged at 1000 *g* for 15 min; serum was collected and stored at −80 °C until the day of measurement. The concentration of anti-AT1R IgG antibody in serum was measured by ELISA according to the manufacturer's instruction. The samples were assayed on angiotensin II type 1-receptor-precoated microtiter plate. Standards and diluted 1:100 samples were added into the wells and incubated for 2 h at 2–8 C. After washing steps, anti-AT1R antibody was detected with POD-labeled anti-human IgG antibody (1:100) followed by color development with TMB substrate solution and measured at 450 nm, with the correction wavelength set at 630 nm. Optical densities were then converted into concentration by standard curve.

The detection range of the test was >2.5 U/ml with positive value set at >9 U/ml and negative ≤9 U/ml. The threshold of anti-AT1R Abs was estimated on the basis of the statistical analysis (see Statistics).

The presence of anti-HLA antibodies was tested by Flow-PRA method (One Lambda). Using solid-phase immunoassay technology (Luminex, Wroclaw, Poland), we retrospectively retested HLA donor-specific antibody for all available sera from patients who developed biopsy-proven AR episode, collected at the time of transplantation and at the time of rejection. The diagnosis of acute rejection was assessed prospectively and based on Banff 2009 criteria. Pathologists were unaware of the antibody status. C4d deposition was assessed by immmunohistochemical method performed on paraffin sections using polyclonal antibody (Biomedica, Vienna, Austria).

The ethical commission of the Wroclaw Medical University approved all study protocols, and informed consent was obtained from all the patients.

Statistics

The cutoff calculation for anti-AT1R Abs was essential for further investigation. We did not have precise data from relevant literature. The analysis of literature showed a wide range of thresholds: 10 U/ml [6], 15 U/ml [7], and 16.5 U/ml [5]. We decided to perform our own analysis to find the most exact cutpoint, being aware that it may provoke discussion like in the case of the MFI level determined by solid-phase immunoassay technology (Luminex).

The selection of cutoff points was based on two methods. We performed receiver operating characteristic (ROC

curve) [15,16] using the area under the curve (AUC) additionally with Youden's index. Youden's index has a connection to ROC analysis as the height above the chance line, and it is also equivalent to the AUC subtended by a single operating point [17]. We also applied Horthorn and Zeileis method which is used for the estimation of simple cutpoint models [18]. Graft survival was the most important event which we estimated using the listed methods. Additionally, we also assessed other events: acute rejection episodes and more precisely acute rejection diagnosed as IIB or antibody-mediated rejection (AMR). We received numerous cutpoints between 5.7 and 10.7 with various statistical significance (*P*-value between <0.0001–0.1) and the highest sensitivity of 80% and specificity of 87%. To make sure which cutpoint is most precise within the 5.7–10.7 range, we performed multivariate logistic regression to assess the association of graft failure with the presence of anti-AT1R antibodies for different cutpoint within the 5.7–10.7 range. We decided to accept 9 U/ml as the best cutoff for further analysis.

R for Windows version 3.0.1 (The R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis. Continuous data were presented as the mean ± SEM. The comparison between the groups was performed using the Student's *t*-test and the Mann–Whitney *U* test for metric variables, while the chi-square test and the Fisher exact test were used to identify the connection between acute rejection and the presence of antibodies. Univariate and multivariate logistic regression analyses were performed to evaluate the association of graft failure risk factors with anti-AT1R antibodies. The Cox model was adjusted for risk factors. The Fisher exact test was performed to assess the influence of anti-AT1R antibodies level on biopsy changes, acute rejection, and arteritis in the performed renal biopsies.

P below 0.05 was considered significant.

Results

Patient characteristics

Anti-AT1R antibodies were observed in 27/117 (23%) of the analyzed recipients already before transplantation. The patients were divided into two groups according to the level of anti-AT1R Abs: >9 U/ml anti-AT1R positive(+) (n = 27) and ≤9 U/ml anti-AT1R negative(−) (n = 90).

The patient characteristics have been presented in Table 1. The immunosuppression consisted of the following: tacrolimus or cyclosporine, mycophenolate mofetil, steroids, and occasionally basiliximab (Table 2.). In case of acute rejection (AR), the recipients received steroids and in

Table 1. Patient population characteristics.

	AT1R Abs >9 U/l n = 27	AT1R Abs ≤9 U/l n = 90	*P*-value
Recipients age (years)	45.6 ± 15.3	48.3 ± 13.9	NS
Male, *n* (%)	19 (70.3%)	59 (65.5%)	NS
Time on dialysis before transplantation (days)	1015 ± 923	1184 ± 904	NS
Cause of chronic renal failure			
Chronic glomerulonephritis	30	27	NS
Diabetic nephropathy	11	10	
Hypertonic nephropathy	11	10	
Polycystic kidney disease	22	19	
Pyeloneohritis	15	10	
Others	11	24	
First transplant	37	63	NS
Retransplant	2	5	NS
Anti-HLA class I and II testing by Flow PRA	38.9%	38.6%	NS
No. of presensitized patients	10/27	34/90	NS
No. of presensitized patients			
PRA <10%	6	19	NS
PRA 10–50%	3	13	NS
PRA >50%	1	2	NS
No. of HLA mismatches	3.4 ± 1.4	3.5 ± 1.1	NS
Donor gender (%)			
Female	32	36	NS
Male	68	64	NS
Donor age (years)	46.7 ± 13.5	45.4 ± 16.5	NS
CIT (hours)	22.9 ± 7.3	23.5 ± 7.3	NS
DGF	25%	30%	NS

PRA, panel reactive antibodies; CIT, cold ischemia time; DGF, delayed graft function.

Table 2. Initial immunosuppression.

	AT1R Abs >9 U/l $n = 27$	AT1R Abs ≤9 U/l $n = 90$	*P*-value
TAC-MMF + S	17	64	NS
CsA-MMF + S	10	26	NS
Simulect + TAC-MMF + S	2	4	NS

TAC, tacrolimus; CsA, cyclosporin; MMF, mycophenolate mofetil; MPA, mycophenolic acid; S, steroids.

AMR additionally plasmapheresis and IVIG. There was no statistically significant difference considering the recipients' and donors' age or gender, cold ischemia time, the number of HLA mismatches, the number of presensitized patients, immunosuppressive regiment, or patients with the presence of anti-HLA antibodies between the groups.

Variability of anti-AT1R Abs level

The median value of pretransplant anti-AT1R Abs was 12 U/ml in anti-AT1R(+) group and 5.7 U/ml in anti-AT1R(−) group. The anti-AT1R Abs levels varied at different measurement intervals within the 1-year follow-up. The mean value before transplantation in anti-AT1R(+) group was 15.01 ± 9.1 U/mL and significantly less, varying from 7.85 ± 5.5 to 10.3 ± 6.5 U/ml at different times after transplantation (Fig. 1). It is difficult to present the mean level in negative patients; in their case, it amounted to <2 U/ml or 2–8.9 U/ml. Thirty-two patients had <2 U/ml level, and in patients with 2–8.9 U/ml, the mean level before transplantation was 5.5 ± 1.7 U/ml, 3.8 ± 1.4 U/ml in the 1st month, 4.8 ± 2.3 U/ml in the 3rd month, and 5.9 ± 1.7 U/ml in the 12th month.

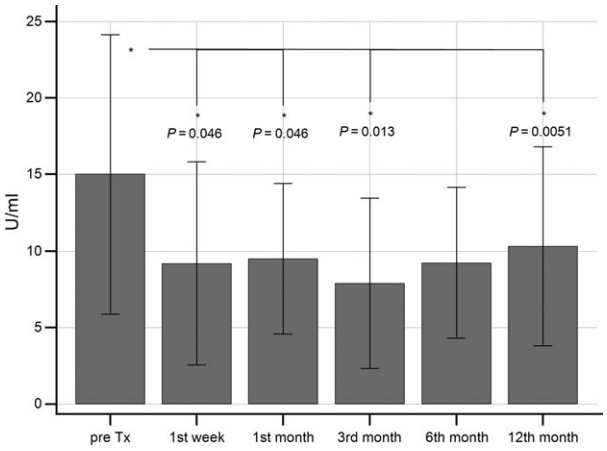

Figure 1 Anti-AT1R Abs mean level in pre- and post-transplant monitoring in AT1R-positive patients.

Pretransplant anti-AT1R Abs>9 U/ml is an independent risk factor for graft failure

The function of renal transplant was significantly worse in anti-AT1R(+) group compared to anti-AT1R(−) group during the first post-transplantation year (Fig. 2). Although in the 1st month, the difference between anti-AT1R(+) and anti-AT1R(−) was not statistically significant, 1.81 ± 0.7 mg/dl vs. 1.64 ± 0.7 mg/dl ($P = 0.28$), in the 3rd month, it reached the significance with 1.76 ± 0.5 vs. 1.50 ± 0.4 ($P = 0.021$). In the 6th and 12th months after transplantation, the significant difference between the anti-AT1R(+) and anti-AT1R(−) groups increased amounting to 1.94 ± 0.9 vs. 1.45 ± 0.4 ($P = 0.039$) and 1.71 ± 0.5 vs. 1.38 ± 0.4 mg/dl ($P = 0.0076$). The association of serum creatinine in the 3rd, 6th, and 12th months with the presence of anti-AT1R Abs >9 U/ml was confirmed using univariate logistic regression with statistical significance in each time (Table 3). Multivariate logistic regression showed a statistically significant association of serum creatinine with the presence of anti-AT1R antibodies in the 3rd, 6th, and 12th months after transplantation (Table 4).

The association of graft failure risk factors with the presence of anti-AT1R was checked by univariate and multivariate logistic regression analyses. We checked the influence of the recipient's age or gender, a donor's age or gender,

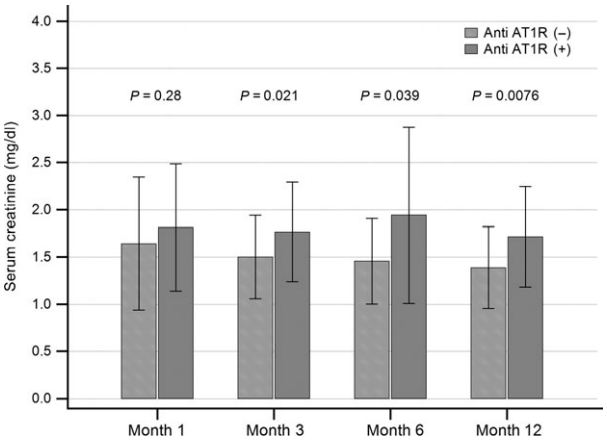

Figure 2 Renal transplant function (mean serum creatinine).

Table 3. The association of serum creatinine (3th, 6th and 12th month) with the presence of anti-AT1R Abs >9 U/ml (univariate logistic regression).

Serum creatinine and anti-AT1R Abs	Odds ratio	95% CI	*P*- value
3 months	3.01	1.13–8.03	0.028
6 months	3.28	1.28–8.45	0.014
12 months	3.72	1.27–10.87	0.016

Table 4. Multivariate logistic regression shows association of serum creatinine with the presence of anti-AT1R antibodies.

	Odds ratio	95% CI	*P*
Retransplantation	1.8693	0.1400–24.9645	0.6362
Donor age	1.0231	0.9853–1.0623	0.2353
PRA max	1.0036	0.9556–1.0539	0.887
No of HLA MM	0.8928	0.5684–1.4022	0.6224
3 m Scr	3.7676	1.3077–10.8546	0.014
	Odds ratio	95% CI	*P*
Retransplantion	1.9512	0.1189–32.0341	0.6396
Donor age	1.0328	0.9892–1.0783	0.1425
PRA max	1.0097	0.9448–1.0791	0.7757
No of HLA MM	0.8175	0.4988–1.3397	0.424
6 m Scr	4.7252	1.5217–14.6727	0.0072
	Odds ratio	95% CI	*P*
Retransplantion	1.62	0.0961–27.2983	0.7378
Donor age	1.0168	0.9774–1.0578	0.4079
PRA max	1.0094	0.9425–1.0811	0.7888
No of HLA MM	0.7472	0.4553–1.2263	0.2489
12 m Scr	4.9011	1.5720–15.2802	0.0061

No of HLA MM number of human leukocyte antigen mismatches.
3 m, 6 m,12 m Scr serum creatinine in the 3rd, 6th, and 12th months.

max panel reactive antibodies (max PRA), cold ischemia time (CIT), and the number of HLA mismatches on the presence of anti-AT1R Abs (Table 5).

Graft survival in anti-AT1R-negative patient is higher

One-year graft survival of patients who were negative (anti-AT1R <9 U/ml) was higher ($P = 0.011$) (Fig. 3). Although the number of patients who lost graft was not large in anti-AT1R Abs >9 U/ml (3/27, 11.1%) and anti-AT1R ≤9 U/ml (1/90, 1.1%), the difference between the groups was statistically significant.

Table 5. Risk factors for anti-AT1R antibodies.

	Univariate			Multivariate		
Variable	Odds ratio	95% CI	*P*	OR	95% CI	*P*
Recipient age	0.98	0.95–1.01	0.23	0.99	0.95–1.04	0.91
Male recipient	1.25	0.49–3.17	0.64	1.29	0.37–4.53	0.68
Donor age	1.01	0.97–1.03	0.745	1.01	0.97–1.05	0.53
Male donor	1.25	0.45–3.43	0.66	1.46	0.47–4.50	0.50
PRA Max	1.00	0.96–1.03	0.89	1.01	0.96–1.06	0.64
CIT (h)	0.98	0.92– 1.05	0.71	1.00	0.93–1.08	0.85
No. of HLA ABDR MM	0.91	0.63–1.31	0.63	0.99	0.62–1.60	0.99

OR, odds ratio; PRA, panel reactive antibodies; CIT, cold ischemia time; MM, mismatch; No, number; HLA, ABDR human leukocyte antigen A, B, DR.

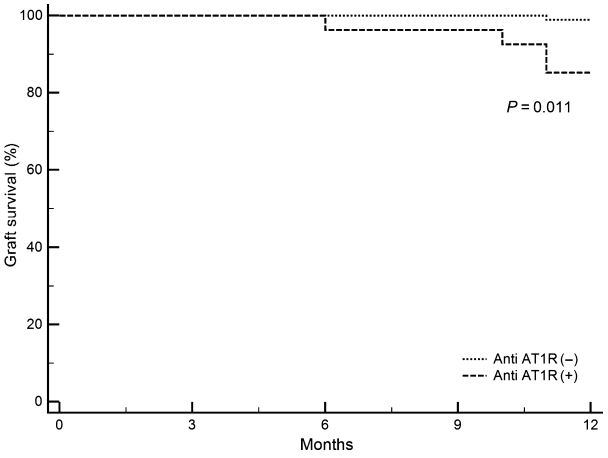

Figure 3 Graft survival (12 months).

Histological acute rejection classification and anti-AT1R Abs

Biopsy-proven acute rejection (BPAR) was described in 4/27 (15%) pts in the anti-AT1R(+) group and 13/90 (14.4%) in the anti-AT1R(−) group. When only ARs without borderline changes were included in the analysis, they were more frequently found in anti-AT1R(+) 4/27 (15%) vs. 9/90 (10%) in anti-AT1R(−) Fig. 4. All patients with BPAR in anti-AT1R (+) developed Banff IIB or AMR, while only 1/90 (1.1%) in anti-AT1R(+) ($P = 0.009$) presented similar features (Fig. 5). Univariate and multivariate analyses (Cox regression analysis) of risk factors for acute rejection IIB or AMR occurrence show that anti-AT1R Abs before transplantation are an independent risk factor for acute rejection IIB or AMR (Table 6). Patients in whom AMR was diagnosed had donor-specific antibodies. One anti-AT1R(+) patient with AMR had DSA, which appeared

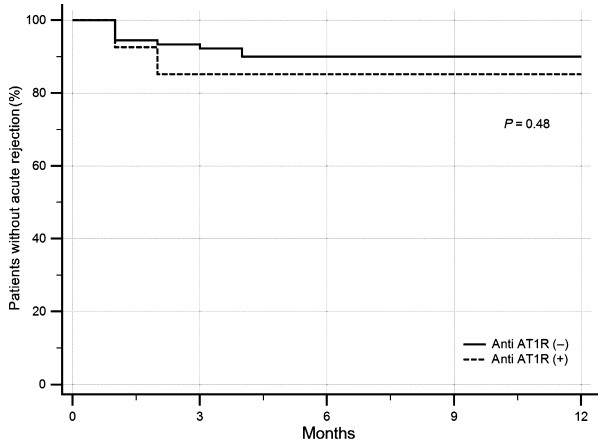

Figure 4 The occurrence of acute rejection and anti-AT1R Abs analyzed as the Kaplan–Meier graft survival curves.

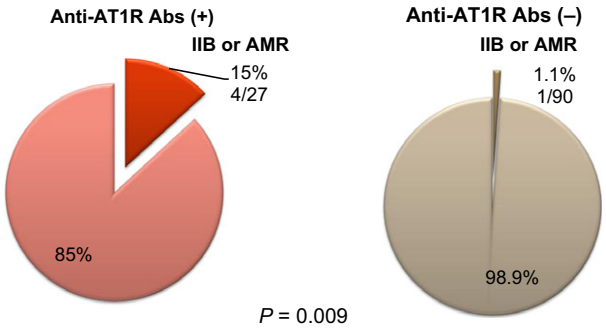

Figure 5 Renal biopsy injury – Banff IIB or antibody-mediated rejection.

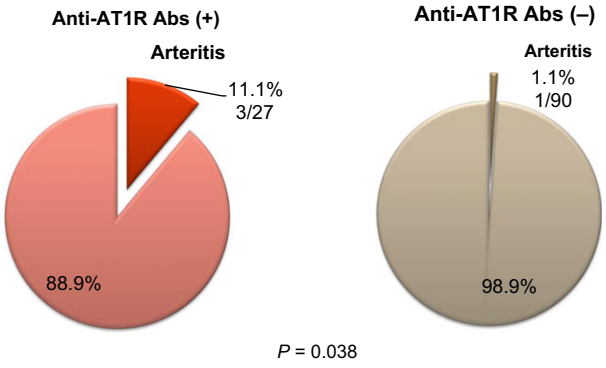

Figure 6 Renal biopsy – arteritis.

de novo, and also one anti-AT1R(−) patient had DSA, which appeared *de novo*. Arteritis was more often observed in anti-AT1R(+) patients: 3/27 (11.1%) vs 1/90 (1.1%) in anti-AT1R(−) group ($P = 0.038$) (Fig. 6). Histological acute rejection classification and anti-AT1R Abs level before and at AR time have been presented in Table 7.

Blood pressure, antihypertensive medications, and proteinuria

The primary report on anti-AT1R informed about the association of acute rejection with severe hypertension [2]. Therefore, we analyzed the presence of hypertension in the 3rd and 12th months. 80% of patients with anti-AT1R Abs had hypertension compared to 75% without anti-AT1R in the 3rd and 90% compared to 85% in the 12th month (P = NS). In the 3rd and 12th months, the median systolic and diastolic blood pressure was 150/80 mmHg and 140/80 mmHg in the anti-AT1R positive vs. 130/80 mmHg and 140/80 mmHg (P = NS), respectively. None of the patients developed malignant hypertension. The mean number of antihypertensive medications in the 3rd and 12th months was similar in both groups: 1.35 and 1.4 in the anti-AT1R(+) vs. 1.43 and 1.48 (P = NS). Proteinuria over 0.5 g/dl was present in 17.3% in the anti-AT1R(+) vs. 10.4% in the 3rd and 9.5% vs. 7.1% in the 12th months (P = NS), respectively.

Table 6. Univariate and multivariate analyses (Cox regression analysis) of the risk factors for acute rejection IIB or AMR occurrence shows that anti-AT1R Abs before transplantation are an independent risk factor for acute rejection IIB or AMR.

Univariate analysis	HR	95% CI of HR	*P*-Value
Anti-AT1R Abs	13.6931	1.54 to 121.17	0.0193
Retransplantation	0	1.01E-198 to 655.06E + 186	0.9631
Historical peak PRA	1.4919	0.25 to 8.85	0.6613
HLA-A-B-DR mismatches >5	0.0001	2.92E-207 to 1.83E + 198	0.9682
Multivariate analysis	**HR**	**95% CI of HR**	***P*-Value**
Anti-AT1R Abs	13.3803	1.49 to 119.38	0.0208
Retransplantation	0	4.95E-194 to 7.71E+183	0.9612
Historical peak PRA	1.1447	0.19 to 6.84	0.8828
HLA-A-B-DR mismatches >5	0.0001	0.00 to 10.14E+303	0.9871

PRA, panel reactive antibodies; HLA ABDR, human leukocyte antigen A, B, DR; Abs, antibodies; AMR, antibody-mediated rejection.

Discussion

We demonstrated that renal transplant recipients possess preformed non-HLA anti-AT1R antibodies that influence renal transplant function during the 1st year after transplantation. From the univariate and multivariate analyses, we found out that patients with anti-AT1R Abs level >9 U/ml run a higher risk of graft failure independently of

Table 7. Histological acute rejection classification and anti-AT1R Abs level before and at acute rejection time.

Pts	AR Banff grading	Pretranspl anti-AT1R Abs level	AR anti-AT1R Abs level	Pretrans PRA last, max	AR anti-HLA class I, II (%)
WM	IIB	11.1	2.9	0; 0	0; 0
WA	IIB	10.4	6.4	4; 4	0; 0
GA	AMR+borderline	15.6	<2.5	19; 64	30; 90
TJ	IIB	12.3	9.3	0; 0	0; 0

AR, acute rejection; AMR, antibody mediated rejection.

classical immunological risk factors such as PRA or HLA mismatches, but also a donor's and recipient's age or gender and cold ischemia time. Moreover, the patients with anti-AT1R Abs level >9 U/ml developed more severe acute rejections described as IIB or AMR in Banff classification. Graft loss in the recipients with anti-AT1R Abs level >9 U/ml was higher. The study suggests monitoring of anti-AT1R Abs before renal transplantation for assessment of immunologic risk profiles and the identification of patients highly susceptible to immunologic events, graft failure, and graft loss.

The humoral theory of transplantation seems to be more and more important and complicated [14,19–23]. We are considering whether predicting a patient's clinical outcome on the basis of HLA presensitization alone is insufficient. Our own recent analysis showed that anti-HLA donor-specific antibodies had a significant disadvantageous influence on graft function, but in more than one-third of patients with the presence of DSA, the deterioration of graft insufficiency was not observed during the 5-year study [24]. Pretransplant detection of complement-fixing DSA may be a valuable tool for risk stratification [25]. The presence of C1q testing in pretransplant sera with DSA class II but not DSA class I may predict acute antibody-mediated rejection or early graft loss [26]. An increased post-transplant sCD30 serum concentration and positive pre- and post-transplant anti-HLA class II reactivities may be useful biomarkers for post-transplant immune monitoring predicting BPAR in pediatric renal transplant recipients [27]. Regulatory T cells (Tregs) were also shown to be involved in the pathogenesis of acute rejection [28]. The determination of the HLA-DR MFI of the HLA-DR(+)-Treg subset allows to discriminate between patients with clinically relevant borderline rejection and patients with subclinical rejection or other causes of transplant failure. On the other hand, non-HLA may also play an important role in graft failure prediction [3,6,7,29]. The detection of anti-AT1R Abs seems to be a complementary risk factor for the identification of patients with higher immunological risk.

We showed the kinetics of anti-AT1R before and after transplantation (Fig. 1). The pretransplant level was significantly higher than the post-transplant one. Additionally, all patients with acute rejection and anti-AT1R >9 U/ml had a lower level of anti-AT1R Abs at the time of biopsy (Fig. 5). Such a regularity was observed in Giral *et al.* analysis, but also in HLA Abs study and may be described as intragraft antibody adsorption [6,30].

We are aware that the exact level is evolving and may also change during discussion in the future similarly to MFI determined by solid-phase assay (Luminex technology). We showed that the 9 U/ml cutoff determined worse graft function, more cases of arteritis, more severe AR, and also higher graft lost. We studied the outcomes at different times: before transplantation and then in 1st week, 1st, 3rd, 6th, and 12th months post-transplantation. The threshold of anti-AT1R Abs was estimated during the investigation on the basis statistical analysis (see Statistics).

In a recently published study of renal transplant recipients, the cutoff of anti-AT1R Abs was determined similarly at 10 U/ml. Hiemann *et al.* [5], considering anti-AT1R in a heart transplant patient to be a significant pretransplant prognostic rejection cutoff, suggested the value >16.5 U/ml. Taniguchi *et al.* [7] in a more recent pre- and post-transplant assessment of the association of anti-AT1R with renal graft failure considered ≥15 U/ml as positive. Although the exact threshold has not been established yet, the results of all the presented research indicate a significant role of anti-AT1R Abs in transplant patients.

We proved that the occurrence of pretransplant anti-AT1R Abs >9 U/ml is an independent risk factor for graft failure. The function of renal transplant was significantly worse in anti-AT1R(+) group compared to anti-AT1R(−) group during the first post-transplantation year (Fig. 2.). In the 1st month, the difference between the groups was not statistically significant, but from the 3rd month, the discrepancy in renal function became significant. The association of serum creatinine at the 3rd, 6th, and 12th months with the presence of anti-AT1R Abs was confirmed using univariate logistic regression with statistical significance at each time. More patients lost the graft in AT1R(+) group in the first post-transplant year. These results support Giral *et al.*'s suggestion that pretransplant anti-AT1R Abs are an independent risk factor for long-term graft loss, but also Taniguchi *et al.* who showed a significant association of anti-AT1R with graft failure [6,7].

Biopsy-proven acute rejection was similar in anti-AT1R (+) [4/27 (15%)] and anti-AT1R(−) patients [13/90 (14.4%)]. When we remove cases with borderline changes, the difference in AR was more relevant, but not statistically significant: 15% vs. 10% in anti-AT1R(+) and anti-AT1R (−), respectively.

The comparison of Banff IIB or AMR changes revealed a disadvantageous result for anti-AT1R(+) patients. All patients with BPAR in anti-AT1R(+) 4/27 (15%) developed Banff IIB or AMR, while only 1/90 (1.1%) in anti-AT1R(−) ($P = 0.009$) had similar features. Arteritis was more often observed in anti-AT1R(+) patients: 11.1% vs. 1.1% in anti-AT1R(−) patients ($P = 0.038$). More cases with AMR (71.4%) had anti-AT1R antibody level >10 U/ml in Giral *et al.* study [6].

Arteritis is stressed as an important criterion for AMR and widely discussed during the last Banff 2013 Meeting Report [31,32]. Even recently, Lefaucheur *et al.* [33] have put forward a proposal of antibody-mediated vascular rejection in Lancet. Therefore, we decided to emphasize a statistically significant presence of arteritis in our analysis.

What seems to be the most interesting is the cause and effect relationship between anti-AT1R and graft injuries. We proved that the level of anti-AT1R Abs is significantly lower after transplantation. It means that anti-AT1R Abs may bind to the graft immediately after transplantation and start inflammatory injuries on vascular cells [34]. We do not know whether other factors influence the features of anti-AT1R, changing their agonistic affinity. It may be modified by the level but also ischemia, inflammatory events, microbiome or anti-HLA influence [35,36]. *De novo* expression of autoantigens and breakdown of B cell self-tolerance may play an important role [9].Mechanisms of tolerance loss to AT1R differ from other non-HLA. Anti-AT1R Abs are involved in pathophysiology of autoimmune vascular disease of pregnancy – pre-eclampsia, autoimmune vasculopathy, and systemic sclerosis [9,37,38]. Epitopes for anti-AT1R Abs in pre-eclamptic and transplant patients are directed against amino acids contained within the second extracellular loop (ECL2) of the receptor. The mechanisms of sensitization are different from those described for neoantigens [9].

Anti-AT1R Abs are of the IgG class requiring T cell help, and T cell self-tolerance may be broken by an infection or inflammatory event [39]. The natural balance of the endothelium may be altered, and the susceptibility of antibody attack on AT1R may increase. These may generate a cascade of events leading to the pathogenesis of vascular rejection. Anti-AT1R Abs may amplify local inflammation, which increases antigen expression and the production of Th1 cytokines and inflammatory chemokines, which may even stimulate cellular rejection [2,10,40].

The mechanism of vascular injury mediated by anti-AT1R Abs seems to be essential. Anti-AT1R Abs stimulate endothelial and vascular smooth-muscle cells inducing Erk 1/2 signal transduction cascade. During incubation with nuclear extract of vascular smooth-muscle cells, anti-AT1R Abs activate the transcription factor activator protein 1 (AP-1). DNA-binding activity of nuclear factor-KB (NF-KB) transcription factor was also amplified. It was noticed that the expression of NF-KB proinflammatory target genes (chemokines MCP-1 and RANTES) increased [13].

Many factors may influence the long-term outcome of renal transplantation. They can be divided into immunological and nonimmunological ones [41]. There is consensus as to a significant role of anti-HLA antibodies in acute and chronic rejection of renal transplant [27], [42–46], although the interpretation of the current DSA results is difficult and may lead to many discussions [47].

Giral *et al.* [6] (France and Germany) were the first to show that pretransplant anti-AT1R Abs are an independent risk factor for long-term graft loss and a higher risk of early acute rejection. The French group consisted of 599 patients and received a kidney between 1998 and 2007. Serum samples in their analysis were collected and assessed retrospectively for the presence of anti-AT1R Abs. The samples of our 117 patients were collected and assessed prospectively between 2011 and 2013. The cutoff of in the French–German study was established at 10 U/ml. In our analysis, the threshold was similarly determined at 9U/ml, which we have discussed earlier. The essential difference concerned the percentage of patients qualified as positive. Giral *et al.* noticed a positive anti-AT1R Abs level in 47.2% patients, which means that almost half of the transplant population had anti-At1R Abs, whereas our analysis showed a positive result in 23% recipients. The French–German group showed that among 37 patients with biopsy-proven AR, 22 had pretransplant anti-AT1R Abs level of >10 U/ml and among 14 with AMR, 71.4% had anti-AT1R antibody level of >10 U. We also observed more biopsy-proven AR in patients with anti-AT1R Abs: 15% vs. 10%, but without statistical significance. Our analysis showed more severe cases of BPAR described as IIB or AMR in the anti-AT1R (+) patients. Among the anti-AT1R(+) patients, 4/27 (15%) developed Banff IIB or AMR, while only 1/90 (1.1%) in anti-AT1R(−) ($P = 0.009$) had similar features. Arteritis in our study was more often observed in the anti-AT1R(+) patients: 11.1% vs. 1.1% in the anti-AT1R(−) patients ($P = 0.038$).

At the oral session of the last ESOT 2013 meeting, we presented the outcomes of 65 consecutive renal transplant patients at the time of transplant biopsy which was performed because of the deterioration of graft function. We evaluated the presence of non-HLA Abs (Anti-AT1R and/or anti-ETAR Abs). A high level of non-HLA antibody activity was found in 7/65 (10.7%) renal recipients. Graft loss was detected in 5/7 (71%) patients in non-HLA Abs(+) 7.8 ± 2.6 months after biopsy. These patients were qualified for renal biopsy by serum creatinine of 2.34 ± 0.6 mg/dl, and biopsy was performed 7.7 ± 3.9 years after transplantation in six patients and 40 days in one patient. Biopsy revealed AR IIB in one patient early after transplantation and chronic allograft injury criteria in six patients late after transplantation (cg1-3, cv1-2, ci1-2, and ct1-2). C4d was present in 3/7 patients. The control group consisted of 44 patients with low level of non-HLA Abs. The serum creatinine in the Abs(−) group was 2.4 ± 1.1 mg/dl in the 3rd month and 2.3 ± 0.9 mg/dl in the 6th month after biopsy, and graft loss was 11% in the 6th month after biopsy. We concluded that a high level of anti-AT1R and/or anti-ETAR antibodies is associated with tissue injury criteria and graft loss [48].

There is more and more evidence proving the importance of non-HLA anti-AT1R Abs. However, further research is required before we modify and establish diagnostics or maybe even targeted therapies in the future. AT1 receptor is one of the most successful cardiovascular

targets of new drug therapies. Plasmapheresis or immunoadsorption is known and used in the reduction of antibody titers [9]. Antihumoral therapy with IVIG and rituximab may significantly reduce or stabilize the progressive loss of transplant function by lowering circulating DSA and reducing intrarenal complement activation [49].

Our observation showed that patients with anti-AT1R Abs level >9 U/ml run a higher risk of graft failure independently of classical immunological risk factors. The recipients with anti-AT1R Abs developed more severe acute rejections described as IIB or AMR in Banff classification. More anti-AT1R positive recipients lost the graft. Our study suggests monitoring anti-AT1R Abs before renal transplantation for the assessment of immunologic risk profiles and the identification of patients highly susceptible to immunologic events, graft failure, and graft loss.

Authorship

MB: designed and performed the research, collected and analyzed the data, and wrote the paper. MBo, MP, AH and PC: analyzed the data. KK-K: performed the research, analyzed the data, and wrote the paper. DK, MM, SZ and BN: collected the data. DB: performed the research and collected the data. MŻ: collected and analyzed the data. MK: designed the research, analyzed the data, and wrote the paper.

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