Human Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Pre-transplant screening for non-HLA antibodies: Who should be Tested?

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ARTICLE INFO

Keywords:
Anti-endothelial cell antibodies
Non-HLA antibodies
Angiotensin II type 1 receptor antibody
High immunologic risk patients
Kidney allograft dysfunction

ABSTRACT

Retrospective studies of angiotensin II type 1 receptor antibodies (AT1R-Ab) and anti-endothelial cell antibodies (AECA) have linked these antibodies to allograft injury. Because rising healthcare costs dictate judicious use of laboratory testing, we sought to define characteristics of kidney transplant recipients who may benefit from screening for non-HLA antibodies. Kidney recipients transplanted between 2011 and 2016 at Johns Hopkins, were evaluated for AT1R-Ab and AECA. Pre-transplant antibody levels were compared to clinical and biopsy indications of graft dysfunction. Biopsies were graded using the Banff 2009–2013 criteria. AT1R-Ab and AECA were detected using ELISA and endothelial cell crossmatches, respectively. AT1R-Ab levels were higher in patients who were positive for AECAs. Re-transplanted patients ($p < 0.0001$), males ($p = 0.008$) and those with FSGS ($p = 0.04$) and younger ($p = 0.04$) at time of transplantation were more likely to be positive for AT1R-Ab prior to transplantation. Recipients who were positive for AT1R-Ab prior to transplantation had increases in serum creatinine within 3 months post-transplantation ($p < 0.0001$) and developed abnormal biopsies earlier than did AT1R-Ab negative patients (126 days versus 368 days respectively; $p = 0.02$). Defining a clinical protocol to identify and preemptively treat patients at risk for acute rejection with detectable non-HLA antibodies is an important objective for the transplant community.

1. Introduction

Repeated injury to a transplanted kidney due to rejection is one significant predictor of graft loss [1–3]. The development of highly sensitive and specific assays for detecting and characterizing HLA antibodies has allowed recognition of the role of non-HLA antibodies in graft injury in patients negative for donor specific HLA antibody (HLA-DSA). Defining an appropriate clinical protocol to identify and preemptively treat kidney transplant recipients who are at risk for acute rejection with detectable non-HLA antibodies is an important objective for the transplant community. One such antibody, specific for angiotensin II type 1 receptor (AT1R-Ab), has been shown to be involved in poor transplant outcomes. Multiple single center retrospective studies have documented acute rejection and in some cases, graft loss associated with presence of AT1R-Abs [4–13].

Anti-endothelial cell antibodies (AECAs) detected in the endothelial cell crossmatch (ECXM) have also been identified in patients who developed severe antibody mediated rejection in the absence of HLA-DSA [14–16]. AT1R is expressed on endothelial cells [17,18] and we have shown that most patients who are positive for AT1R-Ab also have a positive ECXM [19].The ECXM also detects antibodies against targets other than AT1R [20]. Biopsy evaluations at our center have shown histological evidence of increased microcirculation inflammation in patients with positive AT1R-Ab and/or AECAs both in the presence or absence of HLA-DSA [14,19].

In 2013, two of the largest studies in kidney transplantation reported conflicting results on a correlation between pre-transplant AT1R-Ab and kidney allograft outcome. Giral et al. [21] reported an increased incidence of graft loss at 3 years in patients with a pre-transplant AT1R-Ab level > 10 units/ml. In contrast, Taniguchi et al.

Abbreviations: ABOi, ABO incompatible; AECA, anti endothelial cell antibody; AMR, antibody mediated rejection; AT1R, angiotensin II type 1 receptor; AT1R-Ab, anti-angiotensin II type 1 receptor antibody; HLA-DSA, donor specific HLA antibody; HLAi, HLA incompatible; ECXM, endothelial cell crossmatch; FCXM, flow cytometric crossmatch; FSGS, focal segmental glomerulosclerosis; MFI, mean fluorescent intensity; MPGN, membranoproliferative glomerulonephritis

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https://doi.org/10.1016/j.humimm.2018.02.001
Received 21 November 2017; Received in revised form 16 January 2018; Accepted 2 February 2018
0198-8859/ © 2018 Published by Elsevier Inc. on behalf of American Society for Histocompatibility and Immunogenetics.

[22] did not find a correlation between pre-transplant AT1R-Ab and graft outcome but did find a significant association between post-transplant AT1R-Ab and graft failure. However, in the study by Taniguchi, 84% of patients who developed abnormal biopsies tested positive for AT1R-Ab prior to transplant and maintained the antibody post-transplant.

Transplant opportunities for sensitized patients have increased through desensitization treatment, kidney paired donation, and changes in the kidney allocation system [23]. The incidence of antibody mediated rejection in the presence of antibodies other than HLA was estimated to be between 10 and 40% [24], however, since centers are not testing for presence of non-HLA antibodies in patients who are positive for HLA antibodies, the true incidence of antibody mediated rejection associated with presence of non-HLA antibodies in highly sensitized patients is not known. In this study, we evaluate the value of pre-transplant assessment of AT1R-Ab and other AECAs in patients with increased immunological risks and explore the possibility that additional screening for non-HLA antibodies may provide an opportunity for pre-emptive intervention that could improve long term allograft survival.

2. methods

2.1. Study population

Pre-transplant AT1R-Ab test results were available for 170 recipients of a kidney transplant performed between May 1st, 2011 and July 1st, 2016, at the Johns Hopkins Comprehensive Transplant Center. We excluded 26 recipients whose serum date was > 2 months prior to their transplant date. The remaining 144 recipients had a specimen obtained < 50 days prior to transplantation. The study group included 3 categories of patients based on the time of pre-transplant serum evaluation and the reason for testing: group 1: 40 patients whose pre-transplant sera were tested retrospectively for AT1R-Ab and/or AECA to investigate allograft dysfunction that was not explained by HLA-DSA; group 2: 71 consecutive high risk patients transplanted with a living donor; and group 3: 33 consecutive highly sensitized kidney transplant recipients who received a deceased donor organ. An ECXM was performed within 30 days prior to transplantation for 74 of the 144 recipients. All data were obtained under an approved IRB protocol.

2.2. Pre-transplant and post-transplant treatment

Induction treatment consisted of Thymoglobulin® (1.5 mg/kg per day for 5 days), basiliximab (2 mg/kg), or alemtuzumab (30 mg single dose at time of transplantation). Desensitization for HLA and/or ABO incompatibility consisted of single-volume plasmapheresis, and either cytomegalovirus immune globulin (Cytogam®; 100 mg/kg) [25] or human immune globulin (Gamunex®-C; 100 mg/kg). The number of plasmapheresis treatments varied based on the level of HLA-DSA, and ABO antibody prior to transplantation. Recipients received rituximab prior to transplant if their current donor had HLA mismatches repeated from a previous donor and/or in cases where the patient was to receive a kidney that was ABO and HLA incompatible (n = 47). Maintenance treatment consisted of mycophenolate mofetil (2 g/day) and tacrolimus (serum level of 8–10 ng/ml). AMR was treated with plasmapheresis and immune globulin (n = 35), eculizumab (n = 5) and/or splenectomy (n = 5).

2.3. Laboratory evaluations

HLA typing, HLA antibody detection, ECXM and AT1R-Ab testing were performed as previously described [19]. Briefly, recipient and donor HLA-A, B, C, DQ, DR and DP typing were performed by reverse sequence specific oligonucleotide assay (One Lambda LABType®). HLA-DSA was identified using multianalyte bead based assays performed on the Luminex® platform (Immucor-Lifecodes, Stamford, CT and One Lambda, Canoga Park, CA). HLA antibody levels were assigned as cytotoxic positive crossmatch level (CDC +), flow cytometric crossmatch positive (FCXM +), and FCXM- Luminex + (Lum +) using MFI values of ≥10,000; 4000 to < 10,000; and 2000 to < 4000 MFI respectively for HLA-A, HLA-B, and HLA-DR, and MFI values of ≥20,000; ≥16,000; and ≥4000 respectively for HLA-C, HLA-DQ and HLA-DP. MFI values of 1000 or lower that lacked specificity patterns were considered negative. Cytotoxic crossmatch tests were performed prior to transplantation, with positively selected T and B lymphocyte targets [26]. FCXM tests were performed as previously described [27] and were acquired on BD FACSCanto II using FACSDIVA software (BD Bioscience, Franklin Lakes, NJ). Hypotonic dialysis was performed to remove IgM autoantibodies and IgG immune complexes [28] from sera and sera for crossmatch testing were back-dialyzed to achieve isotonicity.

Angiotensin II type 1 receptor antibody (AT1R-Ab) testing was performed using quantitative ELISA (One Lambda, ThermoFisher). Anti-endothelial cell antibodies (AECAs) of the IgG isotype were detected using a flow cytometric crossmatch test (ECXM) performed with angiopoietin receptor positive peripheral blood endothelial cell precursors (ECPs) (XM-ONE; Absorber AB, Stockholm, Sweden).

2.4. Biopsy interpretation

Allograft biopsy was performed either per protocol at 1, 3,6 and 12 months or for cause when serum creatinine increased ≥20% over baseline. Biopsies were graded using the Banff 2009–2013 criteria. Biopsy descriptions were extracted from the patient electronic records and were reviewed by a pathologist. The first biopsy for each recipient, with one or more of the characteristics described in Table 3 was labeled “Abnormal”. Patients who had sequential biopsies, up to the end of follow up that did not show any of the characteristics listed in Table 3 were considered to have “normal biopsies”.

2.5. Statistics

We examined the change in serum creatinine over time using a multilevel, mixed-effects generalized linear model with random effects at patient level. Because the distribution of serum creatinine is skewed to the right, we modeled the logarithmic form of serum creatinine. Our dependent variables were time (number of months since transplant) and AT1R-Ab status. We made spline terms of time period of 0 days – 1 week, 1 week – 1 month, 1 month – 3 months, and after 3 months since transplant. We performed both unadjusted and adjusted models. Covariates in the adjusted model included age at transplant, race, gender, pre-transplant treatment, presence of HLA-DSA prior to transplant and post-transplant treatment for rejection. Specifically, pre-transplant treatments (the use of plasmapheresis and IVIG and/or rituximab) were considered as having an effect on baseline serum creatinine levels and early post-transplant, so we adjusted for these treatments at baseline and within the first 7 days post-transplant. Regressions were performed using Stata 14.1/MP for Windows (College Station, Texas). Comparisons between groups were assessed by one way analysis of variance. Continuous variables were compared using Student’s t test and categorical variables were compared using the χ^2 test and/or Fisher’s exact test. Kaplan-Meier survival curves were generated using GraphPad Prism 6 software. The p-values for the Kaplan-Meier survival curve were determined using the Gehan-Breslow-Wilcoxon method and p-values < 0.05 were considered statistically significant.

Table 1
Characteristics of patients with positive and negative AT1R-Ab prior to transplantation.

	AT1R-Ab ≥ 17 units/ml (n = 32)	AT1R-Ab < 17 units/ml (n = 112)	p value
Patient characteristics			
Age mean (SD)	44.5(2.6)	50.5(1.3)	0.04
Male sex (%)	23(72)	53(47)	0.008
Race (African American)	9(28)	25(22)	0.38
Mean number of transplants	2.09(0.15)	1.43(0.07)	< 0.0001
Primary causes of renal failure			
Hypertension	14(42)	59(52)[1]	0.3
FSGS	7(22)	10(9)	0.04
SLE	3(0.09)	3(0.02)	0.1
Diabetes	2 (0.06)	11(0.09)	0.5
Multiple diagnoses	6(18)	26(23)	0.5
Others[2]	7(21)	29(25)	0.5
Sensitization			
Pre tx HLA antibody positive (%)	27(84)	79(71)	0.11
Pre tx HLA-DSA Negative	15(47)	65(58)	0.34
Pre tx HLA-DSA Lum +	12(37)	38(34)	
Pre tx HLA-DSA FCXM+	5(16)	9(8)	
Pre tx ECXM positive	14(44)	24(21)	0.02
Pre tx ECXM negative	4(13)	27(75)	
ABO incompatible donor	4(13)	23(21)	0.3
Donor characteristics			
Age mean(SD)	40.9(2.4)	40.5(1.3)	0.8
Male sex (%)	14(44)	52(46)	0.07
Deceased	8(25)	50(45)	0.12
Organ Cold ischemia time (hours)	22.08	23.87	
Living related	7(22)	16(14)	
Living unrelated	17(53)	47(41)	
HLA ABC mismatch (SD)	3.4(0.2)	3.6(0.1)	0.5
HLA DR DQ DP mismatch (SD)	3.12(0.2)	3.14(0.1)	0.9

[1] 31/59 patients with reported hypertension had AT1R-Ab levels between 10 and 17 and 28/59 patients had AT1R-Ab levels < 10 units/ml.
[2] Other diseases include MPGN, PKD, renal carcinoma; Wegeners granulomatosis, Recurrent Utitis, and ESRD due to unknown etiology.

3. Results

3.1. Characteristics of patients with AT1R-Ab and AECA testing performed on a pre-transplant sample

Because AT1R-Ab was shown to confer a risk for graft dysfunction, we sought to determine the characteristics of patients who present with high AT1R-Ab concentrations prior to transplant and to assess whether presence of this antibody prior to transplantation precipitates graft dysfunction. Based on our results from a previous study [19] we defined AT1R-Ab positive as concentrations ≥17 units/ml. The characteristics of 144 consecutive kidney transplant cases for which AT1R-Ab testing was performed on a pre-transplant serum are listed in Table 1. Among the kidney transplant recipients included in this study, 22% (n = 32) had AT1R-Ab levels ≥ 17 units/ml. The concentration for those patients with negative AT1R-Ab (n = 112) ranged between 2.5 and 16.84 units/ml. Prior to transplantation, kidney recipients who were positive for AT1R-Ab were younger (mean age 44 years old) than those negative for AT1R-Ab (mean age 50 years old; p = 0.04). The AT1R-Ab positive group also included a larger number of patients who had more than one kidney transplant (p < 0.0001); more males (72% vs. 47%, p = 0.008) and more patients diagnosed with focal segmental glomerulosclerosis (FSGS) (22% vs. 9%, p = 0.04).

Table 2
Patient characteristics based on source of kidney donor.

	Deceased donor (n = 58)	LR[1] donor (n = 23)	LUR[2] donor (n = 63)	p-value
Patient and donor demographics				
Mean patient age	48	50	50	0.2
# of male recipients (%)	23 (40)	11 (48)	42 (67)	0.01
Mean donor age	33	44	48	< 0.0001
# of male donors (%)	32 (55)	8 (35)	26 (41)	0.15
Pre-Tx sensitization				
HLA-DSA positive	19 (33)	8 (35)	37 (59)	0.009
ABO incompatible	0 (0)	5 (22)	22 (35)	< 0.0001
AT1R-Ab positive	8 (13)	7 (30)	17 (27)	0.1
ECXM positive (of #s tested)	3 of 6 (50)	13 of 18 (72)	22 of 50 (44)	0.1
Re-transplanted patients	23 (40)	5 (22)	34 (54)	0.02
Mean # of previous transplants	2.1	2.4	2.5	0.05
Post-Tx Outcome				
Post-Tx HLA-DSA positive	23 (40)	7 (30)	30 (48)	0.33
Increase in HLA-DSA	3	1	6	0.29
Decrease in HLA-DSA	6	4	15	
No change in HLA-DSA	40	16	36	
No post-Tx HLA-DSA data	9	2	6	
Mean days to abnormal biopsy	176	260	174	0.5
Rejection (of # biopsies)	11 of 19 (57)	5 of 16 (31)	20 of 50 (40)	0.2
Pre-Tx Treatment				
Rituximab	2 (0.03)	10 (43)	34 (54)	< 0.0001
Plasmapheresis and IVIG	0 (0)	13 (59)	43 (68)	< 0.0001

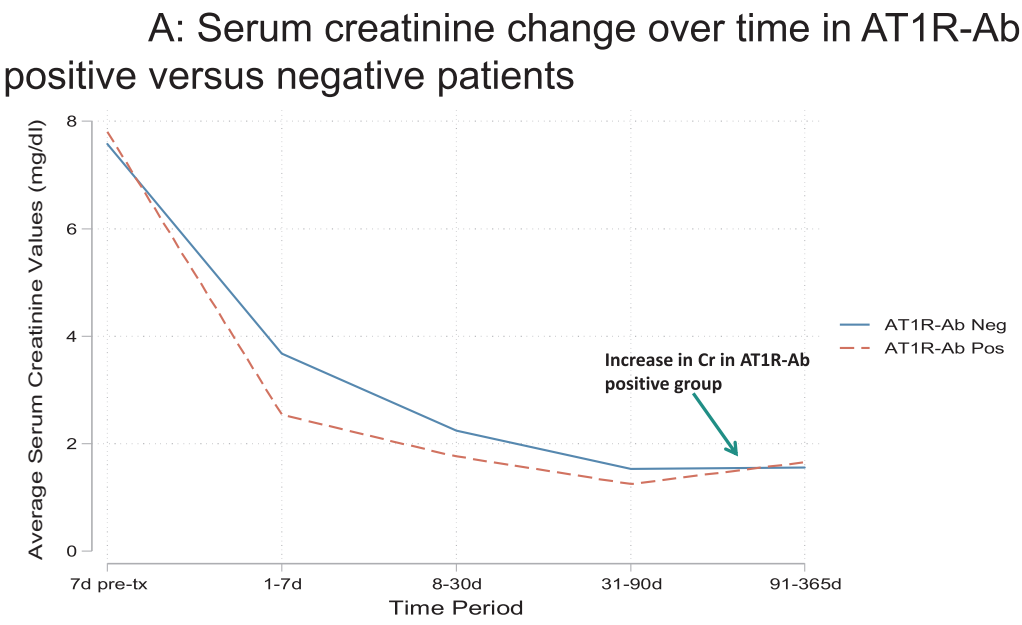
[1] LR: Living related.
[2] LUR: Living unrelated.

The 144 kidney transplant recipients included patients with increased immunologic risk factors for rejection. A substantial number of patients were sensitized against HLA antibodies prior to transplantation (84% in the AT1R-Ab positive group and 71% in the AT1R-Ab negative group). Patients who received a transplant from a living unrelated donor were more sensitized prior to transplantation (p = 0.009) and received more preemptive treatment (p < 0.001) (Table 2). Eighty patients (55%) had no detectable HLA-DSA prior to transplantation (47% in the AT1R-Ab positive group and 58% in the AT1R-Ab negative

Table 3
Summary of biopsy phenotypes.

	N (%)
Abnormal biopsies	52 (61)
Antibody mediated rejection	8 (15)
Concerning for AMR (g[1] + ptc[2] > 3)	9 (17)
Cell mediated rejection Banff 1A/1B/2A	12 (23)
Vascular rejection	4 (8)
Early transplant glomerulopathy	4 (8)
Disease recurrence ([3]MPGN and [4]FSGS)	4 (8)
Tubular injury, interstitial fibrosis	11 (21)
Normal biopsy (no rejection/no abnormalities)	33 (39)
No biopsy available	59 (40)

[1] g = glomerulitis.
[2] ptc = peritubular capillaritis.
[3] MPGN: membranoproliferative glomerulonephritis.
[4] FSGS: focal segmental glomerulosclerosis.



1B. Unadjusted model

Post Tx	AT1R-Ab groups	Coefficient of time	Std Error	p-value	95% CI	
Time 1	Negative	-2.787	0.097	<0.0001	-2.97	-2.59
	Positive	-4.426	0.292		-4.99	-3.85
Time 2	Negative	-0.851	0.039	0.053	-0.92	-0.77
	Positive	-0.601	0.122		-0.84	-0.36
Time 3	Negative	-0.596	0.017	0.001	-0.09	-0.03
	Positive	0.111	0.046		0.02	0.2
Time 4	Negative	-0.001	0.005	<0.0001	-0.01	0.009
	Positive	0.046	0.008		0.02	0.06

1C. Adjusted model

Post Tx	AT1R-Ab gro	Coefficient of time	Std Error	p-value	95% CI	
Time 1	Negative	-2.109	0.099	0.538	-2.304	-1.914
	Positive	-1.929	0.289		-2.497	-1.361
Time 2	Negative	-1.033	0.042	0.022	-1.116	-0.949
	Positive	-1.327	0.129		-1.581	-1.073
Time 3	Negative	-0.084	0.019	0.005	-0.122	-0.045
	Positive	0.054	0.271		-0.042	0.151
Time 4	Negative	-0.012	0.006	<0.0001	-0.025	0.001
	Positive	0.054	0.009		0.036	0.072

Fig. 1. Sequential serum creatinine measurement in patients with pre-transplant positive versus negative AT1R-Ab: Serum creatinine levels were followed from one week prior to transplantation up to one year post transplantation (Fig. 1A). Two patients under the age of 18 were excluded from this analysis. The y-axis in Fig. 1A shows the average creatinine levels (mg/dL). When more than one creatinine measurement was made on a day, the values were averaged. The x-axis shows times post-transplantation. Times 1 through 4 were the spline terms (time1: from day of transplant to 1 week post-transplantation; time 2: from 1 week to 1 month post-transplantation; time 3: from 1 month to 3 months post-transplantation; time 4: from 3 months to 1 year post-transplantation). In Table 1B and C, we modeled the logarithm of serum creatinine levels using multilevel generalized linear regressions with person-level random effects. Data in Table 1B show unadjusted values and data in Table 1C were adjusted for age at transplant, race, gender, HLA-DSA, and pre and post-transplant treatments (such as use of rituximab and total plasma exchange with IVIG). A negative coefficient of time indicates that creatinine decreases. A positive coefficient of time means that creatinine increases; the larger this coefficient of time, the more rapid creatinine changes. In the unadjusted model, difference between the two groups was significant at times 1, 3, and 4 ($p < 0.001$), but not at time 2 ($p = 0.05$). In the adjusted model, the difference between the two groups was not significant at time1 ($p = 0.5$), which coincides with time immediately post induction treatments, but was significant at time 2 ($p = 0.02$), 3 ($p < 0.001$), and 4 ($p < 0.001$).

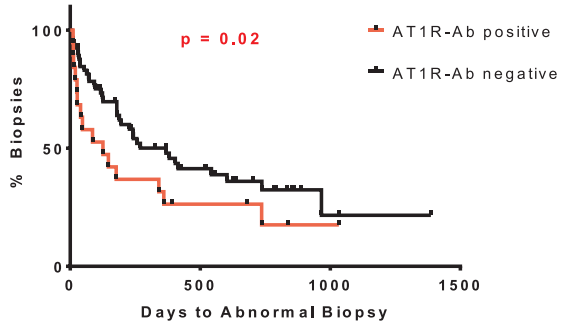
group). Among patients positive versus negative for AT1R-Ab, there was no significant difference in HLA sensitization ($p = 0.11$); level of HLA-DSA prior to transplantation ($p = 0.34$) or frequency of ABO incompatible transplants ($p = 0.3$) (Table 1).

In addition to AT1R-Ab, a pre-transplant ECXM was performed for 74 (51%) of the 144 recipients; 5 ECXMs were reported as uninterpretable due to presence of interfering substances in the patients' sera [15]. AT1R-Ab levels were higher in patients who were positive for ECXM compared to those with a negative ECXM (mean 16.3 units/ml versus 10.3 units/ml; $p = 0.003$). There were also more patients who were positive for ECXM in the AT1R-Ab positive group compared to the AT1R-Ab negative group (44% versus 21%; $p = 0.02$).

3.2. Change in creatinine level over time based on pre-transplant AT1R-Ab levels

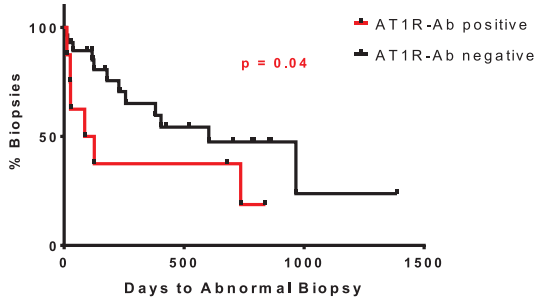
Since serum creatinine is used to monitor kidney function, we compared changes in serum creatinine in the first year after transplantation in patients with pre-transplant AT1R-Ab positive versus negative. The creatinine levels one week prior to transplantation were averaged for each patient and treated as baseline. As shown in Fig. 1, serum creatinine decreased rapidly for both AT1R-Ab positive and negative patients in the first week post transplantation, with a greater decrease observed in the AT1R-Ab positive group (unadjusted slope for AT1R-Ab positive $= -4.426$ versus AT1R-Ab negative $= -2.787$; $p < 0.0001$). From 1 week to 1 month post-transplantation, the decrease in serum creatinine slowed for both AT1R-Ab positive and negative patients (-0.601 versus -0.851 for AT1R-Ab positive versus negative respectively; $p = 0.053$). From 1 month to 3 months, creatinine continued to decrease for AT1R-Ab negative patients, (-0.059) but began to increase for AT1R-Ab positive patients (0.111); $p < 0.001$. After 3 months, the creatinine levels reached a plateau for the AT1R-Ab negative patients (-0.001) but there was a slow increase in creatinine for the AT1R-Ab positive patients (0.046); ($p < 0.0001$). After adjusting for age, sex, race, presence of HLA-DSA prior to transplantation, pre-transplant plasmapheresis and rituximab treatments, the difference between the 2 slopes remained similar to the unadjusted measurements in that compared to AT1R-Ab negative patients, those with AT1R-Ab positive had a statistically significant increase in the slope after 3 months post transplantation ($p < 0.0001$) (Fig. 1). Interestingly, while in the unadjusted model, there was a significant difference between the slopes of the AT1R-Ab positive versus negative in the first 7 days after transplantation ($p < 0.0001$); when adjusted for pre-transplant treatments, the decrease in creatinine for the AT1R-Ab positive group was not statistically different compared to the AT1R-Ab negative group significant ($p = 0.05$). Taken together, the data show that AT1R-Ab positive in this study population was an independent predictor of creatinine increase after 3 months.

A: Time to development of an abnormal biopsy for all patients



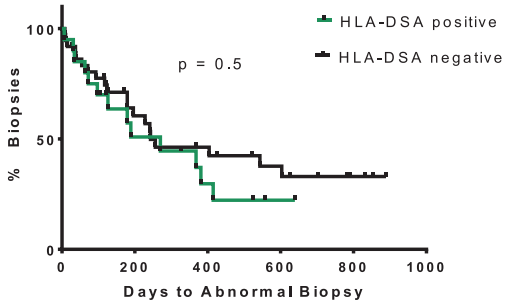
	AT1R-Ab Positive (n=19)	AT1R-Ab Negative (n=66)
N° of Abnormal Biopsies	15	37
N° of Normal Biopsies	4	29
Median Days to Abnormal Biopsy	126	368

B: Time to development of an abnormal biopsy in patients with pre-transplant HLA-DSA negative



	AT1R-Ab Positive (n=8)	AT1R-Ab Negative (n= 29)
N° of Abnormal Biopsies	6	12
N° of Normal Biopsies	2	17
Median Days to Abnormal Biopsy	106	603

C: Time to development of an abnormal biopsy based on post-transplant HLA-DSA levels in patients with pre-transplant negative AT1R-Ab



	HLA-DSA positive (n=24)	HLA-DSA negative (n= 38)
N° of Abnormal Biopsies	17	20
N° of Normal Biopsies	7	18
Median Days to Abnormal Biopsy	270	256

Fig. 2. Time to development of a first abnormal biopsy for AT1R-Ab positive versus negative patients. An abnormal biopsy is defined based on characteristics listed in Table 3. The time to development of an abnormal biopsy was compared among patients with AT1R-Ab positive (≥17 units/ml) or negative (< 17 units/ml) prior to transplantation. Table 1 shows no significant difference in the presence/absence (p = 0.11) or strength of HLA-DSA as measured by crossmatch strength (p = 0.34) between the two groups.85/144 patients in this cohort had one or more biopsies performed post transplantation. The median time to development of an abnormal biopsy for AT1R-Ab positive patients was 126 days versus 368 days for patients with AT1R-Ab negative prior to transplantation (2A). Patients who were negative for HLA-DSA prior to transplantation developed an abnormal biopsy at a mean time of 106 days for AT1R-Ab positive versus 603 days for AT1R-Ab negative patients (p = 0.04) (2B). To further confirm that the development of an abnormal biopsy was not due to pre-transplant HLA sensitization alone, we compared time to development of abnormal biopsy in patients with positive versus negative HLA-DSA and negative AT1R-Ab. Patients with positive HLA-DSA and negative AT1R-Ab developed an abnormal biopsy later (mean time = 270 days; Fig. 2C) than patients with positive AT1R-Ab and negative HLA-DSA (mean time = 106 days; Fig. 2B).

3.3. Pre-transplant non-HLA antibody status and development of abnormal biopsy or clinical phenotypes

To further determine whether pre-transplant AT1R-Ab levels could predict poor outcome and to confirm that the increase in creatinine was associated with allograft dysfunction, we calculated the time from transplantation to a first abnormal biopsy in patients who were positive versus negative for AT1R-Ab. An abnormal biopsy was defined as evidence of antibody, cellular or vascular rejection, microcirculation inflammation (g + ptc scores > 3), or transplant glomerulopathy (Table 3). At least one biopsy was performed as protocol or for cause, in 19 (59.2%) patients with AT1R-Ab positive and 66 (58.8%) patients with AT1R-Ab negative.

Patients who were positive for AT1R-Ab prior to transplantation developed an abnormal biopsy at a median time of 126 days post-transplantation (IQR = 29–375 days) while those with negative AT1R-Ab developed their first abnormal biopsy at a median of 368 days post-transplant (IQR = 82–522 days; p = 0.02) (Fig. 2A). The time to development of an abnormal biopsy for patients whose biopsy was performed to investigate allograft dysfunction was 63 days for AT1R-Ab positive group (n = 8) versus 256 days for AT1R-Ab negative group (n = 24); p = 0.16. For patients with scheduled surveillance biopsies, the time to development of an abnormal biopsy was 177 days for AT1R-Ab positive (n = 12) versus 381 days for AT1R-Ab negative (n = 41); p = 0.08. Of the 80 patients who had no HLA-DSA prior to transplantation, 37 biopsies were performed; 8 from the AT1R-Ab positive group, 6 of 8 (75%) were abnormal biopsies and 29 from the AT1R-Ab negative group in which 12 of 29 (41%) were abnormal biopsies. The median time to development of an abnormal biopsy in the AT1R-Ab positive/HLA-DSA negative group was 106 days post-transplantation versus 603 days post transplantation for AT1R-Ab negative/HLA-DSA negative (Fig. 2B; p = 0.04).

Of 38 recipients with a positive ECXM prior to transplantation, 14 (37%) also had a pre-transplant AT1R-Ab ≥ 17 units/ml of which 6 (43%) had an abnormal biopsy. The remaining patients (n = 24) with a positive ECXM were negative for AT1R-Ab and of these, 4 (17%) had an abnormal biopsy.

There were 3 cases of delayed allograft function (patient 1: AT1R-Ab 19.5 units/ml, ECXM negative, HLA-DSA negative; patient 2: AT1R-Ab = 12.1 units/ml, ECXM positive, HLA-DSA negative and patient 3: AT1R-Ab = 11.6 units/ml, ECXM not tested and HLA-DSA negative). One patient was nephrectomized one week post-transplantation (AT1R-Ab = 13 units/ml, ECXM positive; HLA-DSA negative). This data suggests that for patients with multiple immunologic risk factors, the additional presence of a non-HLA antibody can increase the likelihood of allograft dysfunction.

3.4. Effect of pre-transplant non-HLA antibody levels on post-transplant HLA sensitization

To determine whether the level of AT1R-Ab or AECA prior to transplantation correlated with a rebound in HLA-DSA or development of *de novo* HLA-DSA, we compared HLA-DSA levels at time of transplantation to levels at time of a biopsy or at end of follow-up. After transplantation, 127 of the 144 patients included in this study had longitudinal HLA antibody data. Only 10/127 (8%) of these patients had an increase in HLA-DSA. Five of the 10 patients developed *de novo* HLA-DSA. Additionally, 2 of the 5 patients had a positive pre-transplant ECXM. Moreover, 25 of 127 (20%) had a decrease in HLA-DSA and 92 of 127 (72%) had no change in HLA-DSA levels post-transplantation.

We then assessed whether post-transplant HLA-DSA alone resulted in development of an abnormal biopsy earlier in patients with negative AT1R-Ab and AECA. There were 62 biopsies for patients with negative AT1R-Ab and/or AECAs with post-transplant HLA-DSA testing available. Fig. 2C shows that time to development of an abnormal biopsy based only on the presence of HLA-DSA post-transplantation (in the absence of non-HLA antibodies) was not statistically significantly different (270 versus 256 days for HLA-DSA positive versus negative patients; $p = 0.5$). Therefore, post-transplant HLA-DSA changes did not significantly contribute to the development of abnormal biopsies in this study group.

4. Discussion

Antibody-mediated injury has been clearly established as a cause of graft dysfunction that leads to premature graft loss [1,3,29,30]. Current solid phase assays have increased the detection and characterization of HLA antibodies that, in turn, have resulted in significant improvement in graft survival through the avoidance of donor antigens to which a patient makes antibodies, desensitization treatment to reduce or eliminate HLA-DSA, and early detection and treatment to remove post-transplantation HLA-DSA [31,32]. Because the presence of antibodies to non-HLA antigens has also been implicated in allograft injury, we sought to identify characteristics of patients who might be at increased risk of developing antibody mediated injury and could be targeted for pre-transplant non-HLA antibody testing and post-transplant monitoring. An analysis of patients transplanted at Johns Hopkins and included in this study shows that a greater number of kidney transplant recipients, who are re-transplanted, male, younger, and those with FSGS at time of transplantation were positive for AT1R-Abs and AECAs prior to transplantation. Our data also supports an additional risk of allograft injury in highly sensitized patients with detectable non-HLA antibody.

Using a cutoff of ≥ 17 units/ml, we reported a 22% incidence of positive AT1R-Ab prior to transplantation. Other studies have reported higher incidences of pre-transplant AT1R-Ab positivity; 47% [21] and 59% [33] using a cutoff ≥ 9 units/ml. Despite selecting a lower cutoff for positivity, Lee et al. [33] noted an increase in microcirculation inflammation mostly in patients with higher AT1R-Ab levels (≥22.7 units/ml). In our study, patients with AT1R-Ab ≥ 17 units/ml, exhibited clinical (Fig. 1) and biopsy indication of allograft dysfunction (Fig. 2A) earlier compared to those with AT1R-Ab < 17 units/ml; and the median time to development of an abnormal biopsy in the AT1R-Ab positive group (126 days or approximately 4 months) coincided with the rise in serum creatinine at 3 months. On the other hand, Pinelli et al. [34]found no correlation with pre-transplant AT1R-Ab concentrations ≥ 17 units/ml and transplant outcomes. One difference between our study and Pinelli et al., is the increased number of highly sensitized patients in our group. While it is helpful to determine an appropriate level of reactivity that would constitute increased risk, our experiences with determining MFI cutoffs for HLA antibodies teaches us that the cumulative risk characteristics of a patient must always be considered.

Patients in both AT1R-Ab positive and negative groups were transplanted across similar levels of HLA-DSA (Table 1). Patients with pre-transplant AT1R-Ab positive with various levels of HLA-DSA prior to transplant developed abnormal biopsies sooner compared to those with negative AT1R-Ab and similar pre-transplant HLA-DSA levels (Fig. 2A). Several studies have demonstrated that presence of HLA-DSA and AT1R antibodies together result in a worse outcome compared to either one of these antibodies alone [19,22]. Injury that can be caused by presence of HLA-DSA may also promote inflammation that results in increased development of autoantibodies [35–38]. Therefore, patients with increased immunologic risk factors and with circulating non-HLA antibodies may experience early injury if treatment to reduce the antibody levels is not considered early post-transplantation.

The reverse effect, whereby presence of non-HLA antibodies could lead to development of HLA-antibodies has also been suggested. A few studies have reported development of HLA antibodies in patients with positive AT1R-Abs [22,39]. We found a small number of patients (n = 5) who developed *de novo* HLA- DSA with varying levels of pre-transplant non-HLA antibodies. Cuevas et al. [39] noted a significantly higher number of patients with *de novo* DSA (46%). The patients in our current study represent high risk cases with increased monitoring protocols and use of additional induction treatments particularly for cases of repeated mismatch, or ABO incompatibility with donor. This may account for the lower incidence of *de novo* HLA-DSA. Furthermore, this group was selected particularly because the level of post-transplant HLA-DSA did not explain poor allograft function; therefore only patients who had low levels of *de novo* HLA-DSA were considered for non-HLA antibody testing. In fact, we showed that post-transplant HLA-DSA levels in this group did not account for the earlier development of an abnormal biopsy (Fig. 2C). It is possible that we missed opportunities to detect a true correlation between development of *de novo* HLA-DSA and presence of non-HLA antibodies.

Recipients of one or more previous transplants had an increased incidence of positive AT1R-Ab prior to transplantation, similar to other studies [22]. Angiotensin II type 1 receptor, the target for antibody binding, has been associated with tissue remodeling and wound repair [40]. Tissue remodeling and fibrosis are consequences of chronic inflammation and can expose cryptic self-antigens that become neoantigens, and are therefore targets for the host immune system [41]. AT1R and other endothelial targets are expressed in the peritubular capillary [42] where injury scores are increased in cases with antibody mediated rejection associated with AT1R-Ab [19] and AECAs [43].

The increased incidence of pre-transplant AT1R-Ab positivity in male recipients was intriguing. Animal studies show evidence that sex hormones, testosterone and estradiol play a role in AT1R expression and activation. The female hormone, estradiol, decreases expression of the angiotensin II type 1 receptor (AT1R) in female rat brain, [44] and appears to offer protection against renal diseases by decreasing endogenous ligand binding ability in renal cortex [45]. Menopause or diabetes appears to reduce the protective effect of estradiol in females. On the other hand, female rat models of preeclampsia with increased levels of testosterone, expressed higher AT1R mRNA in rat mesenteric arteries [46]. Therefore if there are more targets expressed on the endothelium, when injury occurs, there are more antigens presented to the host immune system.

The range of pathological diagnoses in the abnormal biopsies includes rejection-related findings, recurrent MPGN and FSGS, and less specific changes such as interstitial fibrosis. While the impact of HLA and non HLA-DSA on recurrent glomerulopathies is not clear, we observed higher AT1R-Ab levels in patients diagnosed with FSGS, also described in previous studies [47,48].

Treatment with plasmapheresis and IVIG has been shown to reduce AT1R-Ab and AECA levels as well as HLA antibodies levels [49]. Patients who received organs from a living donor were more likely to receive plasmapheresis and IVIG as well as rituximab treatments prior to transplantation either for ABO incompatibility or to reduce HLA-DSA. As a result, there were slightly fewer early rejections among the

living donor recipients (Table 2).

We acknowledge some limitations in this study. With fewer data from the ECXM, we could not correlate positive AECAs with changes in serum creatinine and therefore used AT1R-Ab levels as a surrogate for other non-HLA antibodies. Furthermore, unlike previous publications that found a correlation between hypertension and positive AT1R-Ab [5], in this cohort there was no correlation with hypertension. It is important to note that our data evaluates presence of hypertension that contributes to chronic kidney disease (CKD) and is commonly observed in the vast majority of patients with CKD while Dragun et al. reported development of accelerated hypertension associated with vascular rejection [5]. There are also many areas that deserve further investigation. Despite the presence of AT1R-Ab prior to transplantation, most patients shows signs of dysfunction after 3 months post-transplantation, suggesting that non-HLA antibodies may contribute to slower chronic rejection overtime. More work is needed to understand the role of certain post-transplantation events such as inflammation, use of maintenance immunosuppression, on non-HLA target upregulation and consequences on circulating non-HLA antibodies.

In this study, patients with very low level HLA-DSA and either positive AT1R-Ab and/or AECAs developed rejection. The presence of non-HLA antibodies are not an absolute contraindication to transplantation, based on data available to date, but rather may suggest previous or ongoing tissue injury, and may be useful in identifying patients who should be treated either prior to transplantation or post-transplantation to avoid graft injury. Furthermore, identifying presence of non-HLA antibodies may help with treatment selection that may be different from what would be used to reduce or inhibit the activity of HLA antibodies.

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Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or non-for-profit sectors.

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