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Pretransplant angiotensin II type 1-receptor antibodies point to an increase in renal graft sub-intimal fibrosis in living- donor kidney transplant recipients

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ABSTRACT

The association between anti-AT1Rabs and microvascular injury observed in antibody-mediated rejection has been described in kidney graft Biopsies (KGBx).

Methods: We herein describe the histopathologic findings of KGBx performed during the first year of transplantation (Tx) in 134 patients tested for pre-Tx anti-AT1Rabs in cryopreserved sera (04/2009 to 09/2013). Protocol KGBx before implantation (time-zero), 1 year after Tx and for cause KGBx were included. 21/134 Tx patients were anti-AT1Rab positive (≥17 U/mL); 7/21 experienced acute rejection. For comparison a control group with anti-AT1Rabs < 17 U/mL, with (n = 16) and without (n = 31) acute rejection was included.

Results: Preimplantation KGBx showed no differences in inflammatory and chronic findings, nor in subintimal fibrosis (25 *vs* 12.8%, p = .42) between patients with anti-AT1Rabs ≥17 U/mL and those with < 17 U/mL. Follow-up KGBx revealed a significantly greater proportion of arterial sub-intimal fibrosis (52.3 *vs.* 27.6%, p = .049) and extension (15.7 *vs.* 5.3, p = .015) in anti-AT1Rabs ≥17 U/mL compared to anti-AT1Rabs < 17 U/mL KGBx. No differences were observed in microcirculation inflammation, nor in interstitial fibrosis or tubular atrophy between groups. Also, anti-AT1Rabs ≥17 U/mL (β 10.1, 2.3 to 17.8, p = .012) and more importantly anti-AT1Rabs ≥ 30 U/mL (β12.1, 3.1 to 20.9, p < .01), were independent risk factors associated with vascular occlusion resulting from sub-intimal fibrosis.

Conclusion: Our study findings have shown that anti-AT1Rab values ≥17 U/mL are significantly associated to sub-intimal fibrosis and a greater percentage of vessel occlusion in kidney graft biopsies obtained during the first year posttransplant, particularly in coexistence with inflammation and *de novo* DSA.

1. Introduction

The pathogenicity of donor-specific antibodies (DSA) against renal graft HLA antigens has been thoroughly demonstrated [1–5]. Tissue injury resulting from the interaction of HLA-DSA and target antigens has also been recognized and classified [6]. Moreover, the injuring potential of these antibodies (Abs) is in great measure, determined by their capacity to activate the complement cascade [7–9]. Additionally, the main recognized cause of long-term graft loss is firmly linked to the histopathological lesions resulting from the injury mediated by these Abs [3–5].

However, histologic evidence of antibody-mediated injury has been described in the absence of HLA-DSA and even in transplant recipients sharing 2 haplotypes with their donor. A humoral response elicited against non-HLA antigens expressed on endothelial and epithelial cells has been suggested. Recently, Abs directed against the angiotensin II

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type 1-receptor (AT1Rabs) have been associated with renal allograft antibody-mediated rejection [10–13] and shown to promote severe vascular rejection [10], microvascular renal infarcts [10], and arterial thrombosis [14] of the renal graft. Two other studies have also reported that anti-AT1Rabs are an independent risk factor for graft loss [12,13], while another recent publication showed that functional graft loss was most significant in patients with non-HLA Abs (anti-AT1Rabs and/or anti-type A endothelin-1 receptor) when compared with patients that were negative for non-HLA Abs (71% *versus* 11%) [15].

Interestingly, C4d positive staining in biopsies obtained from anti-AT1Rab positive patients with histologic findings of antibody-mediated rejection (AMR), has been found in a limited number of biopsies [11]. Previous reports have described an increase in arteritis during acute rejection (AR) in anti-AT1Rab positive kidney transplant recipients (KTR) [16]. Also, two histopathological features of endothelial injury, glomerulitis and peritubular capillaritis, have been detected much more frequently in patients with significantly increased anti-AT1Rab serum levels and in the absence of HLA-DSA [17]. Li et al. evaluated the effect of anti-AT1Rabs in a murine model with two different methods: 1) with the infusion of a peptide inducing the Abs development and 2) with the direct infusion of preformed rabbit antibodies to ApoE−/− mice. They found a higher expression of inflammatory markers (CRP, TNF-α, NF-κB) and oxidative stress (H_2O_2), and an increase of aortic sclerosis. With these experiments, they established the relationship between anti-AT1Rabs and vascular sclerosis [18].

2. Objective

The aim of this study was to compare the histologic findings, with particular emphasis on vascular changes, in all graft biopsies (bx's) performed during the first 18 months posttransplant -whether for a specific cause or per protocol- in a group of patients who were tested for AT1Rabs in pretransplant serum.

3. Materials and methods

This is an observational and comparative study. We included living-donor kidney transplant recipients (KTR) whose transplant was performed between March 2009 and September 2013 at the *Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán,* and in whom anti-AT1Rabs had been measured; a graft biopsy had to be obtained within the first 18 months posttransplant. Biopsy material had to be sufficient for histopathological analysis.

Patients were divided into two groups: Group 1 (POSITIVE Group) patients with anti-AT1Rab values ≥17 U/mL and no HLA DSA and Group 2 (NEGATIVE Group) patients with anti-AT1Rab values < 17 U/mL.

3.1. Anti-AT1Rab measurement

Anti-AT1Rabs were measured by ELISA (CellTrend GMBH, One Lambda Canoga Park, CA, USA) according to the manufacturer instructions, in serum samples obtained immediately before the kidney transplant. Serum samples were cryopreserved at −70 °C in the Histocompatibility Laboratory until measurement.

3.2. Anti-HLA antibody determination

HLA-DSA assessment was performed with Luminex Labscreen Single Antigen beads, class I and class II (One Lambda, Canoga Park, CA, USA), and were systematically determined pretransplant as part of the patient's immunological risk profile, as well as in the posttransplant period in patients with graft dysfunction and in whom a graft biopsy was performed; they were also determined as a correlate to the 12-month protocol biopsy. DSA ≥ 1000 MFI were considered positive.

3.3. Graft biopsies

In this transplant center, patients undergo protocol biopsies before implantation (time-zero), and one year after transplantation. The time-zero biopsies were obtained post-nephrectomy, after hypothermic perfusion with HTK (Custodiol®) solution.

For-cause biopsy: if acute rejection is suspected during follow-up (defined as a ≥ 25% verified increase in serum creatinine when compared to baseline and in the absence of obvious causes - infection, urinary tract obstruction, dehydration, calcineurin inhibitor toxicity), a percutaneous biopsy of the renal graft is obtained. In addition, when *de novo* proteinuria or *de novo* DSA are identified, a graft biopsy is also indicated.

The biopsies obtained from the included groups were blindly evaluated by the Nephropathologist. Direct immunofluorescence for IgG, IgM, IgA, C1q, C3c, fibrinogen, albumin, kappa and lambda chains was performed on all biopsies. For validation purposes, an initial intra-observer study yielded the following results: Glomerulitis: 0.647; Peritubular capillaritis: 0.619; Vasculitis: 0.642; Chronic glomerulopathy: 0.642; Sub-intimal fibrosis:1.0; Tubulitis: 0.418. Biopsies were classified according to the 2013 BANFF criteria. Sub-group analysis was conducted on the basis of HLA-DSA positivity and inflammation in the biopsies. Inflammation was defined as any degree of tubulitis (t), glomerulitis (g), peritubular capillaritis (ptc) or vasculitis (v).

The percentage of sub-intimal fibrosis and the value of the vascular diameter were morphometrically analyzed with the Image-Pro Express version 6.3 program (Media Cybernetics, Inc., 2008) in samples stained with Masson's trichrome; the area limited by the internal elastica lamina was measured and the area defined by the endothelium was subtracted from it thus allowing the calculation of the proportion of fibrosis. The percentage of interstitial fibrosis was evaluated with the Leica QWin, Leica Imaging Systems Ltd. (Cambridge, Engl.) system.

Histologic findings of all pre-implantation biopsies were reviewed to determine if the donated kidney was diseased *per se,* with particular emphasis on sub-intimal fibrosis. In the analysis of posttransplant findings, we used the first biopsy obtained from each patient and the gradient between biopsy zero and the first biopsy was compared in both groups.

3.4. Clinical information

Relevant demographics and clinical information to the analysis were obtained from the patient charts.

3.5. Statistical analysis

Results are expressed as frequencies and percentages in the case of categorical variables, means and standard deviations for variables with a normal distribution, and medians and interquartile ranges for variables with an abnormal distribution. Between-group comparisons were analyzed with the Chi^2 test or Fisher's exact test in the case of categorical variables, while continuous variables were analyzed with Student's *t*-test or the Mann Whitney *U* test, if the comparison is between two groups, while with more than two groups comparisons with a normal distribution was based on Analysis of Variance or the Kruskal Wallis test, if the variable “distribution” was abnormal. Bonferroni correction was applied in between-group comparisons.

We also conducted univariate and multivariate lineal regression analysis to determine the significance of the arteriolar occlusion percentages. For multivariate analysis, variables with a p value < .15 and biological relevance, were chosen. A p value below 0.05 was considered significant.

4. Results

4.1. General description of the study population

Between March 1st 2009 and September 30th 2013, 241 kidney transplants (KT) were performed at the Institute; in 161 cases, the kidney was obtained from a living- donor. Twenty of the patients were excluded from the analysis because there were not sera available. Adittionally, two cases developed primary graft failure, and in five cases the obtained material for histology was considered inadequate for evaluation. The cryopreserved pretransplant sera of 134 living-donor KTR were evaluated for anti-AT1Rabs. Twenty-one patients resulted anti-AT1Rabs ≥ 17 U/mL and conformed the Group 1. Biopsies from these patients were diagnosed as acute rejection ($n = 7$), while 14 cases only reported "non-significant abnormalities". For comparison purposes, 47 patients anti-AT1RAbs < 17 U/mL, as similar as possible in age, sex and proportion of acute rejection compared to the living-donor of Group 1 were included: 16 with acute rejection and 31 patients with "non-significant abnormalities". Then, a total of 68 patients were included in the study: 21 in Group 1 and 47 in Group 2.

The patients´ average age was 30.8 years, there were more male recipients (58.8%) and more female donors (67.6%). Between-group comparison revealed that glomerulonephritis was the most common etiology of chronic kidney disease (CKD) in Group 1 (38.1 *vs.* 12.7%, $p = .017$), specifically lupus nephritis revealed a trend (19 *vs.* 4.2%, $p = .068$). The percentage of class II PRA was also different between groups (3.3 *vs.* 1.85, $p = .043$). Table 1. (See Tables 2–6.)

Posttransplant anti-HLA antibodies were determined in the first 18 months posttransplant in 18 patients in Group 1 (anti-AT1Rabs ≥ 17 U/mL) and in 38 patients in Group 2 (anti-AT1Rabs < 17 U/mL); the percentage of DSA was documented in 33.3% and in 18.4%, respectively ($p = .21$). The remaining general characteristics are shown in Table 1.

4.2. Histologic characteristics: pre-transplant and follow-up biopsies

All available pre-transplant biopsies were accounted for, 16/21 were included in Group 1 and 39/47 in Group 2, 76.2% and 82.9% of all cases, respectively. Among the total 55 pre-transplant biopsies, 16.6% had sub-intimal fibrosis, although those in the anti-AT1Rabs ≥ 17 U/mL group had greater vascular occlusion than the biopsies from patients in the anti-AT1Rabs < 17 U/ml group; this difference was not statistically significant (5.6 *vs.* 1.7%, $p = .238$), Table 2, Fig. 1.

There was no difference in the average posttransplant period until the first graft biopsy between patients in Group 1 and 2: 10.2 (IQR 4.7–13.3) months and 12.3 (IQR 7.4–13.5) months, respectively. The proportion of biopsies obtained before the 10-month time point was also similar between groups (32 *vs.* 43%, $p = .38$). We detected no difference in the percentage of acute rejections, of any kind, between anti-AT1Rabs ≥17 U/mL and the anti-AT1Rabs < 17 U/mL groups (33.3% y 34%, respectively). (Table 1).

Table 3 describes the biopsy findings in all patients and the comparison of biopsies between anti-AT1Rabs ≥17 U/mL and anti-AT1Rabs < 17 U/mL patients. We found no statistical differences in the evaluated inflammatory characteristics (ptc, g, and v).

Among the patients included in the study, 24 (35.2%) had sub-intimal fibrosis and mean vessel occlusion was 8.5% (min-max 0 to 50%).

Table 1
General characteristics of the study population. Comparison between anti-AT1Rabs ≥ 17 U/mL and anti-AT1Rabs < 17 U/mL.

Variables	Entire group $n = 68$ (%)	AT1Rabs ≥ 17 U/mL	AT1Rabs < 17 U/mL	p value
		$n = 21$ (%)	$n = 47$ (%)	
Recipient age (y), mean ± SD	30.8 ± 12.2	27.1 ± 10.2	32.5 ± 12.7	0.097
Recipient sex (M/F)	40/28	14/7	26/21	0.380
Donor age (y), mean ± SD	39.7 ± 10.1	38.2 ± 12.1	40.3 ± 9.1	0.384
Donor sex (M/F)	22/46	8/13	14/33	0.499
CRD etiology	43 (63.2)	11 (52.3)	32 (68.1)	0.215
Diabetes mellitus	5 (7.3%)	1 (4.7)	4 (8.5)	1.000
Glomerulonephritis	14 (20.6)	8 (38.1)	6 (12.7)	0.017
Haplotypes (0/1/2)	14/44/10	3/15/3	11/29/7	0.691
Second KT	3 (4.41)	2 (9.52)	1 (2.13)	0.223
%PRA class I, md (min-max)	5.75 (0–87)	1.95 (0–24)	7.44 (0–87)	0.750
%PRA class II, md (min-max)	2.31 (0–26)	3.33 (0–26)	1.85 (0–26)	0.043
Pre-KT DSA	2 (3.13)	0	2 (4.35)	1.000
High-risk CMV	8 (11.8)	3 (14.3)	5 (10.6)	0.695
Warm isch (min), mean ± SD	3.83 ± 1.79	4.25 ± 1.73	3.64 ± 1.91	0.222
Cold isch (h), mean ± SD	1.24 ± 0.46	1.1 ± 0.31	1.31 ± 0.51	0.094
Thymoglobulin induction	4 (5.8)	0	4 (8.5)	0.303
Anti-IL2R induction	57 (83.8)	18 (85.8)	39 (82.9)	1.000
de novo DSA*	13/56 (23.2)	6/18 (33.3)	7/38 (18.4)	0.217
Baseline BMI, mean ± SD	23.4 ± 3.5	23.1 ± 3.8	26.6 ± 3.4	0.565
12 mo BMI, mean ± SD	24.3 ± 4.2	24.6 ± 4.7	24.2 ± 4.1	0.733
12 mo Hypertension	27 (39.7)	7 (33.3)	20 (42.5)	0.520
12 mo Tacrolimus (TL), mean ± SD	9.6 ± 3.1	9.9 ± 3.3	9.4 ± 2.9	0.537
Acute rejection	23 (33.8)	7 (33.3)	16 (34)	0.95
Acute cellular rejection	14 (20.5)	5 (23.8)	9 (19.1)	0.66
Acute antibody mediated rejection	7 (10.2)	2 (9.5)	5 (10.6)	0.88
Mixed rejection	2 (2.9)	0	2 (4.2)	1.00

CRD: chronic kidney disease.
KT: kidney transplant.
PRA: panel reactive antibodies.
DSA: donor specific antibody.
isch: ischemia.
BMI: body mass index.
md: median.
SD: standard deviation.
* DSA determinations were obtained in 18 patients in Group 1 and in 38 patients in Group 2.

Table 2
Histologic findings in pre-transplant biopsies and comparison between Groups 1 and 2.

	Entire group	AT1Rabs ≥ 17 U/mL	AT1Rabs < 17 U/mL	p value
	n = 55 (%)	*n* = 16 (%)	*n* = 39 (%)	
Sub-intimal fibrosis (SIF)	9 (16.3)	4 (25.0)	5 (12.8)	0.422
SIF, mean ± SD	2.9 ± 7.8	5.6 ± 12.1	1.8 ± 5.1	0.238
Vessel diameter w/SIF, mean ± SD	119.6 ± 41.3	110.1 ± 39.7	127.4 ± 45.4	0.462
Interstitial fibrosis, mean ± SD	8.8 ± 6.7	10.1 ± 5.2	8.3 ± 7.3	0.135

Both groups had an increased proportion of compromised arteries and percentage of vascular occlusion when compared with the pre-implant biopsy (Fig. 1); however, both the proportion of sub-intimal fibrosis in arteries (52.3 *vs.* 27.6%, *p* = .049) and its extension (15.7 *vs.* 5.3, *p* = .015) in the first follow-up biopsies in patients with anti-AT1Rabs ≥17 U/mL (Group 1), were significantly greater than in the anti-AT1Rabs < 17 U/mL group (Group 2).

Due to the referred significant difference in the proportion of patients with sub-intimal fibrosis in the presence of anti-AT1Rabs ≥17 U/mL, two types of analyses were performed to adjust for probable confusing variables.

4.3. Histologic inflammation

Table 3 describes each inflammatory variable in the studied biopsies. In order to evaluate the contribution of each of these variables on the percentage of sub-intimal fibrosis, we conducted a sub-group analysis, revealing that histologic inflammation is related to the percentage of sub-intimal fibrosis. When patients with and without histologic inflammation were grouped together, the biopsies from those in the anti-AT1Rabs ≥ 17 U/mL more frequently displayed sub-intimal fibrosis, (*p* = .042). Additionally, upon comparison of the average vascular occlusion between both groups (anti-AT1Rabs ≥17 U/mL and AT1Rabs < 17 U/mL), the difference is clear (*p* = .037), Figs. 2 and 3. (See Fig. 3.)

4.4. HLA Donor-specific antibodies

During the first 18 months posttransplant, HLA-DSA were determined in 56 patients (96.5%); 10 patients shared two haplotypes with their donor. Thirteen (23.21%) patients developed *de novo* DSA. In one patient, there was no temporal relationship between the point the biopsy was obtained and a positive HLA-DSA determination, so he was not included in this analysis. The proportion of biopsies with sub-intimal fibrosis was greater in patients with *de novo* DSA (58.3% *vs.* 27.9% *p* = .05). When grouping patients with anti-AT1Rabs ≥17 U/mL and those with *de novo* DSA, a synergic effect was detected although it was not statistically significant (Fig. 4).

However, when evaluating the proportion of vascular occlusion in patients with anti-AT1Rabs ≥17 U plus *de novo* DSA *vs.* anti-AT1Rabs ≥17 U without *de novo* DSA, the difference was statistically significant (*p* = .019), Fig. 5.

4.5. Anti-AT1R values and Sub-intimal Fibrosis

In order to explore different cutoff limits for anti-AT1Rabs when associated to sub-intimal fibrosis, a sub-group analysis was performed (anti-AT1Rabs < 17 U/mL, anti-AT1Rabs ≥ 17- < 30 U/mL, and anti-AT1Rabs ≥ 30 U/mL). The proportion of patients with sub-intimal fibrosis was greater in the group with anti-AT1Rabs > 30 U, although the result was not significant. However, the group with anti-AT1Rabs ≥ 30 had a significantly greater degree of vascular occlusion resulting from sub-intimal fibrosis (OR 3.48, *p* = .048). Likewise, the risk and percentage of vascular occlusion in the anti-AT1Rabs ≥ 30 U group was also greater (Table 4, Fig. 6).

4.6. Proportion of arteriolar occlusion

The clinical and laboratory variables relating to a greater proportion of arteriolar occlusion by sub-intimal fibrosis are shown in Table 5.

Two models were created for analysis of vascular occlusion (Table 6). In the first model, AT1Rabs ≥17 U/mL (β 10.1, 2.3 to 17.8, p = .012) remained significant. In the second model, as in the univariate analysis, anti-AT1Rabs ≥ 30 U/mL values also remained significant. In both models, a history of systemic lupus erythematosus as the underlying disorder and the percentage of interstitial fibrosis also remained statistically significant (β 12.1, 3.1 to 27.5), *p* = .008).

5. Discussion

The main objective of this review was to evaluate the histopathologic characteristics of graft biopsies obtained during the first year

Table 3
Histologic characteristics of the first biopsy obtained during the first 18 months posttransplant. IF: Interstitial fibrosis SIF: Sub-intimal fibrosis.

	Entire group	AT1Rabs ≥ 17 U/mL	AT1Rabs < 17 U/mL	p value
	n = 68 (%)	*n* = 21 (%)	*n* = 47 (%)	
Glomerulitis	31 (45.5)	10 (47.6)	21 (44.6)	0.822
Glomerulitis (g1/g2/g3)	21/8/2	7/3/0	14/5/2	0.908
Peritubular capillaritis	20 (29.4)	4 (19.1)	16 (34.1)	0.259
Peritubular capillaritis (ptc 1/2/3)	11/8/1	3/0/1	8/8/0	0.080
Vasculitis	2 (4.2)	0	2 (4.2)	0.547
Vasculitis (V 1/2/3)	2/0/0	0/0/0	2/0/0	1.000
Chronic Glomerulopathy	2 (4.2)	0	2 (4.2)	0.547
CG (grade1/2/3)	1/1/0	0/0/0	1/1/0	1.000
Sub-intimal fibrosis (SIF)	24 (35.3)	11 (52.4)	13 (27.6)	0.049
SIF, mean ± SD	8.5 ± 14.1	15.7 ± 18.7	5.3 ± 10.1	0.015
Vessel diameter w/SIF, mean ± SD	105.3 ± 43.6	108.9 ± 36.3	102.0 ± 50.8	0.698
Interstitial fibrosis, mean ± SD	13.9 ± 11.1	11.6 ± 7.8	14.9 ± 12.2	0.258
Tubulitis	28 (41.2)	8 (38.1)	20 (42.5)	0.730
Tubulitis (T 1/2/3)	15/6/6	5/1/1	10/5/5	0.846

Table 4
Risk of sub-intimal fibrosis and vascular occlusion according to anti-AT1Rabs level.

Variables	Anti-AT1Rabs	Anti-AT1Rabs	Anti-AT1Rabs	p value
	< 17 U/mL	≥ 17 - < 30 U/mL	≥ 30 U/mL	
Sub-intimal fibrosis, (%)	13 (27.6)	3 (42.8)	8 (57.1)	0.128
Risk, OR (95% CI) p value	Reference	1.96 (0.38–9.98) *p* = .41	3.48 (1.01–12.00) p = .048	
% Occlusion m (SD)	5.2 (10.1)	12 (16.8)	17.6 (19.9)	0.041
Risk, β (95% CI) p value	Reference	6.7 (− 4.11–17.51) *p* = .22	12.3 (4.2–20.4) *p* = .003	

Table 5
Clinical and laboratory variables relating to a greater proportion of arteriolar occlusion by sub-intimal fibrosis.

Variable	Beta coefficient (95%CI)	P value
Donor age	−0.02 (−0.36 to 0.32)	0.89
Thymoglobulin	2.89 (−11.7 to 17.5)	0.69
Anti CD25	1.72 (−7.61 to 11.06)	0.71
No shared haplotypes	0.04 (−11.52 to 11.61)	0.99
One shared haplotype	6.38 (−3.39 to 16.17)	0.19
Second transplant	−8.92 (−25.5 to 7.69)	0.28
PRA class I	0.08 (−0.12 to 0.28)	0.43
PRA class II	0.60 (0.02 to 1.19)	0.04
DSA pre-KT	−9.22 (−29.9 to 11.52)	0.37
Warm ischemia	−1.41 (−3.22 to 0.40)	0.12
Cold ischemia	−4.10 (−11.12 to 2.91)	0.24
Tacrolimus mo 1	−0.29 (−1.62 to 1.03)	0.66
Tacrolimus mo 12	−1.11 (−2.26 to 0.03)	0.056
Continuous AT1R	0.02 (−0.02 to 0.07)	0.26
AT1R ≥ 17 U	10.45 (3.46 to 17.45)	0.004
AT1R < 17	Reference	Ref
AT1R ≥ 17 to < 30	6.7 (− 4.11 to 17.51)	0.221
AT1R > 30	12.3 (4.21 to 20.46)	0.003
de novo DSA	11.27 (2.03 to 20.51)	0.018
Any Inflammation	6.52 (−0.57 to 13.61)	0.071
Glomerulitis (0–3)	2.54 (−1.70 to 6.78)	0.236
PTC (0–3)	2.32 (−2.19 to 6.84)	0.308
Vasculitis	−0.78 (−29.04 to 11.47)	0.390
Chronic glomerulopathy	8.71 (−3.97 to 21.40)	0.175
Interstitial fibrosis	0.43 (0.13 to 0.71)	0.005
Tubulitis	3.41 (−3.52 to 10.35)	0.330
Lupus nephritis as cause of ESRD	20.8 (9.81 to 31.81)	< 0.001
DM2	−5.99 (−19.10 to 7.11)	0.364

posttransplant in a patient population with pre-transplant anti-AT1Rabs ≥17 U/mL and compare them with those in transplant recipients with anti-AT1Rabs values < 17 U/mL.

The study revealed that sub-intimal fibrosis was a conspicuous finding in the biopsies from patients with anti-AT1Rabs ≥17 U/mL, both in frequency and extension as well as in the associated resulting degree of vessel obstruction. Analysis of factors associated to the percentage of vascular occlusion revealed that anti-AT1Rabs were independently associated to it and this correlation appeared to be dose-dependent whereby values above 30 U/mL were the most clearly related (Table 6). We recently found and reported that higher levels of

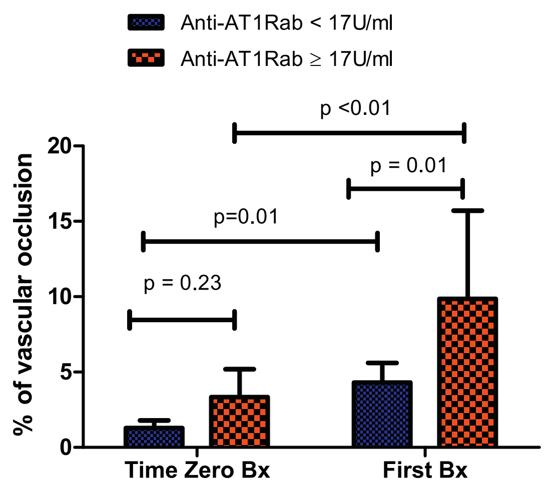

Fig. 1. Graphic representation of the increased proportion of patients with sub-intimal fibrosis in the first biopsy and percentage of vascular occlusion in Groups 1 and 2 [single-column fitting image].

pre-transplant circulating antibodies against AT1R (≥30 U/mL) in kidney graft recipients, constituted an independent risk factor for earlier *de novo* HLA-DSA detection in the posttransplant period, possibly suggesting that certain individuals are prone to develop higher titers of antibodies against different antigenic stimuli including autoantigens [19]. This phenomenon was recently confirmed by other researchers where patients positive for pre-transplant AT1R-Ab were more likely to develop *de novo* HLA-DSA compared to patients that were negative for AT1R-Ab (28% *vs* 10%, $p = .027$) also, pre-transplant positivity for AT1R-Ab was associated with TCMR in the first year post-transplant ($p = .034$) [20].

Aside from anti-AT1Rabs, two important factors also correlated with sub-intimal fibrosis and the degree of vascular occlusion: any grade of inflammation and the detection of *de novo* DSA. Histologic inflammation was additive in conjunction with the anti-AT1Rabs, and greatest in the group with anti-AT1Rabs both with and without inflammation (Figs. 2 and 3). Likewise, the presence of *de novo* DSA in cases with anti-AT1Rabs ≥ 17 U/mL was a risk factor for vascular occlusion when compared to the group with anti-AT1Rabs < 17 U/mL

Table 6
Multivariate analysis of vascular occlusion adjusted to any degree of inflammation and *de novo* DSA.

Variables	Model 1	p value	Model 2	p value
	β (95% CI)		β (95% CI)	
Anti-AT1Rabs ≥ 17 U/mL	10.1 (2.3–17.8)	0.012		
Anti-AT1Rabs < 17 U/mL			REFERENCE	
Anti-AT1Rabs ≥ 17 - < 30 U/mL			6.2 (−5.4–17.9)	0.291
Anti-AT1Rabs ≥ 30 U/mL			12.1 (3.1–20.9)	0.009
Hx of lupus nephritis	15.1 (3.5–13.2)	0.011	15.9 (4.2–27.5)	0.008
Interstitial fibrosis	0.4 (0.1–0.7)	0.006	0.42 (0.1–0.7)	0.007

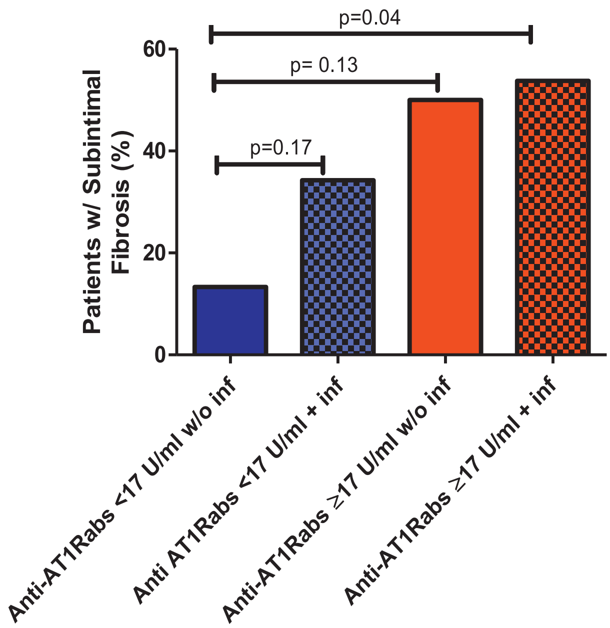

Fig. 2. Percentage of patients with sub-intimal fibrosis according to the pre-transplant anti-AT1R serology (≥17 U/mL ó < 17 U/mL) and the presence of inflammation in the biopsy (p = .113). Patients with anti- AT1Rabs < 17 U/mL without inflammation (n = 15); anti- AT1Rabs < 17 U/mL with inflammation (n = 32); anti-AT1Rabs ≥17 U/mL without inflammation (n = 8); anti-AT1Rabs ≥17 U/mL with inflammation (n = 13); w/o: without inflammation; i: inflammation. Inflammation was defined as the presence of at least one of the following inflammatory indices: glomerulitis (G ≥ 1), peritubular capillaritis (PTC ≥ 1) or tubulitis (T ≥ 1) [Single-column fitting image].

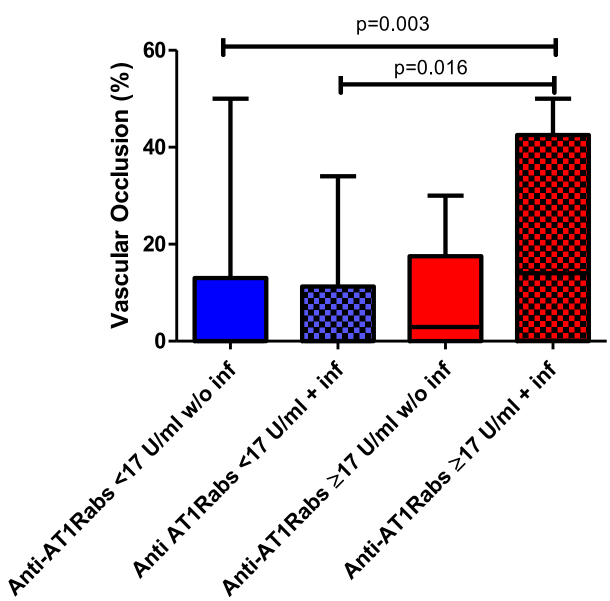

Fig. 3. Box-and-whisker plots of the percentage of vascular occlusion, according to anti-AT1Rabs group and the presence of inflammation (p = .037). Between-group comparison with Bonferroni correction. Inf = inflammation. [single-column fitting image].

and no *de novo DSA* (Figs. 3 and 4). Also, analysis of factors associated to vascular occlusion established that anti-AT1Rabs were independently and significantly associated in models adjusted for the grade of inflammation and *de novo* DSA (Table 6). In a very recent report, the authors examined the relationship of AT1R antibody with clinical outcomes, biopsy findings, inflammatory cytokines, and HLA donor-

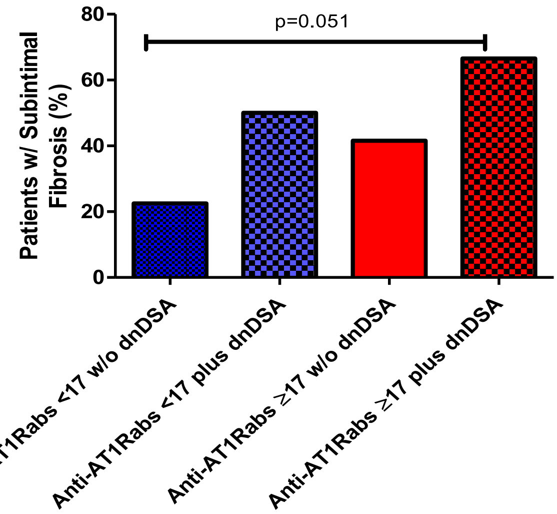

Fig. 4. Percentage of sub-intimal fibrosis in patients with pretransplant AT1Rabs and *de novo* DSA. No between-group comparison was statistically significant. Patients with AT1Rabs < 17 U/mL and without *de novo* DSA (n = 31); AT1Rabs < 17 U/mL plus *de novo* DSA (n = 6); AT1Rabs ≥17 U/mL without *de novo* DSA (n = 12); AT1Rabs ≥17 U/mL plus *de novo* DSA (n = 6). [Single-column fitting image].

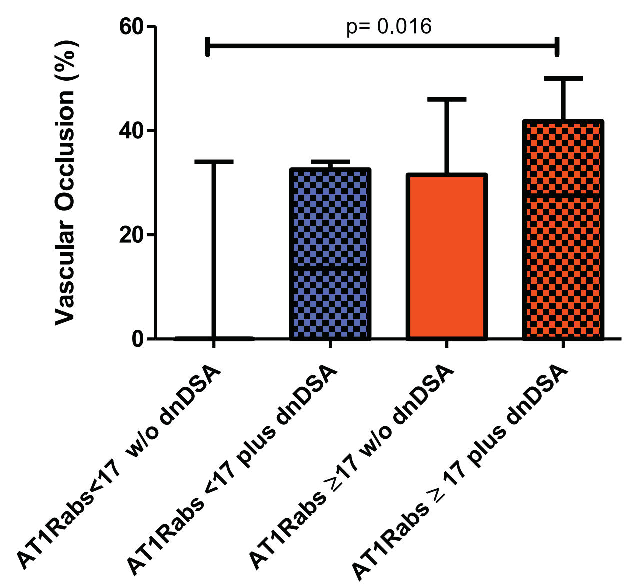

Fig. 5. Box-and-whisker plot of the percentage of vascular occlusion, divided by the presence of AT1R ≥ 17 U/mL and *de novo* HLA- DSA (p < .01). Between groups, only in patients with AT1Rabs ≥ 17 plus dnDSA *versus* AT1Rabs < 17 w/o dnDSA was the analysis statistically significant (p = .016). [Single-column fitting image].

specific antibodies (DSA) in a cohort of pediatric renal transplant recipients. AT1R antibody was associated with significantly greater declines in eGFR in patients both with and without rejection. AT1R antibody was also associated with vascular inflammation in the allograft, and allograft loss [21].

To date, the histologic abnormalities of graft biopsies in cases with positive anti-AT1Rabs have shown a greater frequency of acute antibody-mediated events, [11,] most significant in those with anti-AT1Rabs titers ≥17 U/mL [17], severe vascular rejection and malignant hypertension in individuals without HLA-DSA [10] and an increased risk of developing early acute rejection, both cellular or antibody-mediated [12]. Additionally, an increased frequency of arteritis has also been described in individuals with a diagnosis of antibody-mediated acute

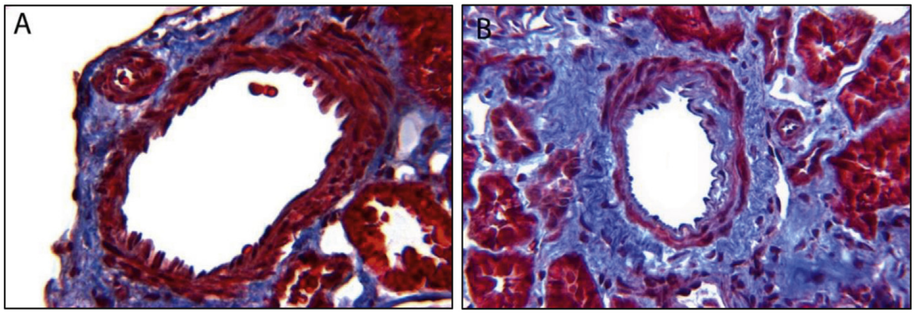

Fig. 6. A) Normal medium size artery from a patient with AT1Rab negative and no acute rejection. B) Medium size artery from a patient with AT1Rab positive (≥ 17 U/mL) and acute rejection. [2-column fitting image].

rejection and anti-AT1Rabs titers ≥17 U/mL [16]. Transplant glomerulopathy has also been described in patients with anti-AT1Rabs ≥17 U/mL, in the absence of HLA-DSA [22]. The association between anti-AT1Rabs and sub-intimal fibrosis is controversial; a recent publication evaluated the presence of anti-AT1Rabs and their association with pathologic findings in graft biopsies, and specifically reported no correlation between the anti-AT1Rab concentrations and intimal arteritis, interstitial fibrosis, tubular atrophy, intimal thickening, C4d staining, and chronic glomerulitis [17]. Other authors have however, reported chronic kidney graft injury associated to AT1RAbs (cg1–3, cv1–2, ci1–2, ct1–2) [15].

It is interesting to underscore the fact that lupus, as a cause of chronic kidney disease in the presence of AT1Rabs ≥17 U/mL, and its association with sub-intimal fibrosis in biopsies obtained during the first year posttransplant, suggests that injury may result from the noxious activity perpetrated by the pre-existing anti-AT1R antibodies, regardless of the concomitant presence of immune complexes since immunofluorescence detection was negative in all cases [23]. A recent review of the kidney biopsies of patients with lupus nephropathy at our Institute, revealed that approximately 44% of them had arteriolosclerosis characterized by sub-intimal fibrosis (manuscript submitted). Very similar findings were recently reported by a group of investigators in China, whose study documented arteriolosclerosis in about 63% (50/79) of biopsies in patients with lupus nephritis [24]. The significance of these lesions in terms of kidney graft functional survival remains to be evaluated.

One limitation of this study is that the cutoff value used in this study was according to the manufacturers protocol which is similar to that used in several studies, however this cutoff value is based on HLA non-sensitized male control. Therefore, we performed an additional analysis with anti-AT1Rab values between 17 and 30 U/mL and > 30 U/mL compared to < 17 U/mL, to detect a cutoff value more robust, however, the number of patients included does not allow an accurate definition of the titer optimally related with the vascular changes observed.

6. Conclusion

Our study findings have shown that anti-AT1Rab values ≥17 U/mL are significantly associated to sub-intimal fibrosis and a greater percentage of vessel occlusion in kidney graft biopsies obtained during the first year posttransplant, particularly in coexistence with inflammation and *de novo* DSA.

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