ORIGINAL ARTICLE

Is pre-transplant sensitization against angiotensin II type 1 receptor still a risk factor of graft and patient outcome in kidney transplantation in the anti-HLA Luminex era? A retrospective study

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SUMMARY

We aimed to assess the correlation of anti-angiotensin II type 1 receptor antibodies (anti-AT1R-Abs) before transplantation on a multicentric cohort of kidney transplant recipients (2008–2012), under tacrolimus and mycophenolate mofetil (MMF), screened by Luminex technology for anti-HLA immunization. Anti-AT1R antibody levels were measured by ELISA in pretransplantation sera of 940 kidney recipients from three French centers of the DIVAT cohort. Multivariable Cox models estimated the association between pretransplant anti-angiotensin II type 1 receptor antibodies and time to acute rejection episodes (ARE) or time to graft failure. Within our cohort, 387 patients (41.2%) had pretransplant AT1R-Abs higher than 10 U/ml and only 8% (72/970) greater than 17 U/ml. The cumulative probability of clinically relevant (cr)-ARE was 22.5% at 1 year post-transplantation [95% CI (19.9–25.4%)]. The cumulative probability of graft failure and patient death were 10.6% [95% CI (8.4–13.3%)] and 5.7% [95% CI (4.0–8.1%)] at 3 years post-transplantation, respectively. Multivariate Cox models indicated that pretransplant anti-AT1R antibody levels higher than 10 U/ml were not significantly independently associated with higher risks of acute rejection episodes [HR = 1.04, 95% CI (0.80–1.35)] nor with risk of graft failure [HR = 0.86, 95% CI (0.56–1.33)]. Our study did not confirm an association between pretransplant anti-AT1R antibody levels and kidney transplant outcomes.

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Key words
acute rejection, Angiotensin type 1 receptor antibodies, graft outcome, pretransplant

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Introduction

In kidney transplantation, graft survival has improved over the years due to better understanding of the alloimmune response, a corresponding increase in the efficacy of immunosuppressive therapy [1] and the prevention of infectious complications [2,3]. However, acute rejection still occurs in around 10% of kidney transplants and can even occur in transplants between fully HLA-matched identical siblings [4,5]. This raises the possibility of alternative mechanisms such as non-HLA sensitization and the exploration for new alloantibody targets [6,7]. New target antigens capable of initiating cellular injury and organ damage have been identified *in vitro* [8,9]. Among these, angiotensin II receptor type 1 (AT1R) antibodies (Abs) have been implicated in the pathophysiology of immune diseases such as preeclampsia and systemic sclerosis [10,11]. In allograft sensitization, AT1R-Abs act as graft endothelial cell agonists [12,13]. Anti-AT1R-Abs seem to exert direct effects on endothelial and smooth muscle cells via induction of the Erk 1/2 signal transduction cascade. By cross-linking the second extracellular loop of the receptor [14], the bound antibodies induce physiological effects close to those normally observed in the renin–angiotensin system. Dragun *et al.* [8,13] have shown for the first time their involvement in patients presenting acute rejection episodes (ARE) with severe hypertensive crisis. Since this first observation, the correlation of anti-AT1R antibodies and acute rejection and/or graft failure has become more debated in kidney transplantation sometimes due to a lack of power of studies [15–24]. In 2013, we showed on a large monocentric cohort (n = 599) of French kidney recipients that patients who displayed a pretransplant AT1R-Ab level above 10 U/ml had higher risk of acute rejection episodes within the first month following transplantation and graft failure beyond 3 years post-transplantation [25,26]. However, our results had to be interpreted with caution as it was a monocentric study. Patients were transplanted between 1998 and 2007; half treated with cyclosporine who displayed a low incidence of clinically relevant acute rejection episode and with pretransplant HLA sensitization assessed by a complement-dependent cytotoxicity assay.

To overpass the limitations of our first observation [25], we used a recent (2008–2012) large multicentric cohort (three centers) of 940 kidney transplant recipients all treated with tacrolimus and mycophenolate mofetil for maintenance therapy and in whom anti-HLA immunization was assessed by solid-phase assay technologies (Luminex, Austin, TX, USA).

Methods

Patients

Nine hundred and forty kidney transplant recipients from three French University transplantation centers (Nantes, Paris Necker and Lyon) belonging to the CENTAURE Network (www.fondation-centaure.org) and DIVAT cohort (www.divat.fr; approved by the French National Commission on Computing and Liberty DR-2025-087, number 914184; February 15, 2015) were included in the study. Data were prospectively computerized in real time as well as at each transplant anniversary. All recipients provided written, informed consent for the biobanking of samples.

Inclusion criteria were the following: patients older than 18 years who received a first or second kidney and/or a combined kidney and pancreas transplant from heart beating deceased donors between 2008 and 2012. Standard induction treatments were performed with IL-2 receptor antagonist (Anti-IL-2R, Basiliximab, Simulect®; Novartis, Basel, Switzerland) or antithymocyte globulin (ATG, Thymoglobulin®; Sanofi, Paris, France). All patients were screened for anti-HLA sensitization by solid-phase assays Luminex® technology within the 6 months before the transplant. We did not include patients with kidney transplantation combined with lung, heart or liver transplants. The follow-up period extended until March 31, 2015. To note, none of the patients from our initial study [25] were included in this second study (distinct period of transplantation).

Studied parameters

Parameters analyzed for donors were as follows: age, gender, last creatinine measurement, and cause of death. Parameters analyzed for recipients were as follows: age, gender, body mass index, prior history of diabetes, blood pressure, initial kidney disease, duration in dialysis before transplantation, renal replacement therapy, previous transfusion, HLA-A-B-DR incompatibilities, anti-HLA class I and II sensitization at transplantation, transplantation rank, kidney or combined kidney/pancreas transplant, cold ischemia time, type of induction (ATG or anti-IL-2R monoclonal antibodies), maintenance therapy (calcineurin inhibitor CNI, mycophenolate mofetil MMF, and steroids), and pretransplantation anti-AT1R immunization.

Anti-HLA immunization

HLA typing of transplant recipients and their corresponding donors was performed by molecular biology

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(PCR-SSP or PCR-SSO; One lambda Inc., Canoga Park, CA, USA, Bionis, Linkage Biosciences or Olerup, Wien, Austria).

Pretransplant immunization against HLA class I and II antigens was prospectively performed using the Luminex® screening (One lambda® or Gen-probe®) technology in each transplantation center within the 6 months pretransplantation. For Luminex® assay screening, antibodies were detected by the fluorescent signal for each bead coated with HLA antigen, normalized to the value measured with negative control serum. The positivity level depended on the laboratory recommendation. The Paris Necker and Nantes centers performed the test using the LAB Screen Mixed LSM12 assay (One Lambda Inc., Canoga Park, CA, USA). The Lyon center used Gen-probe® technology. In each of the three centers, single-antigen bead (SAB) assays were performed in patients with a positive screening to defined antigen specificity against class I and II. In addition, for some recent patients, results of SAB were available within the 6 months pretransplantation. In those cases, we took into account the results of the SAB since it is currently considered as the most sensitive and specific technology to define anti-HLA immunization. MFI (mean fluorescence intensity) threshold values taken into account to consider antibodies were specific to each center, according to their yearly internal and external validations. In the whole cohort, all pretransplant cross-matches performed by direct complement-dependent cytotoxicity (CDC) assays on total and separated T and B lymphocytes were negative at transplantation.

Anti-AT1R immunization determination

All serum samples were systematically and prospectively collected on the day of transplantation and were stored in each transplantation centers' biobank. For this study, all frozen sera samples from Lyon and Paris were collectively shipped to Nantes. ELISA assays for anti-AT1R-Ab titration (One Lambda®) were performed blinded and in "duplicate" on an immuno-monitoring CIMNA® platform of the Nantes University Hospital. Manufacturer's instructions were followed for dilution and incubation times, on precoated plates, with 1:100 diluted serum.

To note: Since Luminex technology is the method of choice to determine the anti-HLA immunization before the transplantation and is routinely used in all French transplantation centers, we did not perform a new and centralized screening for anti-HLA immunization but used the results provided by each center as it was a main inclusion criteria. In contrast, we centralized the ELISA dosage of anti-AT1R to minimize technical variabilities as this particular ELISA kit had never been used by the participating centers before.

Acute rejection episodes

All ARE were biopsy-proven and classified according to the 2007 Banff classification as: cellular, humoral (including mixed humoral and cellular), or borderline [27]. The three centers routinely performed surveillance biopsies at 3 months and 1 year post-transplantation (only since 2009 for the 3-month biopsy in Nantes) [28,29]. Some patients, however, may have been locally contraindicated by their own medical team if the risk for a surveillance biopsy was judged too high compared to the benefit. Finally, ARE were subdivided into two groups: (i) clinically relevant (cr-ARE), if they required antirejection medication (including treated borderline cases); and (ii) subclinical (sc-ARE), if they were diagnosed on surveillance biopsies without any sign of graft function deterioration and were not considered for treatment by the medical team. Borderline cases were pooled with acute cellular rejection if they received steroid therapy and pooled with acute humoral rejection if they were considered as suspicious humoral rejections and treated by plasma exchange and/or IvIg and/or rituximab or bortezomib.

Statistical analyses

To take into consideration confounding factors, the different times to events were analyzed using event-specific Cox models [30] stratified on the center from the date of transplantation: (i) the time to cr-ARE by censoring return to dialysis, preemptive transplantation, and death with a functioning graft; (ii) the time to sc-ARE by censoring cr-ARE, return to dialysis, preemptive transplantation, and death with a functioning graft; (iii) the time to graft failure (return to dialysis or preemptive transplantation) by censoring death with a functioning graft, or iv) the time to death with a functioning graft by censoring return to dialysis and preemptive transplantation. Considering that there is no consensus on the best threshold of the etiological risk of anti-AT1R before transplantation, we considered three categories of threshold for anti-AT1R ($\leq$10, 10–17, >17 U/ml) to assess the final Cox multivariable model for acute rejection episode and graft survival. In order to study the 1 year post-transplantation creatinine level, only the recipients alive with a functional graft were considered.

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The same modeling strategy ($P < 0.20$ in univariate analyses and $P < 0.05$ in multivariable analyses) was performed, the only difference being the use of multiple linear regressions. Each time to event was studied using the same strategy of covariate selection. A multivariable analysis was conducted by taking into account pretransplant anti-AT1R-Ab levels, pretransplant anti-HLA sensitization, risk factors already described in the literature, and significant characteristics by univariable analysis ($P < 0.20$). These later were removed in a backward selection if $P > 0.05$. Proportional hazard assumption for anti-AT1R-Abs was assessed graphically and checked in the final multivariable by using weighted residuals analysis. In contrast with our previous work, we did not identify any violation of proportional hazard assumption in this new cohort of patients. Thus, we used a proportional hazards tests as classically proposed by P. Grambsch and T. Therneau from 1994 [31]. Finally, we also checked whether the association between pretransplant AT1R sensitization and outcomes differed according to the HLA sensitization. The median follow-up time was estimated using the reverse Kaplan–Meier estimator [32].

The cumulative probabilities for events were estimated by taking into account the competing risks using the Nelson–Aalen nonparametric estimator [33]. All analyses were performed using R software [34].

Results

Description of patient characteristics at transplantation

One thousand seven hundred and five (1705) patients who underwent kidney and/or pancreas transplantation from deceased donors in the three hospitals between 2008 and 2012 were initially considered. We did not include 765 patients (44.9%) for either (i) missed screening for anti-HLA immunization by solid-phase assay technology (Luminex®) within the 6 months pretransplantation ($N = 572$), (ii) missing serum samples ($N = 103$) or (iii) segmental glomerulosclerosis as the initial kidney disease ($N = 90$). Demographic characteristics of these 765 nonincluded patients did not significantly differ from those included in the analysis for most of the demographic characteristics ($P > 0.05$, Table S1), except for the donor marginality (37.0% of expanded criteria donors in nonincluded recipients versus 42.6% in included ones, $P = 0.019$), the recipient age at transplantation (mean 50.2 years old vs. 51.6, $P = 0.028$), the possible recurrence of the initial renal disease (33.4% vs. 20.7%, $P < 0.001$), and the transplantation rank (77.5% first transplants vs. 83.1%, $P = 0.004$). These differences can be partly explained by the exclusion of many patients transplanted before 2010, when Luminex® technology was not routinely used.

Nine hundred and forty (940) patients were included in the study: 36.4% ($N = 342$) from Nantes, 35.3% ($N = 332$) from Lyon, and 28.3% ($N = 266$) from Paris Necker. The clinical and biological characteristics of this cohort are described in Table 1. Briefly, mean age at transplantation was 51.6 (±13.3) years, 59.8% were men, 83.1% were transplanted for the first time, 92.8% received a kidney transplant alone, 7.2% received a simultaneous pancreas–kidney transplantation, and 52.0% had at least 4 HLA-A-B-DR incompatibilities. A total of 35.3% of recipients were anti-HLA class I-sensitized and 37.2% were anti-HLA class II-sensitized. The distribution of patients with pretransplantation anti-AT1R immunization was the following: 41% ($n = 387/940$) patients had AT1R level >10 U/ml and only 8% (72/970) >17 U/ml. Sixty percent received antithymocyte globulin (ATG) as induction therapy (38.8% received IL-2 receptor antagonist). All patients except 2% received maintenance immunosuppression with calcineurin inhibitors (CNI) and mycophenolate mofetil (MMF) while 86.2% of recipients also received corticosteroids.

The mean pretransplant level of anti-AT1R-Abs in the whole cohort was 10.0 (±6.2) U/ml. This corresponds to the level previously reported above which recipients may be considered at risk of both ARE and graft failure [25]. Three hundred and eighty-seven (387) patients (41.2%) had a level exceeding 10 U/ml. Patient characteristics according to the pretransplant AT1R-Ab level are presented in Table 1. Patients with AT1R-Abs >10 U/ml were younger (50.0 vs. 52.8 years old, $P = 0.0016$), had more frequent autoimmune disease (33.6% vs. 26.2%, $P = 0.0145$), received more combined kidney and pancreas transplantation (9.8% versus 5.4%, $p = 0.0105$) and had a less frequent history of hypertension (82.4% vs. 88.8%, $P = 0.0054$). In contrast, it is also noticeable that at the time of transplantation, patients with pretransplantation AT1R-Abs ≤10 U/ml were more highly sensitized against HLA class I than patients with AT1R >10 U/ml (38.5% vs. 30.7%, $P = 0.0142$) and HLA class II (41.4% vs. 31.3%, $P = 0.0015$) using Luminex screening technology, and presented higher donor-specific antibodies (DSA, 18.7% vs. 10.9%, $P = 0.0011$) when a single antigen bead assay was performed. This information could help to unmask

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Table 1. Characteristics of the 940 kidney or kidney–pancreas recipients according to the pretransplant anti-AT1R-Ab level.

	All (n = 940)		AT1R <10 U/ml (n = 553)	AT1R ≥10 U/ml (n = 387)	
	NA	*N* (%) Mean ± SD	*N* (%) Mean ± SD	*N* (%) Mean ± SD	*P*-value
Recipient					
Age (years)	0	51.6 ± 13.3	52.8 ± 13.2	50.0 ± 13.4	0.0016
Male	0	562 (59.8)	331 (59.9)	231 (59.7)	0.9594
Body mass index (kg/m^2)	2	24.5 ± 4.3	24.5 ± 4.2	24.4 ± 4.4	0.7731
Recurrent disease	0	195 (20.7)	112 (20.3)	83 (21.4)	0.6569
History					
Transfusion	41	399 (44.4)	245 (46.6)	154 (41.3)	0.1157
Diabetes	0	219 (23.3)	125 (22.6)	94 (24.3)	0.5474
Hypertension	0	810 (86.2)	491 (88.8)	319 (82.4)	0.0054
Cardiac and/or vascular	0	329 (35)	207 (37.4)	122 (31.5)	0.0616
Dyslipidemia	0	361 (38.4)	218 (39.4)	143 (37)	0.4434
Hepatitis B or C	0	57 (6.1)	27 (4.9)	30 (7.8)	0.0697
Neoplasia	0	108 (11.5)	71 (12.8)	37 (9.6)	0.1209
Duration in dialysis (years)	124	3.59 (3.23)	3.75 (3.27)	3.35 (3.15)	0.0792
Donor					
Age (years)	0	52.4 ± 17.0	53.7 ± 17.4	50.6 ± 16.4	0.0054
Male	0	557 (59.3)	324 (58.6)	233 (60.2)	0.6195
Expanded criteria donor	0	411 (43.8)	270 (48.8)	141 (36.6)	0.0002
Transplantation					
Kidney transplant alone	0	872 (92.8)	523 (94.6)	349 (90.2)	0.0105
First transplantation	0	781 (83.1)	451 (81.6)	330 (85.3)	0.1347
ABDR incompatibilities ≥4	2	488 (52.0)	276 (50.1)	212 (54.8)	0.1570
Cold ischemia time (h)	6	18.1 ± 7.0	18.1 ± 7.1	18.1 ± 6.8	0.9223
Immunization					
Anti-HLA class I	0	332 (35.3)	213 (38.5)	119 (30.7)	0.0142
Anti-HLA class II	0	350 (37.2)	229 (41.4)	121 (31.3)	0.0015
CMV serology	12	592 (63.8)	350 (64)	242 (63.5)	0.8839
EBV serology	15	896 (96.9)	534 (97.8)	362 (95.5)	0.0496
Hepatitis C serology	43	24 (2.7)	13 (2.4)	11 (3.1)	0.5404
Anti-HBs antibodies	21	683 (74.3)	401 (73.6)	282 (75.4)	0.5343
HIV serology	1	16 (1.7)	8 (1.4)	8 (2.1)	0.4659
Treatment					
ATG	1	567 (60.4)	317 (57.4)	250 (64.6)	0.0270
Basiliximab	1	364 (38.8)	229 (41.5)	135 (34.9)	0.0410
CNI	1	921 (98.1)	539 (97.6)	382 (98.7)	0.2422
MMF	1	919 (97.9)	540 (97.8)	379 (97.9)	0.9112
Corticosteroids	1	809 (86.2)	471 (85.3)	338 (87.3)	0.3795

NA, not available; ATG, antithymocyte globulin; CMV, cytomegalovirus; CNI, calcineurin inhibitor; EBV, Epstein–Barr virus; HB, hepatitis B; HIV, human immunodeficiency virus; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin.

the group with the highest biological risk, but has to be interpreted carefully as single antigen bead Luminex technology assays were performed in only 28% of the 1043 patients of the whole cohort.

Description of post-transplantation events

The follow-up period extended until March 31, 2015. A clinically relevant acute rejection episode (cr-ARE), that is, requiring antirejection medication, was diagnosed in 249 recipients. The cumulative probability of cr-ARE was 22.5% at 1 year post-transplantation (95% CI from 19.9% to 25.4%). Among the 249 cr-ARE, 71.1% (N = 177) were classified as cellular and 28.9% (N = 72) as antibody mediated. A subclinical acute rejection episode (sc-ARE), that is, diagnosed on surveillance biopsies (planned at 3 and 12 months post-transplantation) without any sign of renal dysfunction and no established treatment, was diagnosed in 39 recipients.

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During the follow-up, 100 recipients returned to dialysis or had preemptive retransplantation. The cumulative probability of graft failure was 10.6% at 3 years post-transplantation (95% CI from 8.4% to 13.3%). Forty-nine recipients died with a functioning graft. The cumulative probability of death with a functioning graft was 5.7% at 3 years post-transplantation (95% CI from 4.0% to 8.1%).

Relationship between pretransplant anti-AT1R-Abs and ARE

Cumulative probabilities of cr-ARE according to the pretransplant anti-AT1R-Ab level are presented in Fig. 1. The results of the corresponding multivariable analysis are presented in Table 2. A pretransplant anti-AT1R-Ab level higher than 10 U/ml was not significantly associated with a higher risk of cr-ARE (adjusted HR = 1.04, 95% CI from 0.80 to 1.35, $P = 0.783$). Note that this association between pretransplant AT1R sensitization and risk of cr-ARE was not statistically different according to the HLA sensitization ($P > 0.05$).

Pretransplant anti-AT1R-Ab level greater than 10 U/ml was observed in 41.8% ($n = 74$) of the cellular cr-ARE and 40.3% ($n = 29$) of the antibody mediated cr-ARE. The estimated cumulative probabilities of cellular and antibody mediated cr-ARE were close, regardless the pretransplant anti-AT1R-Ab level (Fig. S3).

The results of the multivariable analysis of sc-ARE are presented in Table 3. A pretransplant anti-AT1R-Ab level higher than 10 U/ml was not significantly associated with a higher risk of sc-ARE (HR = 1.67, 95% CI from 0.86 to 3.26, $P = 0.130$), probably due to the low number of observed sc-ARE cases.

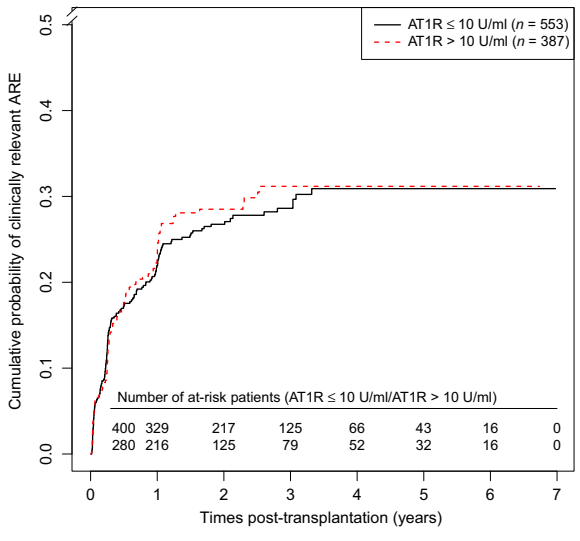

Figure 1 Cumulative probabilities of clinically relevant acute rejection episode (ARE) (Nelson–Aalen estimator, death and return in dialysis as competing events).

Relationship between pretransplant anti-AT1R-Abs and graft failure

Cumulative probabilities of graft failure according to the pretransplant anti-AT1R-Ab level are presented in Fig. 2. The multivariable analysis (Table 4) indicated that a pretransplant anti-AT1R-Ab level higher than 10 U/ml was not significantly associated with a decrease in the time to graft failure (adjusted HR = 0.86, 95% CI from 0.56 to 1.33, $P = 0.507$). This association was not statistically different according to the HLA sensitization ($P > 0.05$).

Considering that there is no consensus on the best threshold of the etiological risk of anti-AT1R before transplantation, we also assess the Final Cox Multivariable model for acute rejection episode and graft survival with by testing three into three categories of thresholds: ≤10, 10–17, and >17 U/ml. We showed that results were similar considering these three cutoffs (see Tables S2 and S3 and Figs S1 and S2).

Despite anti-HLA immunization and immunosuppressive management of combined kidney and pancreas transplantation were similar to single kidney transplantations, we performed the same analyses by removing patients who received combined kidney and pancreas transplantations. We showed a HR of 1.054 (95% CI from 0.804 to 1.383), 1.694 (95% CI from 0.843 to 3.405), and 0.884 (95% CI from 0.567 to 1.378) (Tables S1–S3), respectively, on the time to clinically relevant acute rejection episode, the time-to-event subclinical acute rejection episode and the time to graft failure. These results did not differ with the results observed when recipients of combined kidney and pancreas transplantations remained in the study as shown in Tables 2–4.

Relationship between pretransplant anti-AT1R-Abs and 1-year graft function

Since it is a new cohort of patients, we analyzed not only the etiological role of anti-AT1R-Abs on acute rejection and graft survival but also on 1-year creatinine level as a secondary end point. This analysis only concerned living patients with a functional graft at 1 year post-transplantation. The mean creatinine level for patients with a pretransplant anti-AT1R-Ab level lower than 10 U/ml was 145.0 μmol/l vs. 139.8 μmol/l for

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Table 2. Multivariable analysis of the risk factors associated with the time-to-clinically relevant acute rejection episode (Cox model stratified on center, deaths, and returns to dialysis were right-censored, *N* = 937, three patients removed because of missing values).

	HR	95% CI	*P*-value
Pretransplant anti-AT1R-Abs ≥10 U/ml	1.037	0.798, 1.349	0.783
Positive class I	1.361	1.001, 1.852	0.049
Positive class II	1.021	0.737, 1.416	0.899
HLA-A-B-DR incompatibilities ≥4	1.173	0.907, 1.516	0.224
Previous transplantation	1.158	0.787, 1.704	0.455
Donor age (for 1-year increase)	1.011	1.000, 1.021	0.040
Recipient age (for 1-year increase)	0.985	0.972, 0.997	0.017
Basiliximab as induction therapy	1.512	1.125, 2.033	0.006
History of diabetes	1.406	1.046, 1.891	0.024
History of hepatitis B or C	0.500	0.256, 0.977	0.042

Table 3. Multivariable analysis of the risk factors associated with the time-to-event subclinical acute rejection episode (Cox model stratified on center, deaths, returns to dialysis, and clinically relevant acute rejection episodes were right-censored, N = 938, two patients removed because of missing values).

	HR	95% CI	*P*-value
Pretransplant anti-AT1R-Abs ≥10 U/ml	1.675	0.860, 3.263	0.130
Positive class I	1.263	0.567, 2.814	0.567
Positive class II	1.210	0.516, 2.841	0.661
HLA-A-B-DR incompatibilities ≥4	1.038	0.541, 1.992	0.912
Previous transplantation	0.705	0.257, 1.934	0.497
Donor age (for a 1-year increase)	1.005	0.978, 1.032	0.722
Recipient age (for a 1-year increase)	0.992	0.960, 1.025	0.636

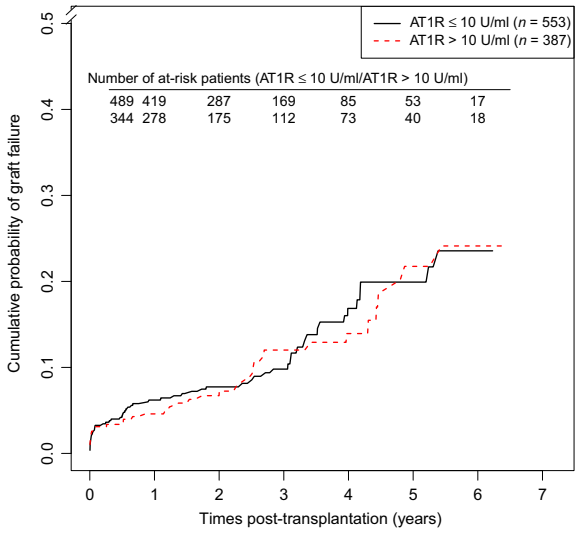

Figure 2 Cumulative probabilities of graft failure (Nelson–Aalen estimator, death as competing event).

patients with AT1R-Ab level ≥10 U/ml (two-sided *t*-test, $P = 0.2305$). By using a multiple linear regression for taking into consideration potential confounders ($P < 0.20$ in univariate analyses: gender of the donor and the recipient, cold ischemia time, age of the donor and the recipient, diabetes history, neoplasia history, cardiovascular history, hypertensive history, EBV serology of the recipient, autoimmune disease, and body mass index of the recipient at the transplantation), the adjusted difference was −2.2 µmol/l ($P = 0.5910$). Therefore, we did not demonstrate any significance difference between patients with pretransplantation anti-AT1R-Abs ≥ or those with anti-AT1R-Abs <10 U/ml. This last observation was in agreement with the non-significant results related to the graft survival.

Discussion

In this study performed on a large multicentric cohort of kidney-transplanted patients maintained under CNI and MMF treatment in the recent era of solid phase assay technology for anti-HLA-A-B-DR screening, we failed to show the etiological role of AT1R immunization before the transplantation on acute rejection, graft survival, and 1-year graft function. By taking into account classical confounders and particularly the pretransplantation anti-HLA immunization in the

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Table 4. Multivariable analysis of the risk factors with the time to graft failure (Cox model stratified on center, deaths were right-censored, $N = 930$, 10 patients removed because of missing values).

	HR	95% CI	*P*-value
Pretransplant anti-AT1R-Abs ≥10 U/ml	0.864	0.561, 1.331	0.507
Positive class I at transplantation	1.644	0.998, 2.707	0.051
Positive class II at transplantation	0.833	0.471, 1.474	0.530
HLA-A-B-DR incompatibilities ≥4	1.208	0.793, 1.840	0.379
Previous transplantation	0.963	0.526, 1.762	0.902
Donor age (for 1-year increase)	1.018	0.998, 1.038	0.070
Recipient age (for 1-year increase)	0.999	0.976, 1.024	0.966
Cold ischemia time (for 1-h increase)	1.024	0.993, 1.055	0.135
Cardiac history	1.945	1.275, 2.979	0.002
History of neoplasia	2.020	1.173, 3.478	0.011
Body mass index ≤18 kg/m^2	2.395	1.251, 4.584	0.008

multivariate analysis, this does not mean that we excluded a possible correlation of pretransplantation anti-AT1R immunization and graft outcome, but that we did not externally confirm our previous results as defined by Justice *et al.* and Royston *et al.* [35,36].

To assess the etiological role of pretransplantation anti-AT1R immunization, we focused on three main times events: clinical ARE, return to dialysis, death with functioning graft and built two Cox regression for each of these events of interest that are not interval-censored. In addition, considering several possible thresholds of AT1R antibodies without difference in the results, we decided to present our results with the threshold of 10 U/ml established in our initial study [25]. In contrast to our previous study, we did not perform an extended Cox model analysis, because there was no significant variation of the hazard ratio according to the post-transplantation time. We did not confirmed our results because we did not find evidence of a difference between patients with pretransplant anti-AT1R-Ab levels greater or less than 10 U/ml on the risk of ARE or on death-censored graft survival. Anti-HLA immunization seems as important in single kidney transplantation as in combined kidney and pancreas transplantation as shown by Cantarovich *et al.* [37] and also by Malheiro *et al.* [38]. Thus, the management of these kinds of grafts is similar to single kidney transplantation. In addition, we showed that removing from the analyses patients who received a combined kidney and pancreas transplantation did not modify the results allowing to pooled both types of transplantation. We voluntarily did not include living donor kidney recipients because they presented scarce immunologic events and surveillance biopsies were less routinely performed, potentially biasing the results through underestimation of subclinical rejection. One-third of the cohort was HLA-sensitized against class I or class II. This finding could be explained by the use of the high-sensitivity solid-phase assay technology performed for all the patients of our cohort within the 6 months before transplantation. We voluntarily limited our study to anti-HLA Luminex® screening technology. While there is no clear consensus on the best method, and interlaboratory variability exits, DSA and anti-HLA screening is currently the only technology admitted by the FDA in the USA. Finally, low-cost Luminex screening is routinely practiced for the large majority of current patients in most transplantation centers.

The incidence of clinically relevant ARE was 30% within the 3 years post-transplantation. This is much higher than in most current published studies [39] on patients under CNI and MMF as maintenance therapy. However, as mentioned in the demographic characteristics section, one-third of our patients were immunized against HLA antigens, a metric possibly resulting from the high sensitivity of the solid-phase assay screening test. In addition, we chose a broad definition for acute rejection, including borderlines as clinically relevant acute rejection episodes if they were considered for a treatment by each medical team (steroid boluses for cellular and IvIG/Plasma exchange/rituximab or bortezomib for Humoral rejection).

While we did not confirm our previous observations on this new cohort of patients, we observed the mean titer of anti-AT1R was the same (10 U/ml). The distribution of patients with a pretransplant sensitization against AT1R above 10 U/ml was close to those of our previous study [25] and that of Taniguchi *et al.* [15] who observed 17% of pretransplant AT1R sensitization >15 U/ml in a study including 351 patients. Other authors have used levels varying from 9.05 to 17 U/ml for AT1R-Abs, sometimes without any scientific basis.

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This cutoff for anti-AT1R antibody levels was recently also observed by Lee *et al.* [16], who showed in a multicenter observational Korean cohort with 166 consecutive kidney recipients that a titer of AT1R-Abs >9.05 U/ml was significantly associated with three times higher risk of biopsy-proven rejection but was not associated with a graft failure. Hernandez-Mendez *et al.* [40] have shown that pretransplant AT1R-Abs was associated with a lower eGFR (estimated glomerular filtration rate) at 12 months post-transplantation, but there was no significant difference for biopsy-proven acute rejection in the AT1R-Ab-positive group. A recent meta-analysis found a significant effect of AT1R-Abs on the allograft outcome, without considering neither the threshold of AT1R-Ab positivity nor the HLA-Ab detection technology. A new meta-analysis considering our new study could be interesting [24].

Our study has several limitations. Firstly, this new study was conducted on patients belonging to three different French transplantation centers. This allowed a greater number of recently transplanted patients to be included, which may be more representative of the total population of kidney transplant recipients. Nevertheless, a multicenter setting entails differences in patient profiles and practices between transplantation centers, especially in defining noncentralized anti-HLA immunization and histological ARE diagnosis that have already been shown to be highly center and operator dependent [41]. As already mentioned, we chose to conduct this study under "real-life" conditions, including all potential biases, and we stratified the analyses by taking into account the center effect. Nevertheless, because anti-AT1R immunization was the principal topic of interest, and to avoid potential technological bias, anti-AT1R measurements were centralized and performed blinded at the Nantes hospital using the commercial ELISA assays (One Lambda®).

Secondly, the multivariable analysis indicated that a pretransplant anti-AT1R-Ab level greater than 10 U/ml tended to increase the risk of subclinical ARE (HR = 1.67, $P = 0.13$), but this association is probably overestimated because the time of sc-ARE was not exactly known (diagnosis made on the histological results of the surveillance biopsy) and some sc-ARE were never identified. If we assume that all cr-ARE result from sc-ARE, we should have observed more than 249 recipients with sc-ARE during the follow-up; however, there were only 39. Finally, we did not analyze post-transplantation anti-AT1R immunization at the time of acute rejection since our hypothesis was to assess the etiological role of AT1R immunization before transplantation. Some studies have reported a significant association between *de novo* anti-AT1R-Abs only and kidney transplant recipient outcomes such as graft dysfunction or risk of ARE [15]. In recent studies, Cuevas *et al.* [42] showed in a cohort of 115 living donor kidney transplant recipients that patients positive for pre- or post-transplantation AT1R-Abs (>17 U/ml) mirrored recipients without any anti-HLA or non-HLA antibodies with a follow-up of 1 year.

As already mentioned, in this study, we contradicted our previous observation [25] and thus we did not confirm the etiological role of AT1R immunization before the transplantation on acute rejection and graft failure as recently. This difference could be explained by the recent cohort that gathered patients from our own center (without overlap with our previous cohort) and patients from two other French centers. In addition, patients of the recent cohort presented differences in demographic characteristics and were also tested for anti-AT1R before the transplantation with a dosage-standardized Elisa kit. Patients were all treated with Tacrolimus and MMF and were screened by Luminex technology for anti-HLA immunization before the transplantation, differing from the patients included in our previous cohort. In addition, another explanation to the discrepancy could be that in the new study, we considered subclinical rejection on 3-month and 1-year surveillance biopsies; this was not the case in our previous study. Moreover, patients of the new study were recruited until 2012, which means that a proportion were followed for only 3 years, partially explaining the difference between both studies. Finally, the distribution of AT1R antibody levels was different in our historical cohort with 47% ($n = 283/599$) of patients presented a level of AT1R >10 U and 19% ($n = 111/599$) >17 U compared with the present study where we observed that 41% ($n = 387/940$) patients had AT1R level >10 U before the transplantation and only 8% (72/970) >17 U. This difference in the distribution of the anti-AT1R immunization could also participate to the discrepancy between the present study and our historical study.

We believe that this negative result may help to participate in better assessing the etiological role of non-HLA sensitization against AT1R-Abs on acute rejection and long-term chronic graft failure [43] in incident kidney transplantation recipients.

Authorship

CD: performed research/study, analyzed data and wrote the manuscript. FG: designed research/study, performed

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research/study, analyzed data, wrote the manuscript. DA: analyzed data. EM: analyzed data Conflict of interest: Chiesi®. KT-L: collected data. FLB: collected data. MR: performed research/study. PG: collected data. SM-C: performed research/study. YF: designed research/study, performed research/study, analyzed data and wrote the manuscript. MG: designed research/study, performed research/study, analyzed data and wrote the manuscript. Conflict of interest: Chiesi® Roche® Novartis®.

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Conflicts of interest

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Comparison of characteristics of kidney transplant recipients included and not included in analysis among eligible recipients.

Table S2. Final Cox Multivariable model for with the time to clinically relevant acute rejection episode with anti AT1R immunization before the transplantation considered into three categories (≤10, 10–17, >17 U/ml).

Table S3. Final Cox Multivariable model for with the time to graft failure with anti AT1R immunization before the transplantation considered into three categories (≤10, 10–17, >17 U/ml.

Table S4. Multivariable analysis of the risk factors associated with the time to clinically relevant acute rejection episode (Cox model stratified on center, deaths and returns to dialysis were right-censored, $N = 869$ patients with a single kidney transplant (no pancreas), 3 patients removed because of missing values).

Table S5. Multivariable analysis of the risk factors associated with the time to sub-clinical acute rejection episode (Cox model stratified on center, deaths, returns to dialysis, and clinically relevant acute rejection episodes were right-censored, $N = 870$ patients with a single kidney transplant (no pancreas), 2 patients removed because of missing values).

Table S6. Multivariable analysis of the risk factors with the time to graft failure (Cox model stratified on center, deaths were right-censored, $N = 863$, 9 patients removed because of missing values).

Figure S1. Cumulative probabilities of clinically relevant acute rejection episode with anti AT1R immunization before the transplantation considered into three categories (≤10, 10–17, >17 U/ml).

Figure S2. Cumulative probabilities of graft failure with anti AT1R immunization before the transplantation considered into three categories (≤10, 10–17, >17 U/ml).

Figure S3. Cumulative probabilities of clinically relevant acute rejection episode (ARE) according to the pre-transplant anti-AT1R-Abs level and by using the Nelson-Aalen estimator: (a) cellular ARE, (b) antibodies mediated ARE.

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