

Received: 2022.05.17


Accepted: 2022.07.15

Available online: 2022.08.01

Published: 2022.08.12

Retrospective Study from a Single Center in Romania of 347 Renal Transplant Patients Treated with Tacrolimus, Mycophenolate, and Steroids to Evaluate the Association Between Anti-HLA Antibodies and 5-Year Graft Survival

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Data Interpretation D
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Financial support: None declared

Conflict of interest: None declared

Background: Kidney transplantation is the most recommended treatment in chronic kidney disease. The recipient's immune system reacts to a kidney graft as to an alloantigen by producing antibodies (anti-human leukocyte antigens [HLAs]). Although immunosuppressive therapy is used to overcome this problem, the long-term survival of a kidney graft after 5 years remains low. This retrospective study from a single center in Romania of 347 renal transplant patients treated with tacrolimus, mycophenolate, and steroids aimed to evaluate the association between anti-HLA antibodies and 5-year graft survival.

Material/Methods: Anti-HLA antibodies were screened and identified using the Luminex method, while tacrolimus levels were monitored using the chemiluminescent assay.

Results: Twenty-seven patients had pre-existing anti-HLA antibodies, while 320 patients did not. Of the 320 patients, 15% developed anti-HLA antibodies following kidney transplantation. The inpatient minimum blood level of tacrolimus (cut-off value: 4.6 ng/mL) after transplantation was significantly associated with the risk of de novo anti-HLA antibodies ($P < 0.001$). In patients with or without de novo anti-HLA antibodies, the 5-year allograft survival rate was 77.1% vs 90.8% ($P = 0.004$). After Bonferroni correction, donor age ($P = 0.001$), and donor type ($P < 0.0001$) were statistically associated with the risk of allograft rejection.

Conclusions: This study showed that anti-HLA antibodies at 5 years after kidney transplantation were significantly associated with graft failure. The findings support previous studies and indicate that monitoring of anti-HLA antibodies should be considered in patients with renal transplant.

Keywords: Allografts • Graft Rejection • Graft Survival • HLA Antigens • Kidney Transplantation • Tacrolimus

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Background

Kidney transplantation significantly improves patient quality of life and allows socio-professional reintegration [1]. Kidney grafts can be transplanted either from brain-dead or living donors. Usually, kidney transplantation is possible when there is no anti-human leukocyte (HLA) donor-specific antibody incompatibility because of a high risk of hyperacute or acute kidney graft rejection [2-4]. However, this approach is associated with a reduced chance of highly immunized patients getting transplants that may prolong their life [5]. Orandi et al observed that patients with HLA incompatibility transplanted from living donors have a longer life expectancy than those who remain on a brain-dead donor waiting list [2]. In the case of transplantation with HLA incompatibility from a cadaveric donor, Krishnan et al have shown that long-term survival is similar to that of patients transplanted from brain-dead but HLA-matched donors [3].

Despite advances made in immunosuppressant development, not all patients have functional renal allografts after approximately 5 years [6]. The recipient's immune system responds to the kidney transplant by producing anti-HLA antibodies, resulting in humoral rejection of the kidney allograft [7] and different outcomes in terms of graft survival rate [8-10]. Analyzing the dynamics of donor-specific anti-HLA antibodies in the immediate posttransplant period, Phillipott et al identified an increase in anti-HLA antibody levels in the first 2 weeks after kidney transplantation, followed by a slight decrease [10]. Reduction in anti-HLA antibodies could be due to strong immunosuppression, their clearance from the circulation by plasmapheresis [11], or prevention of CD20 B lymphocytes from turning into plasma cells using monoclonal antibodies, such as rituximab [12].

To avoid rejection, in addition to low-resolution HLA typing of both recipient and donor for all loci, pretransplantation screening for anti-HLA antibodies and the crossmatch test are performed [13,14]. Crossmatch, which is used to detect the presence of donor-specific alloantibodies in patients' serum, eliminated the phenomenon of hyperacute renal allograft rejection but not chronic rejection [15]. Extensive anti-HLA antibody assessment allowed us to practice more virtual crossmatch in order to shorten the time [16].

Currently, Luminex technology is the most widely used method for the detection and identification of anti-HLA antibodies with high sensitivity (both before and after kidney transplantation) [17,18] and crossmatch testing (before kidney transplantation) [19]. Unlike enzyme-linked immunoassay, Luminex technology allows the simultaneous analysis of a very large number of analytes (up to several hundred) from a small sample volume, with applications in solid organ transplantation, hematopoietic stem cell transplantation, and transfusions [20], infectious diseases [21], autoimmune diseases [22], and cancer [23].

T-cell- and antibody-mediated rejection are 2 main histopathological findings in patients with graft rejection. While T-cell-mediated rejection has little effect on graft survival, antibody-mediated rejection presents microcirculation lesions because of mediated inflammatory cytokine, such as interferon gamma, affecting the graft functions [24].

In our opinion, the factors that influence kidney transplant outcomes are complex. Therefore, this retrospective study from a single center in Romania of 347 renal transplant patients treated with tacrolimus, mycophenolate, and steroids aimed to evaluate the association between anti-HLA antibodies and 5-year graft survival.

Material and Methods

A total of 347 patients (228 men and 119 women aged 18 to 65 years) who underwent kidney transplantation at Fundeni Clinical Institute were included in the analysis based on the following inclusion criteria: age >18 years, no positive crossmatch at the time of the kidney transplantation procedure, screening for the anti-HLA antibody appearance before and after transplantation, no previous donor-specific antibodies, or treatment with tacrolimus. No patient was excluded after applying these criteria. After the kidney transplant, all patients received triple immunosuppressive medication based on tacrolimus, mycophenolate, and prednisone. From the time of kidney transplantation until death or the end of the study period, all patients were monitored for anti-HLA antibodies, immunosuppressive regimens levels, and appearance of acute or chronic graft rejection.

Methods

Our research was undertaken with the approval of the Ethics Committee of the Fundeni Clinical Institute (no. 77883 from 2021), and written informed consent was obtained from all patients. According to the written informed consent, patients who agreed to be included in the present study were free to withdraw their consent without being charged, whenever they decided.

The presence of preformed and de novo anti-HLA antibodies was retrospectively analyzed in serum samples before transplantation and at 1 to 5 years after transplantation.

All the recipients had a compatible donor based on HLA genes. None of the recipients had preformed donor-specific antibodies.

Class I and class II HLA antibodies were screened with LABScreen Mixed12, lot 023 (One Lambda, Inc., Canoga Park, CA, USA) on FlexMap3d (Luminex Corporation, USA). The positive screening was followed by the identification of anti-HLA antibodies with

LS1A04 lot 013 (for class I) and LS2A01 lot 014 (for class II) kits from One Lambda. Screening and identification of class I and class II HLA antibodies were carried out following the manufacturer guidelines, and sample data were collected using xPONENT software version 4.3 (Luminex Corporation, USA) [25] and analyzed with HLA Fusion version 4.3 (One Lambda, Inc.). Based on a mean fluorescence intensity cut-off value of 1500, the anti-HLA antibody was considered either positive or negative. The identified anti-HLA antibodies were then compared with the donor's HLA, which allowed us to identify donor-specific antibodies and non-donor-specific antibodies. Also, we were able to specify if the identified HLA antibodies were preformed (formed before any kidney transplant) or de novo (appeared after a kidney transplant).

Tacrolimus levels in the blood were determined using tacrolimus reagent kits and a chemiluminescent magnetic microparticle immunoassay (Architect i2000, Abbot).

Acute or chronic graft rejection was identified by kidney biopsies that were analyzed based on the Banff criteria [26].

Statistical Analysis

SPSS version 26 (IBM Corp, Armonk, NY, USA) was used for all statistical analyses. Categorical variables are presented as counts and percentages, while continuous variables are reported as means and standard deviations. For categorical and continuous variables, Fisher's exact or the chi-square test were used to compare patients with kidney transplant characteristics in groups differentiated by the presence or absence of anti-HLA antibodies.

Variability of the blood level of tacrolimus was estimated by calculating the coefficient of variation using the following equation: coefficient of variation (%)=(standard deviation/mean concentration)×100 [27]. The cut-off values for the inpatient minimum trough level of tacrolimus at 1 to 5 years after transplantation for predicting the production of anti-HLA antibodies were determined using receiver operating characteristic (ROC) curve analysis. Bonferroni correction was also applied in the case of multivariate analysis.

Data on transplant survival were recorded and stored until either the time of the patient's death or graft loss. Kaplan-Meier analysis was used to plot the survival rates of renal grafts, and the log-rank test was used to compare the groups. A *P* value <0.05 was considered statistically significant.

Results

A total of 347 patients who underwent kidney transplantation between January 2013 and December 2016 were included in

the analysis. Based on pre-existing HLA antibodies, all patients were divided into 2 groups: patients with pre-existing HLA antibodies and patients without pre-existing HLA antibodies. In both groups, chronic glomerulonephritis was the leading cause of chronic renal insufficiency.

In the group without HLA antibodies, 36.9% of patients were women, 63.1% were men, and the average age was 46.7±11.6 years. The follow-up period for the patients included in this group was 4.8±0.7 years. Most kidney grafts were obtained from living-related donors (62.8%).

In the group with previous HLA antibodies, 37.0% of patients were men, and 63.0% were women, with an average patient age of 40.7±12.7 years. The follow-up period for the patients included in this group was 3.4±1.4 years. Most kidney grafts were obtained from cadaveric donors (55.6%).

At 5 years after transplantation, 48 (15%) patients with kidney transplants had de novo anti-HLA antibodies (Table 1). Twenty-two (6.87%) patients had de novo class I anti-HLA antibodies. De novo class II anti-HLA antibodies were observed in 37 (13.07%) patients (Table 2).

As shown in Table 3, antibody-mediated rejection was observed in 10 (20.83%) of the 48 patients from the group with de novo anti-HLA antibodies and in 7 (25.93%) of the 27 patients from the group with pre-existing anti-HLA antibodies. T-cell-mediated rejection was diagnosed in 13 (48.15%) of the 27 patients from the group with pre-existing anti-HLA antibodies and in 19 (39.58%) of the 48 patients from the group with de novo anti-HLA antibodies. Analysis of the possible outcomes showed that T-cell-mediated rejection and antibody-mediated rejection were not related to de novo anti-HLA antibodies.

The association of tacrolimus blood level with de novo HLA antibody production and rejection was studied. The ROC test was used to determine the cut-off value of the tacrolimus blood level. Only a tacrolimus blood level lower than 4.6 ng/mL was associated with de novo HLA antibody positivity. The area under the ROC curve (used to determine the cut-off values of 4.6 ng/mL; Figure 1) was 0.85, with a 95% confidence interval (CI) of 0.80 to 0.88 and *P*<0.05 (Figure 2). The tacrolimus blood level (*P*=0.2376) was not associated with the rate of allograft rejection.

We also analyzed the possible association of donor age, donor type, recipient age, and de novo HLA antibodies with kidney rejection in both groups. In patients without previous sensitization, only donor age (odds ratio [OR]=1.07, *P*=0.001) and donor type "cadaveric donor" (*P*<0.0001) were associated with higher rates of allograft rejection, while recipient age (*P*=0.048), after applying Bonferroni correction, did not remain

Table 1. Characteristics of patients with a kidney transplant.

	Patients with pre-existing HLA antibodies	Patients without pre-existing HLA antibodies	P value
n (%)	n=27 (7.78%)	n=320 (92.22%)	
Age (mean±SD)	40.96±11.65	46.72±11.61	0.0247*
Sex			0.002**
Male n (%)	10 (37.03%)	219 (68.44%)	
Female n (%)	17 (62.97%)	101 (31.56%)	
Donor type			0.311
Cadaveric donor n (%)	15 (55.55%)	139 (43.44%)	
Living related donor n (%)	12 (44.45%)	181 (56.56%)	
Tacrolimus (mean±SD)	5.9±1.55	6.22±1.68	0.0174***

*, a significant association, between ages and presensitization; **, a significant association, between sex and presensitization; ***, a significant association, between tacrolimus and presensitization. HLA – human leukocyte antigen.

Table 2. Prevalence of posttransplant de novo HLA antibodies.

HLA antibodies	Patients n (%)
Total	320 (100%)
Not detected	272 (85%)
Detected	48 (15%)
Class I	
Not detected	298 (93.13%)
Detected	22 (6.87%)
Class II	
Not detected	283 (86.93%)
Detected	37 (13.07%)

HLA – human leukocyte antigen.

statistically significant. In patients with previous sensitization, only donor type “living” ($P<0.001$) was associated with higher rates of allograft rejection, while donor age ($P=0.06$) and recipient age ($P=0.481$) was not.

In the groups of patients without previous anti-HLA antibodies, the death-censored renal graft survival rates were 95.8%

Table 3. Outcome in patients with or without de novo HLA antibodies.

	With preexisting HLA antibodies n=27	With de novo HLA antibodies n=48	P value
T-cell-mediated rejection	13 (48.15%)	19 (39.58%)	0.627
Antibody-mediated rejection	7 (25.93%)	10 (20.83%)	0.775

HLA – human leukocyte antigen.

vs 98.9% (1 year), 89.6% vs 97.1% (2 years), 85.4% vs 95.2% (3 years), 81.3% vs 92.6% (4 years), and 77.1% vs 90.8% (5 years) (Figure 3, Table 4). The graft survival rate was significantly lower in recipients with de novo anti-HLA antibodies 5 years after transplantation ($P=0.004$, hazard ratio=4.0251) (Tables 5, 6).

In the groups of patients with previous anti-HLA antibodies, the death-censored renal graft survival rates were 96.3% (1 year), 92.6% (2 years), 88.9% (3 years), 77.8% (4 years), and 74.1% (5 years).

When comparing both patient groups, we observed that there was a significant difference between survival distributions 5 years after kidney transplantation ($P=0.0286$) (Figure 4).

Only 4 patients with previous anti-HLA antibodies and 8 patients without previous HLA sensitization developed donor-specific HLA antibodies after kidney transplantation. In our patients, there was no significant difference between survival distributions 5 years after kidney transplantation in patients with donor-specific antibodies and those without ($P=0.754$).

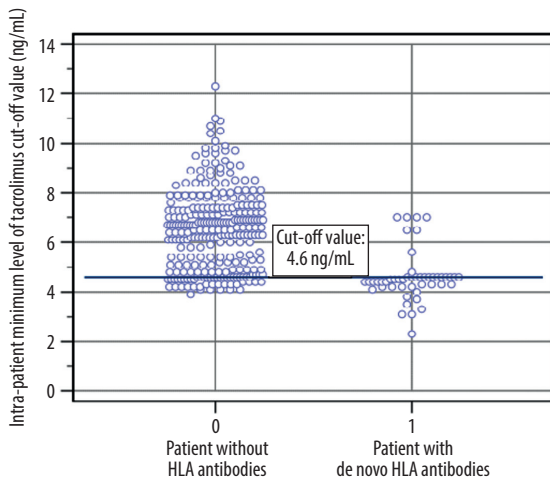


Figure 1. Cut-off value for the appearance of de novo human leucocyte antigen (HLA) antibodies.

Discussion

After kidney transplantation, the incidence of patients with anti-HLA antibodies was relatively small (15%), similar to the incidence observed in a study of 2185 patients by Terasaki and Ozawa (14.7%) [28]. The administration of calcineurin inhibitors in the early posttransplantation period could protect against the development of de novo anti-HLA antibodies [29,30]. However, studies have shown that a lower tacrolimus trough level is associated with the development of de novo anti-HLA antibodies [29,31,32],

Marked inpatient variability in tacrolimus trough levels may be a major risk factor for de novo anti-HLA antibodies [33-35]. In our clinical experience, the inpatient minimum level of tacrolimus (cut-off value: 4.6 ng/mL) after transplantation was associated with the production of de novo anti-HLA antibodies. Two other studies identified tacrolimus trough levels associated with HLA antibody production in different types of organ transplantation. Kaneku et al [36] reported that tacrolimus trough levels <3 ng/mL could be associated with de novo anti-HLA antibodies after liver transplantation. Furthermore, tacrolimus trough levels lower than 3.2 ng/mL after kidney transplantation were found to be linked with de novo anti-HLA antibody presence in a center for kidney disease and transplantation in Japan [37]. Despite health improvements in patients with chronic kidney disease, there are many complex immune aspects that still remain to be solved, such as de novo anti-HLA antibodies and immunosuppressive treatment adverse effects [38].

Five years after kidney transplantation, our patients with de novo anti-HLA antibodies had significantly lower long-term

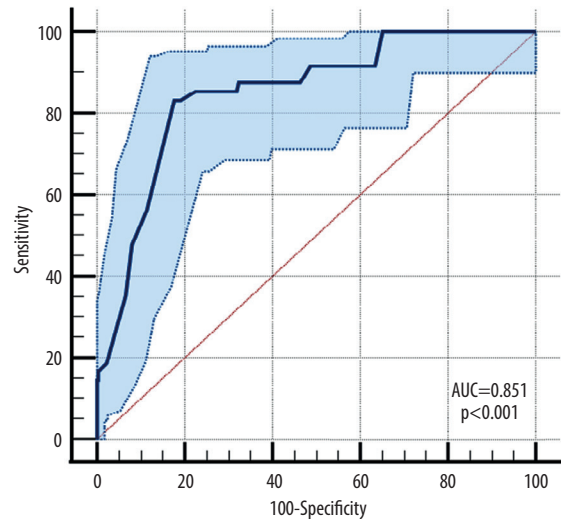


Figure 2. Area under curve (AUC). Because P value is less than 0.05, the area under the receiver operating characteristic (ROC) curve is substantially different from 0.5, implying that the laboratory test has the capacity to identify patients at risk to develop human leucocyte antigen (HLA) antibodies.

death-censored graft survival than those without HLA antibodies ($P < 0.05$). Also, de novo anti-HLA antibodies have been shown to have a significant clinical impact on long-term graft survival in other studies [39-41]. Terasaki et al [41] observed that since 2003 there has been a better rate of graft survival. The graft survival rates were also lower in patients with de novo HLA antibodies than in patients with no anti-HLA antibodies in the study conducted by Süsal et al [42]. These findings could be related to the use of immunosuppressive drugs and improved monitoring guidelines for the prevention and management of posttransplant complications (eg, immunosuppressive drug toxicity, viral infections, diabetes, malignancy, low level of immunosuppressive drugs [43-45], and anti-HLA antibody appearance).

In the present study, between patient groups, there was a significant difference between survival distributions 5 years after kidney transplantation ($P = 0.0286$). However, de Sousa et al did not find any significant differences in terms of graft survival in patients with and without previous HLA sensitization [8].

There are also many factors that can affect the outcomes of kidney transplantation. One such factor is donor age: older age of the donor can adversely affect immediate graft function and long-term outcomes [46]. In our study, age was not linked with HLA antibody production, probably because recipients over 65 years of age are still uncommon in Romania, but age was associated with graft loss 5 years after kidney transplantation.

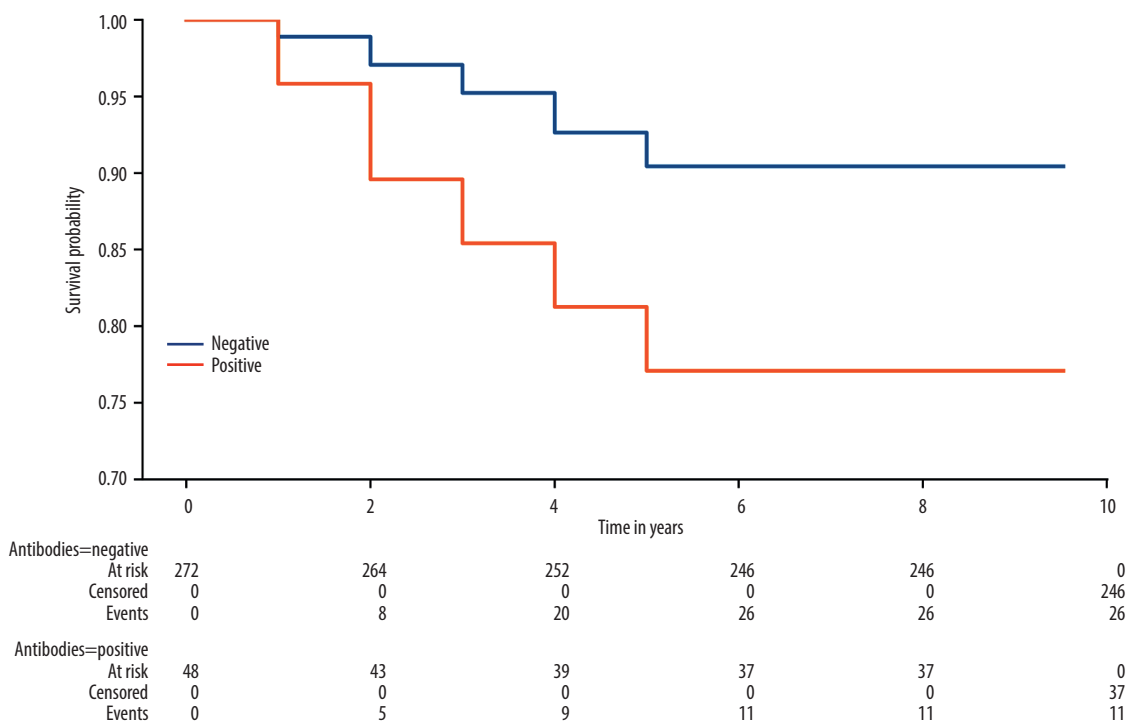


Figure 3. The survival graft rates in patients without preformed anti-human leucocyte antigen (HLA) antibodies.

Table 4. Graft survival rates in patients without preexisting anti-HLA antibodies.

Survival time (years)	Factor				Overall	
	Negative HLA antibodies		Positive de novo HLA antibodies		Survival proportion	Standard error
	Survival proportion	Standard error	Survival proportion	Standard error	Survival proportion	Standard error
1	0.989	0.00633	0.958	0.0288	0.984	0.00693
2	0.971	0.0102	0.896	0.0441	0.959	0.011
3	0.952	0.0129	0.854	0.0509	0.938	0.0135
4	0.926	0.0158	0.813	0.0563	0.909	0.016
5	0.908	0.0175	0.771	0.0607	0.888	0.0177
Endpoint: Observed n	25		11			
Expected n	30.9		5.1			
Observed/Expected	0.8082		2.171			

HLA – human leucocyte antigen. The survival proportions for the groups with and without de novo HLA antibodies, as well as the overall survival proportion, are presented at each year from the moment of transplantation.

Cadaveric donor was another independent factor of the graft loss 5 years after kidney transplantation. Increased recipient age (older than 65 years) or cadaveric donors were also identified as independent risk factors for the development of chronic renal allograft failure in White patients [46,47]. In contrast,

age had no effect on 5-year graft survival in a cohort of 627 kidney transplant patients [48].

The immune system has developed strategies for determining whether cells are “self” or “non-self” (foreign) by evaluating

Table 5. Comparison of survival curves (log-rank test).

Chi-squared	8.2623
Degree of freedom	1
Significance	$P=0.004$

Statistically, the 2 survival curves differ significantly ($P<0.05$). Thus, development of de novo human leucocyte antigen (HLA) antibodies has a significant influence on survival time of kidney graft.

HLA genes which are extremely polymorphic and differ from one individual to another [36].

HLA proteins play key roles in immune function, including antigen presentation proteins or complement system components [49]. For successful kidney transplantation, HLA markers must be matched between the organ donor and recipient [50].

When the immune system detects foreign antigens, a series of processes involving T cells and B cells occur that can lead to anti HLA antibodies production [51]. The appearance of HLA alloantibodies is attributed to the exposure of the recipient's immune system to foreign HLA molecules in multiple pregnancies, transfusions, or previous transplants. Antibody responses begin with the activation of B cells, with specific immunoglobulin-like surface receptors that bind to epitopes

Table 6. Hazard ratios with 95% confidence interval in patients without preformed anti-HLA antibodies.

Factor	Negative HLA antibodies	Positive HLA antibodies
Negative HLA antibodies	–	4.0251 1.5574 to 10.4032
Positive HLA antibodies	0.2484 0.09612 to 0.6421	

HLA – human leucocyte antigen. In the non-HLA previous sensitized group, the estimated relative risk of the event of kidney graft loss occurring in the subgroup with de novo anti-HLA antibodies is 4.0251 greater than in the subgroup with no HLA antibodies.

on immunizing antigens [52]. Their interactions with helper T cells result in proliferation, affinity maturation, and immunoglobulin class switching, and eventually, differentiation into antibody-producing plasma cells [53]. The immunogenetic relationship between the antibody producer and the immunizing allele affects the antibody response to a mismatched epitope [54]. Previous research has revealed that antibodies to HLA are linked to poor kidney transplant outcomes [55,56]. However, this does not fully explain why some patients have a functional graft even after 5 years, while others, transplanted

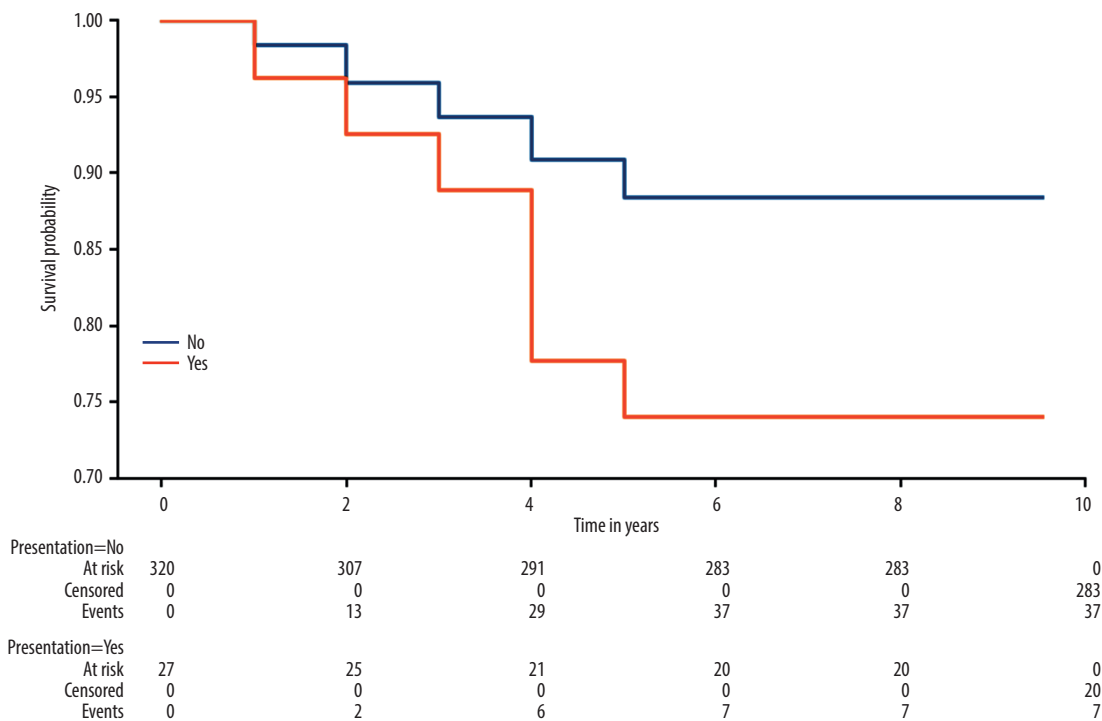


Figure 4. Survivals rate according to status of human leucocyte antigen (HLA) immunization before kidney transplantation.

under the same conditions, reject the graft. Many transplant centers use desensitization methods, such as plasmapheresis, intravenous immunoglobulin, and bortezomib, to remove anti-HLA antibodies [57], but these efforts have often had limited success and are expensive.

The major limitations of our study were the small number of patients and the short follow-up time. As a result, changes in transplant procedures and immunosuppression over time may have led to an overestimation of survival in our study [58]. Other limitations are represented by the kits used to analyze the anti-HLA antibodies that are suitable for identifying the target protein but lack the possibility of quantifying the antibody levels and the mean fluorescence intensity cut-off value, which may differ from values used in other laboratories. However, this is the first report of the association between immunosuppressive therapy and the production of de novo anti-HLA antibodies after transplantation and graft survival in the Romanian population.

Future studies should address the above-mentioned limitations to determine if increasing the number of patients and follow-up time will confirm our findings.

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Conclusions

This study showed that anti-HLA antibodies 5 years after kidney transplantation were significantly associated with graft failure. The findings support previous studies and indicate that monitoring of anti-HLA antibodies should be considered in patients undergoing renal transplantation.

Acknowledgements

We thank Dr. Calenic Bogdan from “Carol Davila” University who helped us to elaborate the responses for the reviewers.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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