Evaluation of antibodies directed against two GPCRs, anti-AT1R and anti-ETAR, on kidney transplant outcome: Own experience and review of the topic

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RESEARCH ARTICLE

Evaluation of Antibodies Directed Against Two GPCRs, Anti-AT1R and Anti-ETAR, on Kidney Transplant Outcome

Jaouad El kaaoui El band[1], Santiago Llorente[2], Pedro Martinez-Garcia[1], Rafael Alfaro[1], Victor Jimenez-Coll[1], Francisco Boix[1], José A. Galián[1], Helios Martinez-Banaclocha[1], Carmen Botella[1], María R. Moya-Quiles[1], Alfredo Minguela[1], Isa Legaz[3,#] and Manuel Muro[1*,#]

[1]*Immunology and* [2]*Nephrology Services, University Clinical Hospital Virgen de la Arrixaca-Biomedical Research Institute of Murcia (IMIB), Murcia, Spain;* [3]*Department of Legal and Forensic Medicine, Biomedical Research Institute (IMIB), Regional Campus of International Excellence "Campus Mare Nostrum", Faculty of Medicine, University of Murcia, 30100 Murcia, Spain*

Abstract: ***Background:*** The role of an alloimmune response against non-self-antigens is established in organ transplantation. HLA incompatibilities are mainly responsible for this recognition between donor and recipient, but they may also be involved in the reactivity against other alloantigens expressed on the allograft resulting from an autoimmune response developed against self-antigens.

Objective: Our study aimed to determine the presence of non-anti-HLA antibodies (anti-AT1R and anti-ETAR) in sera from patients with end-stage renal disease, who underwent kidney transplantation in pre- and post-transplantation samples to study their influence on the development and evolution of acute humoral rejections and DSAs.

Methods: Antibodies (Abs) against two G protein-coupled receptors (GPCRs), angiotensin II type 1 receptor (AT1R) and endothelin-1 type A receptor (ETAR), have been detected in the sera of transplant recipients, who experience allograft dysfunction, patients with coronary heart disease, marginal hypertension and refractory, vascular lesions, myocardial hypertrophy and chronic inflammatory diseases, such as atherosclerosis or sclerosis.

Results: Kidney graft recipients were monitored for anti-ETAR, -AT1R, and -HLA Abs in pre-and post-transplant evolution, and anti-AT1R and/or -ETAR Abs were detected in 24% of recipients (22.4% with anti-AT1R Abs and 9.8% with anti-ETAR Abs). Due to acute humoral rejection, Graft loss was detected in 6.4% of patients with anti-GPCRs non-HLA Abs, and 3.2% had DSA anti-HLA Abs. In this research, we have described how the function of the anti-GPCRs autoAbs and how these Abs that activate GPCRs could influence graft outcome.

Conclusion: In conclusion, there is a high association of non-HLA anti-GPCRs Abs levels with reduced kidney function after transplantation, especially in the presence of DSA anti-HLA Abs. Although more studies are needed, anti-AT1R and anti-ETAR antibodies may be helpful biomarkers that allow the risk of graft loss to be assessed.

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1. INTRODUCTION

The main immunological factor of failure or graft loss in kidney transplant patients has classically been related to the presence of circulating donor-specific HLA antibodies (Abs) (DSA). However, in recent years, several studies have highlighted the importance of non-Ac anti-HLA in graft loss [1-4], especially antibodies against two G protein-coupled receptors (GPCRs), endothelin-1 receptor type A (anti-ETAR) and against angiotensin II receptor type 1 (anti-AT1R).

In this sense, these non-anti-HLA-specific antibodies directed against anti-AT1R and anti-ETAR on vascular cells may be responsible for antibody-mediated rejection (AMR) in vascular cells in the absence of DSA anti-HLA antibodies in kidney transplant patients [2]. Interestingly, not in all

*Address correspondence to this author at the Immunology Service. Instituto Murciano de Investigación Biosanitaria (IMIB), Hospital Clínico Universitario Virgen de la Arrixaca (HCUVA), Murcia, Spain; Tel: +34 968 369599; Fax: (34) 968-349678; E-mail: manuel.muro@carm.es
[#]*The order of these authors is equal and is arbitrary*.

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cases the recipients that present positive antibodies against AT1R and/or ETAR suffer kidney graft damage associated with their presence.

On the other hand, not only do these antibodies seem to play a role in organ transplantation but it has been established in the literature that these two types of anti-ATR1 and anti-ETAR antibodies may be involved in harmful pathological processes in various diseases and also in the development of grafts loss due to rejection. For this reason, the routine post-transplant follow-up may be interesting, and in the case of its appearance, to be able to discuss therapeutic approaches to prevent the eventual harmful effects in the long run and to improve the correct evolution of the kidney transplant, where humoral and cellular processes play important roles [3, 5-7]. The need to be able to evaluate and avoid the development of immunological risk factors for graft loss demands the investigation of the other transplants to direct an overall vision between allogenicity, autoimmunity, and the immune response of tolerance versus rejection where receptor interactions with donor organ are maintained during the functioning of the transplanted organ and, where appropriate, elucidate alternative optional treatments.

This research on kidney transplantation will add more debate to the existing one on this matter.

1.1. G Protein-coupled Receptors (GPCRs)

Autoantibodies that cause the activation of GPCRs have been reported as associated with cardiovascular disease, pre-eclampsia, senescence and aging, arterial hypertension, autoimmune model pathologies, tumor pathologies, and allograft dysfunction. Next, we will focus on ATR1 and ETAR and their transplantation role.

1.1.1. Angiotensin ll Receptors (ATR)

Angiotensin ll receptors (ATR) are receptors composed of 2 subtypes (AT1R and AT2R). Its structure consists of seven transmembrane alpha-helices, linked by three extracellular and three intracellular loops, associated with G proteins [8, 9]. The human AGTR1 gene encoding the AT1R subtype is found on the long arm of chromosome 3 (3q24) and is made up of four exons. AT1R receptor is distributed throughout the body, tissues, and organs, such as blood vessels, heart, liver, and kidney. Its principal function is carried out through the renin-angiotensin-aldosterone system, activated by its ligand, angiotensin II, inducing the secretion of aldosterone from the adrenal cortex that produces the renal reabsorption of sodium ions in the proximal tubule, ascending segments of the Henle loop, and distal tubule [10]. It is also involved in the regulation of blood pressure and is involved in vasoconstriction [11].

Excessive AT1R activity may be caused by its agonist, anti-ATR1 antibodies, due to the high affinity. It normally occurs in vascular smooth muscle and endothelial cells, leading to alloreactive and self-reactive responses [12]. These responses have been detected in patients with coronary heart disease and affect endothelial cells and pathologies, such as autoimmune diseases, marginal hypertension, refractory, vascular lesions, pre-eclampsia, type 2 diabetes mellitus, myocardial hypertrophy, and chronic inflammatory diseases, such as atherosclerosis [12, 13].

Furthermore, endogenous angiotensin II stimulates T cells and NK cells through AT1R (14). Therefore, anti-AT1Rs may play an essential role in the rejection of kidney grafts [14].

The presence of these anti-AT1R Abs in serum samples, specifically the IgG1 and IgG3 subtypes, mimics angiotensin ll actions in patients with kidney grafts suffering from steroid-refractory vascular rejection and malignant hypertension [15]. These antibodies are produced against the second extracellular loop, between amino acids 165 and 191 [15]. However, other studies indicate that anti-AT1R Ab in pre-kidney transplant recipients is independently related to graft loss [2, 16].

The eventual mechanisms of action of anti-ATR1 and ETAR antibodies in activating the gene expression of essential genes for several immune functions are shown in Fig. (**1**).

1.1.2. Endothelin Receptor Type A (ETAR)

Endothelin-1 (ET-1) is a peptide synthesized by different types of cells, such as endothelial cells, smooth muscle cells, and renal epithelial cells. It is considered the most potent vasoconstrictor protein in humans and is even more potent than angiotensin ll [17]. ET-1 acts on two types of high affinity transmembrane 7-domain receptors that are coupled to protein G, endothelin receptor type A (ETAR), and type B (ETBR) [18]. ETBR (peptide of 442 amino acids) is produced through the transcription and translation of the EDNRB gene; in humans, this gene is located on the long arm of chromosome 13 (13q22.3) and acts on endothelial cells producing vasodilation. ETAR is formed by a sequence of 427 amino acid residues encoded by the EDNRA gene [19]; it is composed of 8 exons, and it is located in two positions of the long arm of chromosome 4 (4q31.22-q31.2.3).

ETBR acts on endothelial cells, causing vasodilation, while ETAR is expressed at various sites, such as vascular smooth muscle, hepatic stellate cells and hepatocytes, and cardiac myocytes (Hay *et al*., 1996). Its activation by its ligand, ET-1, in vascular smooth muscle cells generates contraction of muscle fibers, vasoconstriction, and cell proliferation, promoting inflammation [19, 20].

The hyperactivity of ETARs due to their interaction with the ligand, ET-1, can produce pathologies, such as arterial hypertension, atherosclerosis, kidney failure, heart failure, or type 2 diabetes [3]. Although the evidence of its association in patients with kidney grafts is limited, there are indications of its involvement in various diseases [1, 3, 9, 13], which raise the suspicion that they could have clinical importance in kidney rejections transplants through anti-ETAR Abs.

Furthermore, in the follow-up of several patients during pre-transplantation and post-transplantation (in the first 12 months), anti-ETAR Abs have been associated with a worse prognosis in acute rejections due to increased levels of these Abs [21]. The eventual mechanisms of action of ETAR antibodies are shown in Fig. (**1**).

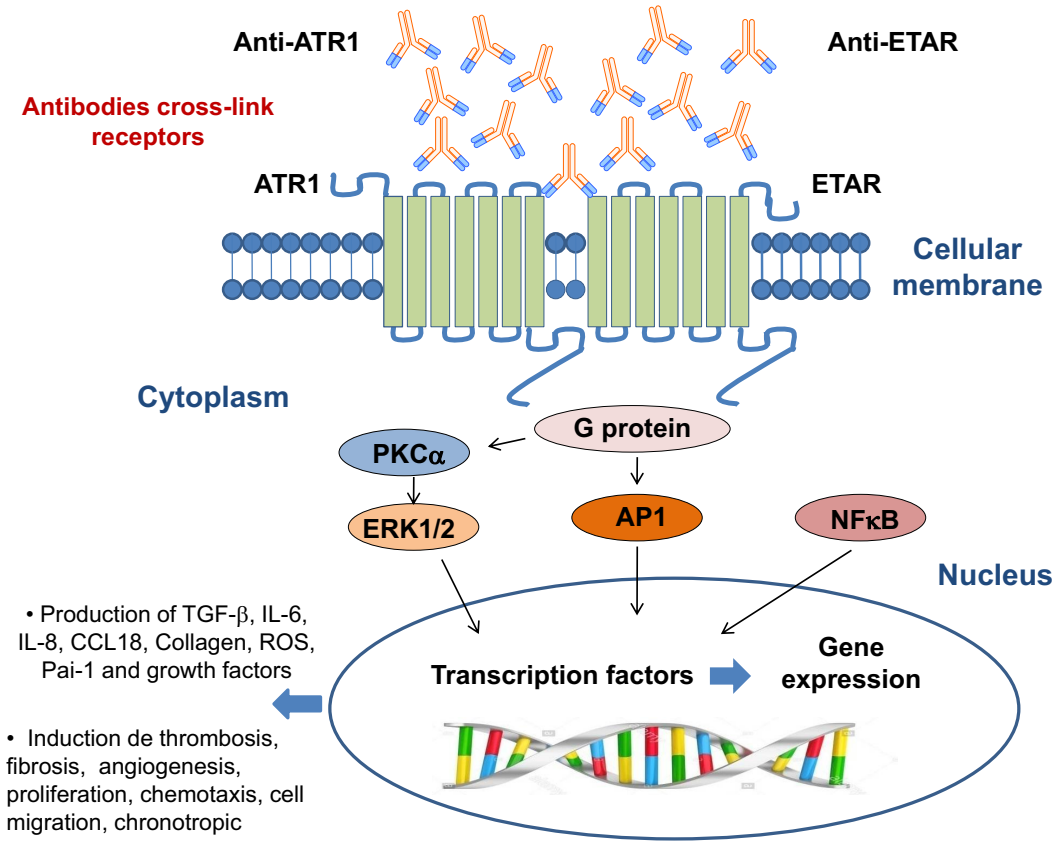

Fig. (1). Mechanisms of action of anti-ATR1 and ETAR antibodies activate the gene expression of important genes for certain immune functions. The binding of antibodies to their receptors promotes the G protein's activation, which activates PKCa and AP-1. ERK1/2 and NFkB are also activated, which promote the transcription of very important genes in immune function, wound repair, or fibrosis. The production of important molecules and the induction of key processes are shown. Abbreviations: ATR1: angiotensin II type 1 receptor, ETAR: endothelin-1 type A receptor, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells, PKCα: protein Kinase C alpha, ERK: extracellular signal-regulated kinases, AP-1: Activator protein 1, TGF-β: Transforming growth factor-beta, IL: interleukin, CCL18: C-C Motif Chemokine Ligand 18, ROS: reactive oxygen species, Pai-1: plasminogen activator inhibitor-1. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

1.2. Levels of Anti-ATR1 and Anti-ETAR Antibodies

Considering the levels of antibodies, a study has shown that high levels of anti-AT1R Abs and anti-ETAR Abs are associated with cellular and humoral rejection that produces vascular disease in heart transplant patients [9]. In other analyses, it has been observed that anti-ETAR and anti-AT1R Abs are related to renal dysfunction in the first 12 months after transplantation, but more cases with mild to severe intimal arteritis were also found [22]. Therefore, the histological lesions found in kidney transplants are generally produced by anti-HLA Abs from the donor [23, 24]. However, a large number of studies have created controversy suggesting that these anti-endothelial cell antibodies, in the absence of anti-HLA Abs, can act as possible activators of immune cells, such as T cells, B cells, or blood monocytes peripheral. Various mechanisms link both the innate response and the immune system's adaptive response, leading to graft rejections [25]. These atypical non-HLA Abs are the topic of interest of recent studies by several researchers due to their possible involvement in many pathologies. As mentioned above, vascular abnormalities induce humoral alloreactivity that causes failure in allogeneic renal transplants [25-27].

The increase in the use of immunosuppressants with greater potency and the increase in rejections in the absence of DSAs against HLA antigens with pathologies in which atypical non-HLA Abs could be involved, play an essential role in endothelial cells and could lead to a vascular lesion. We decided to study the relationship between these Abs (anti-AT1R and anti-ETAR), HLA-DSAs, and rejections in kidney transplants of patients in a pre-transplant and post-transplant situation in a wide series of our Region.

1.3. Own Research

Our study aimed to determine the presence of non-anti-HLA antibodies (anti-AT1R and anti-ETAR) in sera from patients with end-stage renal disease who underwent kidney transplantation in pre- and post-transplantation samples to study their influence on the development and evolution of acute humoral rejections and DSAs.

2. MATERIALS AND METHODS

2.1. Patient Enrollment and Data Acquisition

A total of 125 consecutive medical records of kidney transplant (KT) patients were recruited and analyzed retro-

spectively from 2015 to 2019 at the University Clinic Hospital, "Virgen de la Arrixaca" (Spain). Healthy volunteers were included as negativity controls (n = 20).

The sociodemographic, clinical, and biochemical data of KT patients were also studied. The mean age of the total cohort of recipient KTs was 55.0 ± 12.0 years (mean ± SD), of which 68% (n = 85) were men and 32% (n = 40) were women (Table **1**). The mean age of the donor cohort of recipient KTs was 52.1 ± 19.5 (mean±SD), of which 66.4% were men (n=83) and 33.6% (n=42) were women. Regarding the origin of the transplanted kidney, the majority came from cadaver donors (98.4%), and only 1.6% of the donors were from living donor kidneys.

Only patients whose kidney graft was in operation for at least 1-month post-transplantation and who had DSA Luminex determinations for detecting anti-HLA antibodies (T and B cells) screening before transplantation were included in this study. Allograft losses were estimated as a return to dialysis.

The inclusion criteria for renal graft recipients were primary renal transplant with no history of other types of transplantation, ABO compatibility, negative serological crossmatch test, and immunosuppressive therapy with tacrolimus (TRL) or Cyclosporine (CsA), mycophenolate mofetil (MMF), and HIV negativity.

All patients gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the Ethics Committee approved the protocol of HUVA (PI15/0370).

2.2. Indications and Main Diseases for Kidney Transplant

All the patients in this study had end-stage kidney disease and were transplanted. As shown in Table **1**, the main indications for KTs in our cohort (n = 125) were glomerulonephritis (n = 43, 34.2%), followed by polycystic kidney disease (n = 25, 20.3%), type I diabetes (n = 15, 11.9%), chronic obstructive pyelonephritis (n = 10, 8.4%), unknown renal insufficiency (n = 8, 6.1%), lupic nephritis (n = 5, 3.6%) and reflux nephropathy (n = 3, 2.4%). The rest of pathologies were included as other indications (n = 16, 13.1%).

On the other hand, the recipient's main symptoms were terminal chronic kidney failure (CKF), arterial hypertension, secondary hypertension (HPT2), cytomegalovirus infection, nephrogenic anemia, asymptomatic hyperuricemia, dyslipidemia, ischemic heart disease, and diabetes mellitus type 2.

Estimated glomerular filtration rate (eGFR) and creatinine were analyzed in all transplant patients (normal values between brackets), creatinine (0.7 to 1.2 mg/dl) and eGFR (>90 ml/min/1,73 m^2), according to the National Kidney Foundation ("Kidney basics|National Kidney Foundation"). Our patients' cohort showed the following values before the transplant; creatinine (mg/dl, 2.9 ± 2.1, mean ± SD) and an eGFR less than 60 mL/min/1.73 m^2 for more than three months suggests a chronic kidney disease. All patients were treated before transplantation to decrease creatinine levels and improve graft function.

Table 1. Demographic data and main kidney transplantation indications.

Total of transplantation, n (%)	125
Kidney receptor characteristics	-
Age*	55.0 ± 12.01
Gender, n (%)	-
Men	85 (68)
Women	40 (32)
Kidney donor characteristics, n (%)	-
Age	52.1 ± 19.5
Gender, n (%)	-
Men	83 (66.4)
Women	42 (33.6)
KT come from, n (%)	-
Deceased donor	123 (98.4)
Living donor	2 (1.6)
Transplantation indications, n (%)	125
Glomerulonephritis	43 (34.2)
Polycystic kidney disease	25 (20.3)
Type I diabetes mellitus	15 (11.9)
Chronic obstructive pyelonephritis	10 (8.4)
Unknown renal insufficiency	8 (6.1)
Lupus nephritis	5 (3.6)
Reflux nephropathy	3 (2.4)
Others	16 (13.1)
Main diseases in recipient, n (%)	-
CKF terminal	125 (100)
Arterial hypertension	122 (97.6)
Secondary hypertension (HPT2)	90 (72.0)
Cytomegalovirus infection	71 (56.8)
Nephrogenic anemia	63 (50.4)
Asymptomatic hyperuricemia	33 (26.4)
Dyslipidemia	31 (24.8)
Ischemic heart disease	26 (20.8)
Diabetes mellitus type 2	23 (18.4)

N, number of individuals with a particular disease; SD, standard deviation; KT, kidney transplantation. CKF, Terminal chronic kidney failure.
*Age (years). The mean values were analyzed (mean value ± SD) in all cases.

2.3. Immunosuppressive Treatment

Immunosuppressive therapy consisted of monotherapy, including an anti-calcineurin, such as Tacrolimus (TRL)/ Cyclosporine (CsA) and corticosteroids, or generally dual therapy, based on TRL/CsA, MMF, and corticosteroids. Doses were adjusted for blood concentrations and clinical complications to resolve rejection episodes. Thus, in the first stages of transplantation, induction immunosuppressants, such as Prednisone, TRL/CsA, and MMF, were used. CsA is usually replaced by TRL when certain clinical situations are aggravated (e.g., hirsutism) and is also used during Covid19 . After transplantation, the patient uses these immunosuppressants at specific doses, which are as follows a) Dacortin (Prednisone) 5 mg/24 hours, b) Myfortic (MMF) 360 mg/1 tablet/12 hours; c) Prograf (TRL) 3 mg/1 tablet/12 hours. Ranitidine was also used, with a dosage of 1 tablet/12 hours.

2.4. Kidney Rejection Diagnosis and Treatment

Allograft Acute Cellular Rejection (ACR) was defined as an increase in serum creatinine at least 20% above baseline serum creatinine and as biopsy-proven rejection (specimens were evaluated by light microscopy and immunofluorescence staining with a marker of classical complement activation as C4d and classified according to Banff classification) [28]. The diagnosis of acute AMR or Acute Humoral Rejection (AHR) requires the presence of distinguishable histopathological findings, a positive C4d staining in peritubular capillaries, and the simultaneous presence of DSA [29].

Mild ACR (Banff grade I) was treated with pulse steroids (500 mg methylprednisolone boluses), which improved maintenance immunosuppression. All other ACRs were treated with Anti-Thymocyte Globulin (ATG).

Acute rejection episodes were further classified as steroid-sensitive rejections (ACR Banff grade I) or steroid-insensitive rejections (ACR Banff grade II and III) and AMR. AHR was also treated with pulse steroids and intravenous immunoglobulin (0.25 gr/kg), and the last session 1 gr/kg (maximum 140 g) was divided into two doses associated with plasmapheresis (three sessions a day, every five days). Later, we administered 500 mg anti-CD20 (Rituximab, Roche pharmaceuticals) intravenously. Anti-AMR treatment was also administered in patients receiving anti-proteasome inhibitor Bortezomic (Velcade®, formerly PS-341). No correlation was observed between acute T-cell mediated rejection (TCMR) and pre- and post-transplant DSA (data not shown).

2.5. Determination of the Causes of Kidney Graft Loss

In the case of sudden death, the major causes of kidney graft failure had been examined in all patients and the cause of death was determined from medical death certificates and/or medico-legal autopsy.

2.6. Determination of Anti-AT1R and Anti-ETAR Antibodies

The presence of anti-AT1R and anti-ETAR Abs was evaluated in serum samples of 125 consecutive recipients for kidney pre-transplantation and post-transplantation.

The study of these antibodies was evaluated by ELISA technique using two commercial determination kits, anti-AT1R antibody (EIA-AT1R, One Lambda, Canoga Park, CA) and anti-ETAR antibody (CellTrend, Luckenwalade, Germany). The protocols with all their specific and internal controls were carried out according to commercial protocol. The Quanta Lyser 2 program was used to detect anti-ETAR concentrations and anti-AT1R Abs from 2.5 U/ml to 40 U/ml. All samples were analyzed in triplicate. Levels of concentration larger than 10 U/ml of anti-ETAR and anti-AT1R were considered positive, as published in the literature [2, 9, 21, 26, 27].

2.7. Anti-HLA Antibody Screening

Pre- and post-transplant sera were also analyzed for HLA antibody screening using multiplexed solid-phase-based microbeads array (Mix and Single Antigens Class I and II Kits, OL, CA), performed according to the manufacturers recommended procedure, as previously published [30, 31]. Serial dilutions were also performed in order to avoid an eventual prozone effect that could mask high-level antibodies. In this case, a pre-transplant serum was considered positive when its Panel Reactive Antibodies (PRA) value was higher than 0% (PRA $>$ 0%), and the mean fluorescence intensity (MFI) was higher than 1500 (MFI $\geq$ 1500).

The presence of DSA was determined by comparing particular HLA antibodies detected in the kidney recipient serum with the kidney donor HLA type. DSA to donor HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, -DRB5, and -DQB1 mismatched antigens were analyzed. DSA were subsequently classified according to HLA antigen class, class I only, class II, or both and stratified according to the cumulative normalized MFI, considering positive antibody above 1500.

The presence of anti-HLA antibodies, impaired renal function, and C4d deposits lead us to suspect possible humoral rejections that could also implicate anti-ETAR and anti-AT1R Abs as possible factors in renal failure. Samples were also taken at the time of biopsy in case of suspected graft rejection or malfunction.

For pre-transplantation, a serological cross-match was performed for microlymphocytotoxicity, resulting in a negative crossmatch and an access to the transplant.

2.8. Statistical Analysis

Demographic data and results were collected in a database (Microsoft Access, Microsoft Corporation, Seattle, WA), and the analysis were performed using SPSS 23.0 (SPSS software Inc., Chicago, IL). Quantitative variables were expressed as the mean ± SD and qualitative variables as a percentage. Pearson's $\chi 2$ and two-tailed Fisher's exact tests were used to compare categorized variables between different study groups.

Demographic, clinical, immunological features, and post-transplant antibodies status were compared using Pearson $\chi 2$ test or Fisher´s exact test for categorical data and Student's T-test or Mann-Whitney U test for continuous data. For this purpose, the correlation of DSA data and the outcome of 125 kidney transplants were assessed by the Kaplan-Meier

method. Differences in graft survival were analyzed every ten weeks until 60 weeks after transplantation and determined using the log-rank test. Results were expressed as the Hazard Ratio (HR) at 95% Confidence Interval (CI). A two-sided p-value < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

The patients were divided into two groups (Table **2**) according to the level of non-anti-HLA Abs; equal to or greater than 10 U/ml were considered as positive (n = 30), and less than 10 U/ml were considered negative (n = 95) (in both cases, these were present in a greater number of men than women). Levels of non-HLA antibodies (anti-AT1R and/or anti-ETAR) were positive in 30 (24.0%) out of 125 of the studied patients. The performed tests were positive in 28 (22.4%) patients with anti-AT1R Abs and 12 (9.8%) with anti-ETAR Abs, and 10 of them had the presence of both non-HLA antibodies. In the pre-transplant sera, the average level of positive non-HLA Abs was found to be equal to 16.12 ± 10.61 U/ml in anti-ETAR Abs and 15.39 ± 8.47 U/ml in anti-AT1R Abs, while was equal to 17.61 ± 9.10 U/ml anti-ETAR Abs and 15.10 ± 6.47 U/ml anti-AT1R Abs in post-transplant sera. During the first year post-transplantation, graft loss mediated by AHR was observed in eight patients with a positive result in non-HLA antibodies, although only four showed DSA anti-HLA.

Table 2. General characteristics of transplant patients and donors.

-	**HLA-positive, N=30; n (%) (Mean ± SD)**	**HLA-negative, N=95; n (%) (Mean ± SD)**
Age of patients (years)	52.5 ± 11.9	55.4 ± 12.1
Patient gender	-	-
Women (%)	8 (26.7)	32 (33.7)
Men (%)	22 (73.3)	63 (66.3)
Dialysis time before transplant (mean days ± SD) *	2516 ± 2414	2037 ± 1912
Number of transplants performed	-	-
First transplant	20 (66.6)	80 (84.2)
Second and third transplant	10 (33.3)	15 (15.8)
Cold ischemia time (hours ±)*	10.3 ± 4.7	10.3 ± 5.5
Kidney donor type	-	-
Live	0 (0)	2 (2.2)
Cadaveric	30 (100)	93 (97.8)
Age of donor*	44.5 ± 15.8	52.7 ± 17.3
Donor gender	-	-
Women (%)	11 (36.6)	31(32.6)
Men (%)	19 (63.4)	64 (67.4)
Clinical and immunological characteristics	-	-
Creatinine (mg/dl)	-	-
Pre-transplant**	7.21 ± 3.36	6.71 ± 3.29
Kidney rejection, N=41	10 (33.3)	31 (32.6)
CHR	1 (3.3)	1 (1.1)
ACR	1 (3.3)	12 (12.6)
AHR	8 (26.7)	18 (18.9)
Immunological characteristics	-	-
C4d (+)	2 (6.6)	11 (11.5)
HLA-I (+)	14 (46.7)	29 (30.5)
HLA-II (+)	10 (33.3)	31 (32.6)
DSAs (+)*	7 (23.3)	20 (21.1)

*Median±SD; CHR, chronic humoral rejection; AHR, acute humoral rejection; ACR, acute cellular rejection.

Chronic humoral rejection (CHR) was found in one patient and ACR in another patient.

In the renal biopsy, of the 30 patients with non-anti-HLA positive Abs, in 10 (33.3%) of them, it was found that they had undergone more than one kidney transplant throughout their life, 10 of them presented some rejection, 14 (46.7%) of them presented anti-HLA class I antibodies and 10 (33.3%) presented anti-HLA class II antibodies, 7 (23.3%) of them were DSA positive. The mean serum creatinine level of these 30 patients, before transplantation, was 7.21 ± 3.36 mg/dl, and in the post-transplant, it was 2.90 ± 2.01 mg/dl, and in two of these patients, $^{+}$C4d deposits were detected in the peritubular capillary by immunohistochemical techniques.

Of the total of 125 patients considered in our study, 41 (32.8%) cases of rejection were detected, 24 in women and 17 in men. Twenty-eight had AHR, 11 had ACR, and two recipients developed CHR. Of the total number of patients undergoing kidney transplantation, two of them received an allograft from living donors, and none of them presented non-anti-HLA Abs. However, they did present graft rejection. As previously mentioned, patients with DSAs^{+} and non-anti-HLA^{+} Abs were also detected in post-transplantation: one patient with CRH presented DSAs^{+} and non-anti-HLA^{+} Abs; in those who had ACR, three were DSA-positive, and one was non-anti-HLA Abs positive; in those who presented AHR, 18 of them were DSA^{+}, and eight presented non-anti-HLA^{+} Abs. In the crossover analysis of the variables, DSAs^{+} and non-anti-HLA^{+}, seven patients with non-anti-HLA^{+} Abs were detected out of the 27 patients with DSAs^{+}; in the patients with negative DSAs, 23 were detected with non-anti-HLA^{+} Abs.

When determining the three variables (DSAs, non-HLA, and type of rejection), anti-non-HLA Abs was observed in five patients with different rejections in the presence of positive DSAs and another five rejection patients in the absence of positive DSAs (Table **3**). The value of P between types of rejections was 0.305.

In this study, a total of 55 samples with non-anti-HLA positive Abs were detected in 10 recipients with different types of rejection developed (Table **4**); 37 were positive for anti-AT1R Abs, and 18 were positive for anti-ETAR Abs. 10 patients were observed who shared both non-anti-HLA positive Abs and 3 of them had AHR. Of the positives, 20 appeared in some type of renal allograft rejection, and 35 appeared in patients with decreased renal function.

In the analysis of the non-anti-HLA positive Abs separately and, together with the three types of rejection observed, in the presence and absence of the positive DSAs (Table **5**), in the AHR, the presence of eight anti-AT1R and anti-ETAR positive patients with the absence of DSAs and six non-anti-HLA Abs with the presence of positive DSA was observed.

When making the survival curves using Kaplan-Meier analysis in the course of post-transplantation in all patients with AHR, a greater survival was observed (Fig. **2**) in those patients with AHR and without anti-ETAR and anti-AT1R Abs, with an average of 44.59 weeks, than in non-anti-HLA positive patients, who presented an average of 39.4 weeks. However, the differences were not statistically positive ($p = 0.483$).

On the other hand, when comparing the survival curves between the three variables (DSAs, non-anti-HLA Abs, and

Table 3. Comparison of 41 rejections, non-anti-HLA, and DSAs Abs detected in patients.

-	No Anti-HLA (+)		No Anti-HLA (-)	
Rejection Types, N=41	**DSAs (+) N=5 (50.0)**	**DSAs (-) N=5 (50.0)**	**DSAs (+) N=17 (54.8)**	**DSAs (-) N=14 (45.2)**
CHR, n=2	1 (20.0)	0 (0.0)	0 (0.0)	1 (7.1)
ACR, n=11	0 (0.0)	1 (20.0)	3 (17.6)	7 (50.0)
AHR, n=28	4 (80.0)	4 (80.0)	14 (82.4)	6 (4.9)

N, number; DSAs, donor-specific antibodies; CHR, chronic humoral rejection; ACR, acute cellular rejection; AHR, acute humoral rejection.

Table 4. Different types of non-anti-HLA+ Abs in patients with different types of rejection.

Rejection Types	Pre-AT1R N=11, n (%)	Post-AT1R N=26, n (%)	Pre-ETAR N=7, n (%)	Post-ETAR N=11, n (%)
CRH	1 (9.0)	1 (3.8)	1 (14.3)	1 (9.0)
ACR	1 (9.0)	1 (3.8)	0 (0.0)	0 (0.0)
AHR	3 (27.3)	7 (26.9)	1 (14.3)	3 (27.3)
Others*	6 (54.6)	17 (65.4)	5 (71.4)	7 (63.6)

*Decreased kidney function; N, number; DSAs, donor-specific antibodies; CHR, chronic humoral rejection; ACR, acute cellular rejection; AHR, acute humoral rejection.

Table 5. Non-anti-HLA positive Abs types crossed with developed rejections and the presence/absence of DSAs.

-	Pre-AT1R n=5		Post-AT1R n=9		Pre-ETAR n=2		Post-ETAR n=4	
Rejection Types	**DSAs (+)**	**DSAs (-)**	**DSAs (+)**	**DSAs (-)**	**DSAs (+)**	**DSAs (-)**	**DSAs (+)**	**DSAs (-)**
CHR, n=4	1 (50.0)	0 (0.0)	1 (20.0)	0 (0.0)	1 (100.0)	0 (0.0)	1 (50.0)	0 (0.0)
ACR, n=2	0 (0.0)	1 (33.3)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0))
AHR, n=14	1 (50.0)	2 (66.7)	4 (80.0)	3 (75.0)	0 (0.0)	1 (100.0)	1 (50.0)	2 (100.0)

N, number; DSAs, donor specific antibodies; CHR, chronic humoral rejection; ACR, acute cellular rejection; AHR, acute humoral rejection.

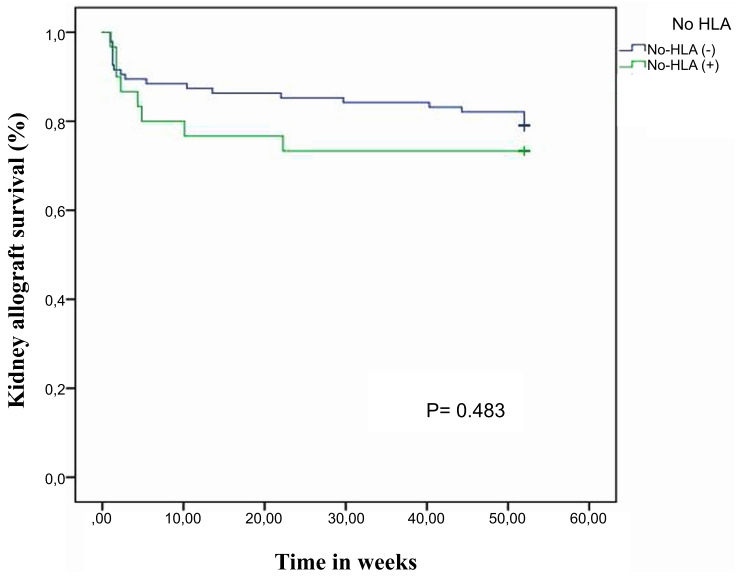

Fig. (2). Comparison of positive and negative non-anti-HLA antibodies for AHR development in allograft survival curves. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

AHR) (Fig. **3**), the average survival of 25.0 weeks in non-anti-HLA negative patients was observed compared to non-anti-HLA-positive patients with 26.5 weeks (p = 0.612). However, in patients with AHR, absence of DSAs and non-Anti-HLA^{+} an average survival of 45.3 weeks was observed, than in patients without non-anti-HLA Abs, who presented an average of 49.9 weeks (p = 0.694). In any case, the results, although highly visual, were not statistically significant, maybe due to the fact that it was a study in a series of transplants limited in terms of the number of transplants considered, requiring subsequent multicenter studies to be able to draw conclusions that allow asserting the observed trends.

In conclusion, a high correlation between the levels of non-HLA antibodies and reduced kidney function during post-transplantation was found, especially in the presence of DSA anti-HLA antibodies, as previously notified by Reismonen *et al.* [32]. Anti-AT1R and anti-ETAR antibodies can be useful to evaluate the risk of allograft loss. Finally, more studies are needed with more number of kidney recipients and more post-transplant monitoring time to elucidate our preliminary results.

4. DISCUSSION

This study monitored kidney graft recipients for anti-GPCRs non-HLA Abs, anti-ETAR, -AT1R, and -HLA Abs in pre-and post-transplantation to establish their relationship with DSA antibodies development and if there are differences between these types of antibodies in transplant outcome.

The primary immune failure or graft loss factor in kidney transplant patients has traditionally been related to circulating DSA HLA antibodies. However, several studies have highlighted the importance of non-anti-HLA Abs in graft loss in recent years, especially against endothelial cells y ATR1 [1, 2, 4, 21].

The results of renal biopsies in recipients with high levels of anti-ETAR and anti-AT1R Abs reveal graft lesions that are accompanied by acute and chronic vascular diseases. There was a significant relationship between patients with DSA HLA Abs and rejection types (p = 0.001). However, the association between the type of rejection and non-anti-HLA Ab did not show statistically significant differences (p = 0.305).

In our study of patients undergoing kidney transplantation, from the uni-variable and multiple-variable analysis of anti-AT1R and anti-ETAR Abs, with a cut-off point equal to or greater than 10 U/ml, we observed positivity in the serum pre-transplantation (preformed) and post-transplantation or *de novo* in 24% of renal recipients. By classifying them into subgroups, we observed 22.4% of patients with positive anti-

AT1R Abs [28], 9 of them already positive in the pre-transplantion. On the other hand, anti-ETAR Abs positivity was detected in 9.8% of patients (12/125), and 6 of them maintained that positivity since pre-transplantation. Therefore, there are more patients with the presence of anti-AT1R Abs than anti-ETAR Abs. Also, the decrease in the levels of concentration of anti-AT1R Abs in 2 patients is noteworthy; one of them had AHR, and also occurred in a patient anti-ETAR Abs after transplant.

Dragun *et al.* related the decrease in levels of anti-AT1R Ab with AT1R binding to the graft immediately after implantation and the initiation of inflammatory lesions in vascular cells, thus making it impossible to detect these increases in blood circulation [33]. We do not know precisely why there is a decrease in non-anti-HLA Ab levels, but it can be hypothesized that it could be due to the time of cold ischemia, the induction therapy received, or the influence of the anti-HLA Abs, as reported by some authors [34, 35].

Our cut-off threshold was established in patients with a non-anti-HLA Ab concentration equal to greater than 10 U/ml. This threshold was established, taking into account the manufacturer's protocols, the number of patients with rejection from that range, and the literature [2, 9, 21, 26, 27]. Therefore, Banasik *et al*. studied 117 kidney transplant recipients by considering the cut-off point of anti-AT1R Abs in patients with a concentration higher than 9 U/m, and was observed that 23% [27] of them were positive [21]. Also, Giral *et al*., in a study of 599 kidney transplant recipients, established the cut-off point of anti-AT1R Abs at levels greater than 10 U/ml and 47.2% of patients were detected with positivity; they also associated the importance of the Abs detected in pre-transplantation as an independent risk factor for long-term graft failure [2]. Nevertheless, Hiemam *et al*., in another study with only 30 heart transplant patients, predicted the anti-ETAR and anti-AT1R Abs cut-off point to be greater than 16 U/ml [9]. Another study by Lim *et al.*, with 27 patients, proposed the cut-off for anti-AT1R Abs in concentrations higher than 17 U/ml, detecting 29% of positivity in transplant patients [36]. If we place our cut-off point in patients who have tested positive in concentrations of non-anti-HLA Abs greater than 17 U/ml, we would have six receptors that exceeded the threshold, of which 2 of them suffered AHR, one in the presence of DSAs and another in the absence of positive DSAs.

In this manner, although the exact threshold is not yet clearly established, we find an existing relationship between the non-anti-HLA Abs and the evolution of renal receptors.

According to the bibliography, other non-anti-HLA Abs could also be related to acute rejection [37]. Also, some studies strongly link malignant arterial hypertension with acute vascular rejection between renal transplants [15] and, in the presence of non-HLA Abs, in our study, high-frequency hypertension was also observed (Table **1**). In fact, in our study of 125 patients, we found 41 patients with different rejection types, and 28 of them had AHR. Therefore, there is a higher frequency of acute rejection produced by Abs than other types of rejection. Of the patients with AHR, 18 were DSA positive, and in eight patients, non-anti-HLA Abs was observed (Table **2**). From this association, we detected a greater number of positives (Table **4**) of anti-AT1R Abs in patients with AHR than the anti-ETAR Abs. In majority of them, DSAs+ was absent (Table **5**).

Patients who suffered from AHR, and both positive non-anti-HLA and DSAs, had a lower survival rate than other patients who were non-anti-HLA-negative and DSA-(Fig. **3**), or those who were only DSA-positive. In patients undergoing renal biopsy after transplantation, mean serum creatinine levels were high (2.90 ± 2.01). Therefore, the damage observed in the biopsy in AHR patients and the absence of DSA could be due to the non-anti-HLA Abs. In this sense, Taniguch *et al*. [4], in a study in pre- and post-transplantion, in a group of 351 renal recipients, found lower graft survival in patients with anti-AT1R Abs and DSA-positive than those who only presented DSAs. Therefore, there is evidence of damage to the grafts by non-anti-HLA Abs, but the exact mechanism that causes this loss of transplanted tissue is still unknown, although what is known for now is the activation of endothelial cells or their lysis in vasculopathy and autoimmune diseases mediated by these non-HLA Abs [38-40].

Of the twenty-five patients who underwent more than one kidney transplant, eight had AHR, and ten were positive in non-anti-HLA Abs. We observed two of the eight patients with AMR positivity in anti-ETAR and anti-AT1R Abs but the absence of DSA. Likewise, the two grafts received by living donors had AHR, but neither was positive for anti-ETAR or anti-AT1R Abs.

This could indicate that grafts received by living donors do not produce non-anti-HLA Abs and, therefore, could have more prolonged survival compared to cadaver donors [9, 40], or perhaps there may also be an eventual relationship with antigens sharing as the donors and recipients are related. We have also detected deposits of C4d in the peritubular capillaries, both in anti-ETAR and anti-AT1R Abs, in two patients who suffered AHR. However, some publications do not associate it with AMR but with ACR [41, 42], suggesting that the complement acts independently of graft injury, although our data differ from those described by other authors [43]. Recently, in a similar manner to our study, Fichtner *et al.* showed an accumulative load of DSA+ Abs and non-HLA Abs in blood was related to the different degrees of microinflammation peritubular capillaries [44]. Non-HLA antibody positivity was an independent non-invasive risk factor for graft function deterioration. Besides, AT1R antibodies are highly prevalent after intestinal transplantation and may be triggered by immune activation associated with the transplant, as recently published [45].

Therefore, there is evidence that high levels of anti-AT1R and anti-ETAR Abs are associated with the characteristics of injuries and kidney graft failures [21]. Extensive studies, preferably multicentric, of these types of Abs are still required to confirm with certainty their eventual implication in AHR and thus may be able to establish a diagnosis method or therapies that reduce this high loss of grafts.

In this sense, there are already some pharmacological antagonists for ETAR, which are used to treat arterial hypertension. There are also successful therapeutic strategies, such as plasmapheresis or immunoadsorption used in AT1 receptor in heart disease or anti-AHR treatment with anti-intravenous immunoglobulin and rituximab, which are help-

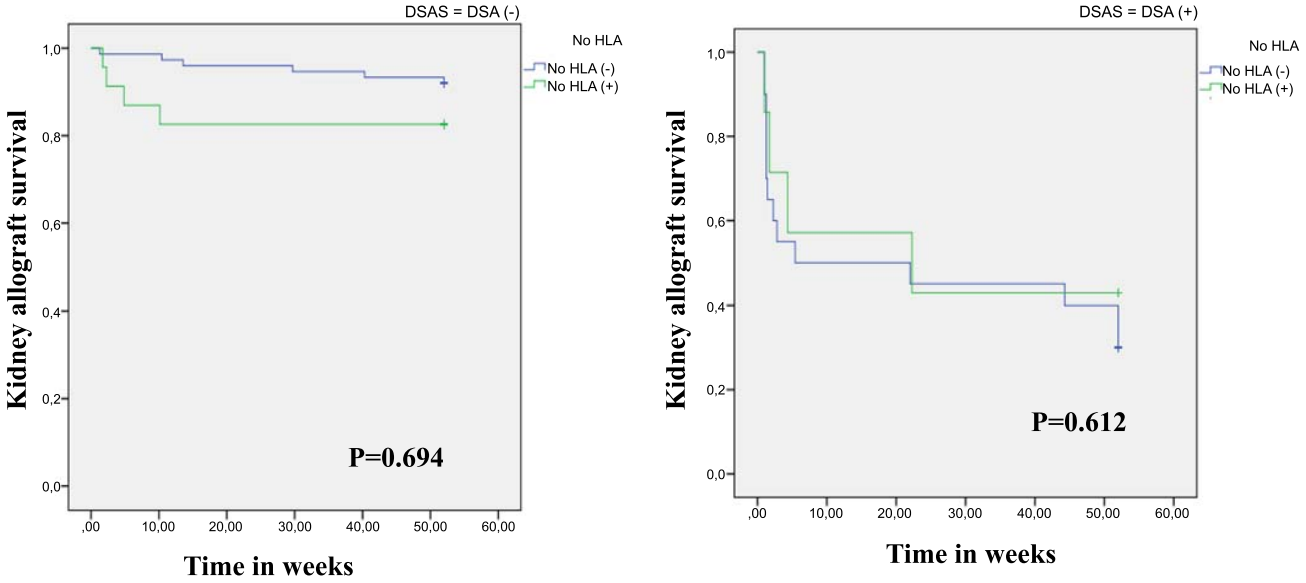

Fig. (3). Comparison of the three variables, DSAs, non-anti-HLA antibodies, and AHR development in allograft survival curves. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

ful for the reduction or stabilization of Abs titers in acute pathologies [23, 24, 46, 47].

Another essential point to study about the role of these antibodies is their role in the B cells [48], the expression of activation or repression molecules of tissue damage in biopsies [49], regulatory cells induction and cytokines [50-54], and the role in other types of transplantation as tolerogenic as liver where even the role of anti-HLA antibodies is not fully demonstrated, as suggested by the literature [24, 31].

4.1. Multiplex Non-HLA Antibody Assays: From Fiction to Reality and Better Future

As the field of organ transplantation advances, the desire to eliminate any type of complication in the course of it may motivate the need to analyze not only one type of non-anti-HLA antibodies by technique and/or method but to be able to analyze more than one simultaneously in order to study their clinical relevance in transplantation. In this sense, the recent appearance of novel multiplex methods used with the Luminex platform has revolutionized auto-antibodies' interest in solid organ transplantation. Due to the ability to test anti-HLA antibodies in the same routine, the presence of this type of antibodies should contribute discovering the role of these antibodies in different types of receptors, different types of transplantation, and different types of post-transplant complications.

Many different approaches have been mentioned in the literature, from home trials to commercial trials. For example, there is an interesting home trial by Kamburova *et al*. [55] that uses fourteen non-HLA targets deemed relevant for kidney transplantation. For commercial trails, there are interesting presentations as ThermoFisher (One Lambda) or Immucor (Lifecodes) that seem to offer an answer for all that antibody detection needs, including non-HLA antibodies. These are multiplex solid-phase essays covering a broad range of targets, including ATR1, ETAR, MICA (classically added in anti-HLA antibody detection kits by Luminex or single), vimentin, tubulin, and many other non-HLA antigens. The analysis of an extensive series of transplanted patients with these mass and multiplex detection approaches should clarify whether this type of non-HLA antibodies is important, achieving the role of anti-HLA antibodies' independently. The future will tell us if they are as important as they may seem *a priori.*

Nevertheless, as the future is being written every day, there are already very recent publications using this multiplex technology that allows us to sweep a battery of non-anti-HLA antigens. For example, a recent article by Betjes *et al*. [56] shows that higher levels of AT1R autoantibody are specifically associated with interstitial fibrosis (Fig. **1**) and graft survival, although another previous article with the same multiplex technology concluded that ATR1 Abs [57] did not contribute to risk stratification and could not explain AMR histology in the absence of DSA antibodies. Other group analyzing transplanted patients that presented early AMR confirmed by biopsy showed anti-LG3 antibodies, two of them with concomitant anti-ATR1 and only one patient developed de novo-DSA antibodies [58] with other studies suggesting the need for further confirmation of the role of these antibodies in transplant in the future [59-61].

CONCLUSION

Our study has demonstrated anti-ETAR and anti-AT1R antibodies' positivity in pre-transplantation and post-transplantation or *de novo* in renal recipients. Within the group of patients with DSAs positive antibodies, those who presented non-HLA antibodies had lower survival than patients who presented only DSAs positive antibodies, although the results were not statistically significant.

We know that the publications in this field are limited and much remains to be done, we hope that future researches will contribute to learn studies to learn more about the role played by this type of Abs in transplantation.

Finally, the detection of this type of non-HLA antibodies by more innovative technology than the ELISA, such as multiplex assays in Luminex technology, should lead us to shed light in the future on a subject that is not completely understood until now.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics Committee of University Clinical Hospital Virgen de la Arrixaca (approval number PI15/0370), Spain, approved this protocol of the study.

HUMAN AND ANIMALS RIGHTS

No animals were used in this research. All human procedures followed were in accordance with the Helsinki Declaration of 1975.

CONSENT FOR PUBLICATION

All patients gave their informed consent for inclusion before they participated in the study.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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