TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Predictive factors of allosensitization in renal transplant patients switched from calcineurin to mTOR inhibitors

Juan Carlos Ruiz San Millán,[1] Marcos López-Hoyos,[2] David San Segundo,[2] Estrella Quintela,[1] Emilio Rodrigo,[1] Carlos Gómez-Alamillo,[1] Iñigo Romón[3] and Manuel Arias[1]

1 Nephrology Department, Hospital Universitario Marqués de Valdecilla-IFIMAV, Universidad de Cantabria, Santander, Spain
2 Immunology Department, Hospital Universitario Marqués de Valdecilla-IFIMAV, Universidad de Cantabria, Santander, Spain
3 Histocompatibility Testing Laboratory, Hospital Universitario Marqués de Valdecilla-IFIMAV, Universidad de Cantabria, Santander, Spain

Keywords
anti-HLA-antibodies, calcineurin inhibitors, everolimus, immunosuppression, kidney transplantation, sirolimus.

Correspondence
Juan Carlos Ruiz San Millán, Nephrology Department, Marqués de Valdecilla Hospital, Santander, Spain.
Tel.: +34 942 202738;
fax: +34 942 315101;
e-mail: ruizjc@humv.es

Conflicts of interest
J.C. Ruiz San Millán has participated in advisory boards with Novartis and Pfizer. M. Arias has participated in advisory boards with Novartis and Astellas. The authors disclose any commercial associations that might pose a conflict of interest in connection with the submitted manuscript.

Received: 2 July 2013
Revision requested: 18 September 2013
Accepted: 4 April 2014
Published online: 17 June 2014

doi:10.1111/tri.12334

Summary

Conversion of kidney-transplant recipients from calcineurin inhibitors to mTOR inhibitors has been suggested to be a risk factor for increased alloimmune response. We have analyzed the development of new HLA-antibodies (HLA-Abs) early after conversion in 184 patients converted in stable phase at our hospital and compared with a control group of nonconverted comparable 63 transplants. Using single-antigen solid-phase immunoassay analysis, a preconversion and a 3–6 months postconversion sera were prospectively analyzed in every patient for the appearance of new HLA-Abs. Renal function at 2 years postconversion and cumulative graft survival were compared between groups. In 16 patients, new HLA-Abs (3-DSA and 13-NonDSA), not present at the moment of conversion, were detected (8.7% vs. 3.1% in the control group). The type of mTORi used, type of CNI preconversion, the presence of steroids, time of conversion, or indication for conversion did not have influence on this effect but the presence of HLA-Abs before conversion highly correlated with the appearance of new specificities. Patients with *de novo* HLA-Abs showed a trend to worst graft function and survival. In conclusion, conversion to mTORi can be followed by early appearance of *de novo* HLA-Abs, especially in patients with HLA-Abs preconversion, and this complication should be screened early after conversion.

Introduction

According to the humoral theory of transplantation, the presence of antibodies against the HLA system (HLA-Abs) is responsible for graft rejection [1]. These HLA-Abs are known as essential players in hyperacute and acute rejection. In addition, HLA-Abs have been associated with the further development of chronic rejection (CR), compromising graft function and survival in renal transplantation [2–5]. In recent years, not only the presence of preformed HLA-Abs before transplantation but also the development of *de novo* HLA-Abs after transplantation has been involved in the development of chronic antibody-mediated rejection (ABMR) [6,7]. In such a process, the presence of donor-specific HLA-Abs (DSA) and also, although at a lesser extent, nondonor-specific HLA-Abs (NDSA) play a crucial role [8].

The importance of the new development of HLA-Abs after transplantation has been probably underecognized in the past years. Recent studies demonstrate that ABMR is probably implicated in a considerable number of transplants that fail [9–11]. When the maximum efforts to find

the cause of graft loss are made in every case of failed transplant (clinical data, renal biopsy, C4d, electron microscopy, molecular analysis and sensitive HLA-antibody testing) an immunological mechanism can be detected as the responsible for graft loss in a considerable number of cases. In a recent report, Sellares *et al.* show similar results, being ABMR responsible for graft loss in more than 60% of cases in a series of 56 failed transplants (all with previous renal biopsy) and interestingly nonadherence could be detected in almost 50% of those cases [12]. This paper raises the question that whether reductions in (global) immunosuppression might trigger the development of new DSA and secondarily ABMR leading to graft damage and finally graft loss. Nonadherence is probably an extreme form of underimmunosuppression but other planned interventions such as maintenance of low calcineurin inhibitor (CNI) levels to avoid CNI toxicity, steroid withdrawal or perhaps conversion to an mTOR-Inhibitor (mTORi) with CNI elimination might have a similar effect and it should be important to evaluate the effects of planned immunosuppression modifications in the risk of development of *de novo* HLA-Abs after transplantation.

As a consequence of the risk of developing HLA-Abs for graft survival, monitoring of these Abs after transplantation has become a routine tool in the clinical follow-up of renal transplant recipients, especially in the context of clinical events or changes in immunosuppression as recommended by several guidelines [13,14].

Conversion from a CNI to an mTORi-based protocol, either Sirolimus (SRL) or Everolimus (EVR) has been proposed in the last years as an option in normal/low immunological risk patients with the dual objective of preventing or treating chronic CNI nephrotoxicity and so preserving renal function in the long term and secondly of preventing malignant neoplasms, specially cutaneous neoplasms [15–18]. This change in immunosuppression is associated with a small risk of acute rejection (about 5% of cases) as it has been observed in most clinical trials of conversion [19] and it has been suggested that such interventions might be associated with an increased risk of developing CR in the long term (due to insufficient organ immunoprotection). Nevertheless, this idea has not been demonstrated in any clinical study, either retrospective or prospective and even more long-term follow-up of patients included in clinical trials using mTORi as main immunosuppression do not seem to show an increased incidence of CR.

The immunological risk after conversion to an mTORi could be evaluated by the production of *de novo* HLA-Abs in the early phase after conversion to an mTORi-based protocol as a biomarker of a humoral immune response against the transplanted organ and as a predictor of the future development of chronic ABMR.

Our center has an extensive experience using mTORi in kidney transplantation, especially in conversion with complete elimination of CNI [20–27]. We have analyzed our series of patients converted from a CNI to an mTORi-based immunosuppressive protocol evaluating the presence of HLA-Abs immediately before and 3–6 months after conversion to evaluate the risk of developing *de novo* DSA or NDSA. Here, we present the results of this study.

Materials and methods

Patients

All patients converted from a CNI-based protocol to an mTORi-based protocol after the third month of transplantation at our center until February 2010 were included in the study. A total of 184 patients were selected and analyzed. Ninety-two patients had been converted to SRL and 92 patients to EVR. Most patients were converted due to a clinical indication and the reasons for conversion were variable, being the most important reasons the demonstration of chronic allograft nephropathy (CAN) on biopsy [*Interstitial Fibrosis and Tubular Atrophy* (*IFTA*) in the 2007 Banff classification] or the recent diagnosis of a malignant neoplasm (see Table 1 for a complete listing of indications). The first patient was converted in October 1999 and the last patient in February 2010. The moment of conversion after transplantation ranged between three and 375 months with a median time of 69 months.

An additional group of 63 patients on CNI therapy and nonconverted to an mTORi with an equivalent post-transplant time of follow-up was selected as a control group to evaluate the risk of developing HLA-Abs in a similar period of time in the absence of any major modification in the immunosuppressive protocol. These patients were selected using the rule of searching those transplants made immediately before or after every case that remained functioning at the moment of conversion. A proportion of 1:3 (one control for every three patients) was considered adequate to compare results in both groups. Demographic characteristics of both groups were comparable and are detailed in Table 2.

Table 1. Indications for conversion to mTORi.

	n	%	Median time after transplant (months)
Chronic Allograft Nephropathy (IFTA)	88	47.8	56.3
Malignant neoplasm	65	35.4	157.7
Prevention	17	9.2	5.4
Vascular disease	3	1.6	46.8
Other indications	11	6.0	6.1

Table 2. Main clinical and demographic characteristics of the patients included in the study.

	mTORi conversion (n = 184)	CNI therapy (n = 63)	P value
Period of study	Oct-1999/ Feb-2010	Oct-1999/ Feb-2010	
Gender (male/female)	121/63	45/18	NS
Ethnicity (Caucasian/other)	183/1	63/0	NS
Donor type (Living/Deceased)	2/182	0/63	NS
Time after Tx (months): median (range)	65 (3–375)	38 (3–236)	NS
Mean age at conversion (years ± SD)	53.4 ± 13.4	53.2 ± 11.8	NS
Indication for conversion			
IFTA	88		
Neoplasia	65		
Pre-emptive	17		
Other	14		
Mean sCr preconversion (mg/dl ± SD)	1.86 ± 0.72	1.74 ± 0.70	NS
Number of Tx (1st/2nd/3rd) (%)	79.3/19.0/1.6	78.9/17.3/3.8	NS
PRA CDC Pre-Tx (<10%/≤10%)	90%/10%	88%/12%	NS
Preconversion immunosuppression			
TCR/CsA	86/93	29/34	NS
MMF/AZA	97/25	41/8	NS
Esteroids/No esteroids	96/88	39/24	NS

PRA CDC, panel reactive antibodies by complement-dependent cytotoxicity; sCr, serum creatinine; Tx, transplantation.

Protocol of conversion to an mTORi

In all cases, a quick conversion to an mTORi with complete elimination of the CNI was made. A short overlapping period between the introduction of the mTORi and the complete elimination of the CNI of 4 days (in patients converted to EVR) or 7 days (in patients converted to SRL) occurred in most cases. The first day of introduction of the mTORi, the CNI (CSA or TAC) doses were immediately reduced by 50% and antiproliferative drugs when present were reduced in those cases receiving more than 1000 mg per day of mofetil mycophenolate, 720 mg of sodium mycophenolate or 50 mg of azathioprine. A first through level of EVR or SRL was scheduled at day 4 after EVR introduction or day 7 after SRL introduction, and when it was in the desired range, the CNI was completely eliminated. The objective through levels (for both drugs) were between 4 and 8 ng/ml in those patients converted after the first year post-transplant and between 8 and 12 ng/ml in those patients converted in the first year post-transplant. A more detailed description of the conversion procedure can be seen in previous publications [26,27]. During the study period, no other major modifications in the immunosuppressive protocol were made in any patient.

Serum samples selection

Serum samples of all transplanted patients at our center are collected and stored at −80 °C every 3 months as a routine in every clinical visit. Frozen serum samples were retrospectively selected to prospectively investigate the presence of HLA-Abs. For every patient, two samples were selected, the one collected immediately before conversion and a second one collected near the third month after conversion (usually between the third and the sixth month after conversion). A total of 494 serum samples were selected and included in the study (368 in the study group and another 126 in the control group).

Sera analysis

Serum samples were screened for the presence of HLA-I and HLA-II Abs by the Luminex-based bead assay (One Lambda, Canoga Park, CA, USA). Specificity of HLA-Abs was determined by Luminex Single Antigen (LSA, *LAB-Screen Single Antigen* beads, One Lambda). All the assays were performed following the manufacturer's protocols. Screening and specificities of HLA-I and HLA-II Abs were qualitatively informed. The cutoff value for considering a positive HLA Ab specificity was set when two of three criteria were fulfilled: More than 1500 Raw mean fluorescence intensity (MFI), more than 1000 Baseline MFI and/or the 25% of the maximum MFI bead [28].

Classification of patients

Patients in the study and in the control groups were initially classified into four groups according to their HLA-Abs status before and after conversion using the screening method (*Screening Groups*). *Group I*: no HLA-antibodies before conversion and no HLA-Abs after conversion (Neg/Neg), *Group II*: the presence of HLA-Abs before conversion and absence (disappearance) of HLA-Abs after conversion (Pos/Neg), *Group III*: the presence of HLA-Abs before conversion and presence after conversion (Pos/Pos) and *Group IV*: the absence of HLA-Abs before conversion and the presence of HLA-Abs after conversion (Neg/Pos).

In the second phase and based on the LSA analysis, patients with postconversion antibodies (groups III and IV) were reclassified into three additional groups

(*Single-Antigen Groups*): *Group A*: those patients with HLA-Abs but without any *de novo* HLA specificity demonstrated in the postconversion sample (No *de novo* Abs; all patients pertaining to Group III), *Group B*: those patients with new HLA specificities but with no antibodies directed to donor HLA antigens (*de novo* NDSA Abs) and *Group C*: those patients with newly developed specificities against donor antigens (*de novo* DSA Abs).

Risk factors for the appearance of *de novo* HLA-Abs after conversion

The presence of HLA-Abs before conversion, the type of mTORi introduced, the type of CNI used before conversion, the presence or absence of steroids at the moment of conversion, the indication for conversion and the time of conversion after transplantation were considered to evaluate their influence on the appearance of new Abs.

Effects of *de novo* HLA-Abs appearance on graft evolution

All patients in study and control groups were followed until February 2012 so all the patients had a follow-up after conversion of at least 24 months. Renal function as well as patient and graft survival were recorded before conversion and at 12 and 24 months after conversion and values compared between patients developing (groups B + C) and patients not developing (group A) *de novo* HLA-Abs after conversion to evaluate the consequences of such Abs formation. This analysis was made in an intent-to-treat basis, irrespective of the continuation of the mTORi or not (ITT analysis) and failed grafts were considered. In those cases, where graft had failed at 2 years, serum creatinine was computed as 10 mg/dl.

Statistical methods

Quantitative variables were compared using the Student′s *t*-test. Distribution of frequencies between groups was compared using the chi-square test. Graft survival was analyzed using the Kaplan–Meier test and comparison between groups was made using the Log-Rank test. Differences were considered significant when P was below 0.05.

Results

Distribution of patients according to the screening test

Preconversion status

Thirty-four patients (18.5%) had HLA-Abs present in serum immediately before conversion, whereas in the remaining 150 patients, no Abs were detected in the preconversion sample (81.5%). Among those patients with HLA-Abs 20 patients had HLA-I Abs (11%), eight patients had HLA-II Abs (4%), and six patients had both class-I and class-II Abs (3.5%). With respect to the control group, 10 patients (15.9%) had HLA-Abs at the equivalent moment before conversion, whereas the remaining 53 (84.1%) did not have any detectable Ab. Both populations were comparable according to preconversion status ($P = 0.641$).

Distribution of the study population according to Screening Groups

One hundred and forty-four patients out of 184 were classified in the Group I (no detectable Abs before and after conversion; Neg/Neg) which accounts for the 78.3% of patients, five patients were included in Group II (Pos/Neg; 2.7%), twenty-nine patients were classified as Group III (Pos/Pos; 15.8%), and finally, six patients were included in the Group IV (Neg/Pos; 3.3%) (Table 3). The distribution of patients according to the presence of only class-I or class-II Abs is also detailed in Table 3.

Distribution of the control population according to Screening Groups

Fifty-three of the 63 control patients were included in the Group I (Neg/Neg; 84%), whereas the remaining 10 patients were included in the Group III (Pos/Pos; 16%). No patients in this group showed changes from positive to negative (Group II) or from negative to positive (Group IV) according to this screening test (Fig. 1).

Table 3. Distribution of patients in the study (Converted) and control (Nonconverted) groups according to the screening test before and after conversion.

	HLA-I Abs (%)	HLA-II Abs (%)	Total (%)
Group I (Neg/Neg)			
Converted	153/184 (83.2)	167/184 (91.9)	144/184 (78.3)
Nonconverted	56/63 (88.9)	56/63 (88.9)	53/63 (84.1)
Group II (Pos/Neg)			
Converted	4/184 (2.2)	1/184 (0.5)	5/184 (2.7)
Nonconverted	0/63 (0)	0/63 (0)	0/63 (0)
Group III (Pos/Pos)			
Converted	22/184 (12.0)	12/184 (5.4)	29/184 (15.8)
Nonconverted	7/63 (11.1)	7/63 (11.1)	10/63 (15.9)
Group IV (Neg/Pos)			
Converted	5/184 (2.7)	4/184 (2.2)	6/184 (3.3)
Nonconverted	0/63 (0)	0/63 (0)	0/63 (0)

Patients were grouped into four categories according to the presence of serum HLA-Abs before and after conversion: negative preconversion/negative postconversion (Group I, Neg/Neg), positive preconversion/negative postconversion (Group II, Pos/Neg), positive preconversion/positive postconversion (Group III, Pos/Pos) and negative preconversion/positive postconversion (Group IV, Neg/Pos). Absolute numbers and frequencies (in parenthesis) of patients with antibodies against HLA-I, HLA-II or total (I and/or II) are shown.

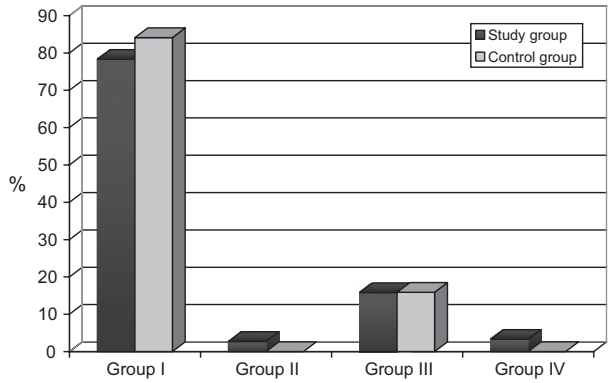

Figure 1 Distribution of patients in study and control groups according to the results of the screening test before and after mTORi conversion. Group I: No HLA-Abs before and after conversion (Neg/Neg), Group II: the presence of HLA-Abs before and absence after conversion (Pos/Neg), Group III: the presence of HLA-Abs before and after conversion (Pos/Pos) and Group IV: absence of HLA-Abs before and presence after conversion (Neg/Pos).

Results of Single-Antigen analysis

Distribution of the study population according to Single-Antigen Groups

Thirty-five patients had HLA-Abs after conversion (irrespective of the preconversion status) (29 from Group III and 6 from Group IV) and were further classified according to the *Single-Antigen Groups*. Nineteen patients did not show any change in HLA specificities with respect to those detected before conversion (Group A, 19/35, 54.3%), whereas in 16 patients, new HLA specificities, not present in the preconversion serum, were detected after conversion (16/35, 45.7%). This represents an 8.7% of the whole-study population (16/184). Those 16 patients included 13 patients with new NDSA (Group B) and three patients with DSA (Group C) (Table 4). A list of the new HLA specificities detected is shown in Table 5.

Table 4. Distribution of patients in the study (Converted) and control (Nonconverted) groups according to the appearance of new HLA specificities. Only patients with post-transplant HLA-Abs are considered.

	Converted (mTORi), *n* (%)	Nonconverted (CNI), *n* (%)
No changes in HLA-Abs specificities	19 (54.3)	8 (80)
de novo HLA-Abs	16 (45.7)	2 (20)
Nondonor-specific (NDSA)	13*	2
Donor-specific (DSA)	3	0

*In five of the 13 patients, the donor specificity of the HLA-Abs could not be determined because they were directed against the HLA-DQ and/or HLA-DP loci and the HLA-DQ/DP donor typing was not available. In one additional patient, HLA A/B/DR typing was not available.

Table 5. List of the new HLA specificities detected after mTORi conversion.

Patient	Class-I specificities	Class-II specificities	DSA
Patient 1	A33, A68	–	No
Patient 2	B37	–	No
Patient 3	A29*, B58*, A26, A34, A69, A80, B8, B13, B37, B44, B47, B48, B51, B52, B53, B58, B59, B60, B61, B63, B77, B81	–	Yes
Patient 4	–	DR53, DQ7, DQ8, DQ9	Unknown**
Patient 5	A11, A25, A26, A43, A66		No
Patient 6		DQ4, DQ7, DQ8, DQ9	Unknown**
Patient 7	A2, A23, A24, A32, A68, A69, B35, B38, B44, B45, B46, B49, B50, B51, B52, B53B, B56, B57, B58, B59, B62, B63, B71, B72, B75, B76, B77, B78, B82		No
Patient 8		DR52	No
Patient 9		DR53, DQ2, DQ7, DP3, DP6, DP9, DP14, DP17	Unknown**
Patient 10	A29, A43		Unknown***
Patient 11	A33*, A29, A30, A31		Yes
Patient 12		DR11, DP10, DP11, DP13, DP18, DP19, DP2, DP20, DP28	Unknown**
Patient 13	B8*, A80		Yes
Patient 14	B42		No
Patient 15	B76		No
Patient 16	A1, A11, A24, A25, A3, A36, A43, A66, A80, B13, B41, B47, B54, B57, B61, B63, B8	DQ2, DQ4, DQ7, DQ8, DQ9	Unknown**

*An asterisk following an HLA specificity indicates a DSA (patients 3, 11 and 13).

**Donor HLA-DQ/DP typing not available.

***In this case, donor HLA-typing was not available so it cannot be established whether the antibodies are DSA or not.

Distribution of the control population according to Single-Antigen Groups

Among the 10 patients with postconversion HLA-Abs (all pertaining to the Screening Group III, that is with HLA-Abs before and after conversion) in two patients new HLA specificities were detected but none of them were donor-specific (Group B, *de novo* NDSA Abs), whereas in the remaining eight patients, no new specificities were detected (Group A, No *de novo* Abs). None of the 63 patients of the control group developed DSA (Group C). The rate of appearance of new antibodies in this control group was a 3.2% of the whole group (Fig. 2).

In three patients of the study population, DSA were detected in the preconversion serum. In two cases, the DSA had disappeared at 3 months postconversion, whereas in one patient, it remained present. Two patients continued with functioning grafts 9 and 10 years after conversion whereas the third patient lost the graft 5 months after conversion, although the DSA was not detected in the postconversion sample.

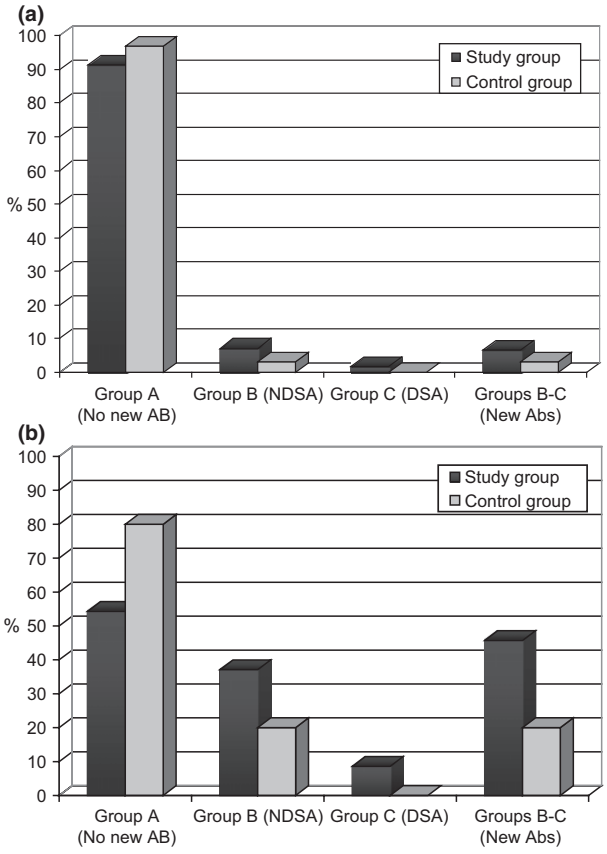

Figure 2 Distribution of patients in study and control groups according to Single Antigen analysis. (a) Percentages with respect to the whole study group. (b) Percentages with respect to the subgroup of patients with postconversion HLA-Abs.

Of 126 patients of group A were tested at 24 months to detect new Abs and in nine cases new specificities were detected (8 NDSA and 1 DSA) which accounts for an additional 7,1% of cases of seroconversion at 2 years.

Risk factors for the appearance of new HLA-Abs after conversion

The presence of HLA-Abs before conversion

Patients were classified according to the presence or absence of any type of HLA-Abs before mTORi conversion and the risk of developing new HLA-Abs after conversion was calculated. Among the 150 patients without HLA-Abs before conversion only six patients developed HLA-Abs in the months following conversion (6/150; 4%) and none developed DSA (0/150). By contrary among the 34 patients with HLA-Abs before conversion 10 patients developed new HLA-Abs (10/34; 29.4%; $P < 0.001$) and the three patients who developed DSA were included in this group (3/34; 8.8%; $P = 0.0035$) (Fig. 3).

Type of mTORi

No differences were observed with respect to the type of mTORi used. Eight patients of the 92 converted to SRL developed new HLA specificities [8/92 (8.7%)], whereas in the group of patients converted EVR new Abs were detected in 10 patients [10/92 (10.9%); pNS]. Two of the three patients with new DSA had been converted to SRL and the other one to EVR.

Type of CNI before conversion

About 179 patients were under CNI therapy before conversion and only five patients were not receiving CNI as main

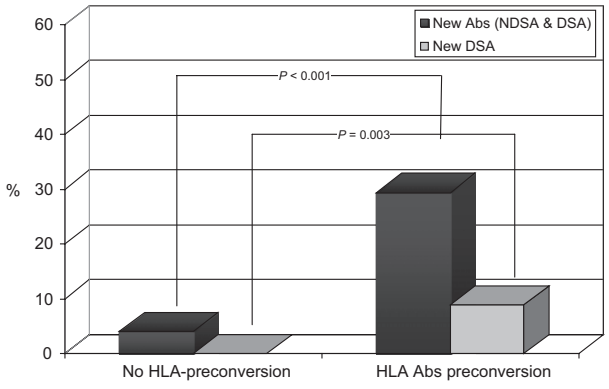

Figure 3 Percentage of patients who developed new HLA-Abs according to the preconversion HLA Ab status. In the group of patients without HLA-Abs before conversion, only 4% of cases developed new Abs and none of them were DSA, whereas in the group of patients with preconversion HLA-Abs one-third of patients developed new HLA specificities and more than 8% of cases developed DSA ($P < 0.001$ for any type of new Abs and $P = 0.003$ for DSA).

immunosuppression before the mTORi introduction (Azathioprine plus steroids). Ninety-three patients were under CSA and 86 under TAC. Seven of the 93 patients in the CSA group (7.5%) and 9 of the 86 in the TAC group (10.5%) developed new Abs (pNS). Additionally, two of the five patients without CNI before conversion developed new Abs. Considering the three patients who developed DSA two cases were receiving TAC and 1 CSA before conversion. It is important to consider that the percentage of retransplants was significantly higher in the TAC group (29 vs. 14%; $P = 0.01$), probably reflecting the different eras of use of CNI.

Steroid-containing immunosuppressive protocol at conversion

About 96 patients were receiving steroids at the moment of conversion (52.2%), whereas in the remaining 88 patients (47.8%), steroids had been previously eliminated, at least 3–6 months before conversion. In the group of patients receiving steroids, seven cases of new antibodies after conversion were detected (7/96, 7.3%), whereas in the group of patients without steroids were included the 11 remaining cases (11/88, 12.5%). This difference was not statistically significant ($P = 0.235$).

Time of conversion after transplant

Mean time from transplant to conversion was 91 months in group A and 104 months in group B + C (pNS), whereas median time was 69 and 65 months, respectively (pNS). One of the 32 patients converted in the first-year post-transplant (1/32, 3.12%) and 15 of the 152 patients converted after the first year (15/152, 9.87%) developed new Abs after conversion (pNS).

Indication for conversion

Eight patients converted due to IF/TA (8/88; 9.1%) and seven patients converted due to malignant neoplasm (7/65; 10.8%) developed new Abs, whereas none of the 17 patients converted for prevention developed new Abs.

Patient and graft evolution among groups

Patient survival at 2 years was 91.7% in group A and 93.8% in group B + C (P = NS), whereas graft survival at 2 years was 82.1% in group A and 62.5% in group B + C ($P = 0.05$). Figure 4 plots graft survival in both groups.

With respect to renal function, mean serum creatinine (sCr) was comparable in both groups (A vs. B + C) at the moment of conversion: 1.85 ± 0.73 mg/dl in group A and 1.89 ± 0.76 mg/dl in group B + C (pNS). Two years after conversion, mean sCr was 1.84 ± 0.93 mg/dl in group A and 1.64 ± 0.61 mg/dl in group B + C (pNS) when lost grafts were excluded and 3.23 ± 3.2 and 4.43 ± 4.1 mg/dl, respectively, when lost grafts were included in the analysis ($P = 0.13$) (Table 6).

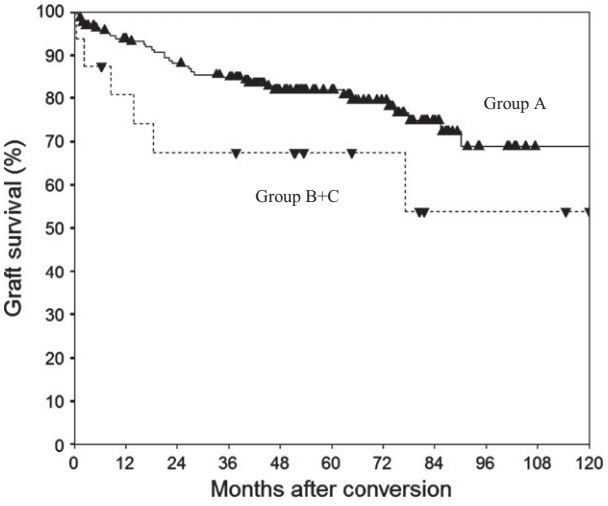

Figure 4 Comparison of graft survival between patients who did not develop new antibodies after conversion (Group A) and patients who developed new antibodies after conversion (Groups B + C). Log-rank: $P = 0.09$.

Table 6. Patient and graft evolution after mTORi conversion according to the development of *de novo* HLA-Abs after conversion.

	Group A	Groups B + C	*P*
n	168	16	
Patient survival (%) at 2 years	91.6	94.4	0.673
Graft survival (%) at 2 years	81.9	66.7	0.123
sCr at conversion (mg/dl)	1.84	1.89	0.856
sCr at 2 years excluding lost grafts	1.83	1.64	0.556
sCr at 2 years including lost grafts*	3.23	4.43	0.135

Group A: patients not developing *de novo* HLA-Abs after conversion.
Groups B + C: patients who developed NDSA (B) or DSA (C) after conversion.
*Lost grafts were considered as having a sCr of 10 mg/dl.

Discussion

This study demonstrates that conversion from a CNI-based to an mTORi-based immunosuppressive protocol in stable renal transplant recipients can be associated with an early humoral response in a small but probably relevant number of patients. This humoral response is detected by the appearance of new HLA-Abs in the first months after conversion that were not present immediately before conversion. Similar results have been recently published by Kamar *et al.* in a smaller series of patients converted to EVL [29]. Although it cannot be definitely established a cause/effect relationship between conversion and the appearance of new Abs the close temporal relationship between both events

makes it highly probable, specially if we consider that most patients were converted after the first and a high number of patients after the third post-transplant year, when the "spontaneous" rate of Abs appearance (without significant changes in immunosuppression) should be considerably lower, as it has been reported previously [30] and as it can be observed in the control group of our study.

There is not clear explanation for this finding, although some authors have hypothesized that CNI appear to significantly decrease the *de novo* formation of T-cell-dependent HLA-Abs responses in previously nonsensitized patients [31,32]. CNI therapy would block T-cell-dependent formation of germinal centers that generate plasma and memory B cells, necessary for the production of HLA-Abs.

Liefeldt *et al.* reported last year similar results in a small cohort of 61 patients recruited from two clinical trials converted to EVR in the first months after transplant and followed by 5 years [33]. Our study confirms those results in a much larger series of patients and shows that the risk is similar with SRL and that persists over time, not only in those patients converted in the early phase post-transplant. Surprisingly, in our series of patients converted for prevention without any clinical indication (17 cases), which highly resembles the cases published by Liefeldt, no new Abs were detected, probably reflecting the selection bias in this group of patients (low-risk patients).

More importantly, our study shows that this risk of developing HLA-Abs is not uniform among all the patients converted and they can be preclassified as low- or high-risk patients for the appearance of new HLA-Abs after conversion according to the presence or the absence of HLA-Abs before conversion. Those patients without any detectable HLA-Abs when conversion is planed have a extremely low risk of developing Abs that do not seem to be different to that observed in the control group whereas in patients with preformed HLA-Abs before conversion (irrespective they were already present before transplant or appeared after transplant) as far as almost one third will develop new HLA-Abs. Thus, the presence of HLA-Abs before conversion is probably selecting the population of highly responder patients.

An important aspect to consider of our results is the high proportion of cases that developed NDSA whereas DSA was detected in a small number of cases. This effect is difficult to explain and it is probably related to the presence of similar epitopes in different antigens [34]. Nevertheless, as it is shown in Table 5, in several cases, Abs were classified as NDSA due to the absence of DP and DQ typing in most donors, but the Abs might really be DSA, this is a limitation of the study. For these reasons, we decided to consider as a whole group all the patients with new HLA specificities irrespective of their DSA or NDSA classification when considering its effect on graft function and survival. In fact, there were no evident differences in these two aspects between patients with DSA and patients with NDSA.

The importance of the appearance of *de novo* Abs after transplantation has not yet been clearly established, but there is a growing body of evidence of the harmful effects of these Abs on the long-term graft survival via the development of chronic ABMR [3,7,8,12,30] and maximum efforts should be made to avoid its appearance and to detect its development following major immunosuppressive changes.

Another interesting finding in our series is that in five patients with preconversion HLA-Abs, these disappeared after conversion. This effect cannot easily be interpreted. It might be a consequence of Abs fluctuation with time [35,36], but it might also be explained by the modifications on regulatory CD25 T-cells induced by mTORi [22] and its possible effects suppressing alloimmune responses as it has been suggested by Salama *et al.* [37].

According to our results, conversion to an mTORi-based protocol with CNI elimination can be associated with an increased risk of developing *de novo* HLA-Abs, especially in patients with preformed Abs before conversion. Further studies are needed to confirm these results. In our opinion, the HLA-Abs status should be checked in all renal transplant patients considered for conversion to an mTORi-based protocol with CNI elimination and in those patients with preformed HLA-Abs the risks of conversion should be carefully balanced with the benefits according to the indication for conversion. In those patients converted to an mTORi, we suggest that they should be routinely screened in the first months after conversion for the appearance of *de novo* HLA-Abs and in those cases where new HLA-Abs are detected a quick reconversion to a CNI should be considered, although the effect of this strategy needs to be evaluated. These recommendations seem to be adequate irrespective of the moment of conversion or the indication for mTORi introduction.

Authorship

JCRSM and MA: designed the study. EQ: collected data. DSS, MLH and IR: performed serum analysis for antibody testing. JCRSM and MLH: performed data analysis. ER, MA and CGA: contributed to analysis and interpretation of results. JCRSM: wrote the paper. All authors reviewed and criticized the final manuscript.

Funding

This work was partially supported by grants of Fondo de Investigaciones Sanitarias-ISCIII (PI1100990 and REDINREN) and Fundación Marqués de Valdecilla-IFIMAV (API 11/24).

Acknowledgements

We are especially grateful to Camino Villa, Maria Eugenia Cuadrado and Asun Riaño for their care in serum samples organization and selection and to Julia Vicente and Luz Valcarcel for the technical help provided in the measurement of HLA-Abs. We also thank Dra. Sánchez-Fructuoso for her critical reading of the final manuscript.

References

1. Terasaki PI. Humoral theory of transplantation. *Am J Transplant* 2003; **3**: 665.
2. Terasaki PI, Cai J. Humoral theory of transplantation: further evidence. *Curr Opin Immunol* 2005; **17**: 541.
3. Terasaki PI, Cai J. Human leukocyte antigen antibodies and chronic rejection: from association to causation. *Transplantation* 2008; **86**: 377.
4. Palomar R, Lopez-Hoyos M, Pastor JM, *et al.* Impact of HLA antibodies on transplant glomerulopathy. *Transplant Proc* 2005; **37**: 3830.
5. Issa N, Cosio FG, Gloor JM, *et al.* Transplant glomerulopathy: risk and prognosis related to anti-human leukocyte antigen class II antibody levels. *Transplantation* 2008; **86**: 681.
6. Claas FH. Clinical relevance of circulating donor-specific HLA antibodies. *Curr Opin Organ Transplant* 2010; **15**: 462.
7. Lee PC, Zhu L, Terasaki PI, Everly MJ. HLA-specific antibodies developed in the first year posttransplant are predictive of chronic rejection and renal graft loss. *Transplantation* 2009; **88**: 568.
8. Hourmant M, Cesbron-Gautier A, Terasaki PI, *et al.* Frequency and clinical implications of development of donor-specific and non-donor-specific HLA antibodies after kidney transplantation. *J Am Soc Nephrol* 2005; **16**: 2804.
9. El-Zoghby ZM, Stegall MD, Lager DJ, *et al.* Identifying specific causes of kidney allograft loss. *Am J Transplant* 2009; **9**: 527.
10. Einecke G, Sis B, Reeve J, *et al.* Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. *Am J Transplant* 2009; **9**: 2520.
11. Gaston RS, Cecka JM, Kasiske BL, *et al.* Evidence for antibody-mediated injury as a major determinant of late kidney allograft failure. *Transplantation* 2010; **90**: 68.
12. Sellares J, de Freitas DG, Mengel M, *et al.* Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. *Am J Transplant* 2012; **12**: 388.
13. Montgomery RA, Hardy MA, Jordan SC, *et al.* Consensus opinion from the antibody working group on the diagnosis, reporting, and risk assessment for antibody-mediated rejection and desensitization protocols. *Transplantation* 2004; **78**: 181.
14. Hernandez D, Sanchez FA, Seron D, *et al.* Chronic transplant nephropathy. *Nefrologia* 2006; **26**(Suppl. 1): 1.
15. Bumbea V, Kamar N, Ribes D, *et al.* Long-term results in renal transplant patients with allograft dysfunction after switching from calcineurin inhibitors to sirolimus. *Nephrol Dial Transplant* 2005; **20**: 2517.
16. Weir MR, Mulgaonkar S, Chan L, *et al.* Mycophenolate mofetil-based immunosuppression with sirolimus in renal transplantation: a randomized, controlled Spare-the-Nephron trial. *Kidney Int* 2011; **79**: 897.
17. Alberu J, Pascoe MD, Campistol JM, *et al.* Lower malignancy rates in renal allograft recipients converted to sirolimus-based, calcineurin inhibitor-free immunotherapy: 24-month results from the CONVERT trial. *Transplantation* 2011; **92**: 303.
18. Budde K, Becker T, Arns W, *et al.* Everolimus-based, calcineurin-inhibitor-free regimen in recipients of de-novo kidney transplants: an open-label, randomised, controlled trial. *Lancet* 2011; **377**: 837.
19. Schena FP, Pascoe MD, Alberu J, *et al.* Conversion from calcineurin inhibitors to sirolimus maintenance therapy in renal allograft recipients: 24-month efficacy and safety results from the CONVERT trial. *Transplantation* 2009; **87**: 233.
20. Ruiz JC, Campistol JM, Grinyo JM, *et al.* Early cyclosporine a withdrawal in kidney-transplant recipients receiving sirolimus prevents progression of chronic pathologic allograft lesions. *Transplantation* 2004; **78**: 1312.
21. Ruiz JC, Alonso A, Arias M, *et al.* Conversion to sirolimus. *Nefrologia* 2006; **26**(Suppl. 2): 52.
22. Segundo DS, Ruiz JC, Izquierdo M, *et al.* Calcineurin inhibitors, but not rapamycin, reduce percentages of $CD4^+CD25^+FOXP3^+$ regulatory T cells in renal transplant recipients. *Transplantation* 2006; **82**: 550.
23. Ruiz JC, Campistol JM, Sanchez-Fructuoso A, *et al.* Increase of proteinuria after conversion from calcineurin inhibitor to sirolimus-based treatment in kidney transplant patients with chronic allograft dysfunction. *Nephrol Dial Transplant* 2006; **21**: 3252.
24. Ruiz JC, Sanchez A, Rengel M, *et al.* Use of the new proliferation signal inhibitor everolimus in renal transplant patients in Spain: preliminary results of the EVERODATA registry. *Transplant Proc* 2007; **39**: 2157.
25. Sanchez-Fructuoso AI, Ruiz JC, Perez-Flores I, Gomez AC, Calvo RN, Arias M. Comparative analysis of adverse events requiring suspension of mTOR inhibitors: everolimus versus sirolimus. *Transplant Proc* 2010; **42**: 3050.
26. Ruiz JC, Sanchez-Fructuoso A, Rodrigo E, *et al.* Conversion to everolimus in kidney transplant recipients: a safe and simple procedure. *Transplant Proc* 2006; **38**: 2424.
27. Sanchez-Fructuoso AI, Ruiz JC, Calvo N, *et al.* Everolimus as primary immunosuppression in kidney transplantation: experience in conversion from calcineurin inhibitors. *Transplantation* 2012; **93**: 398.
28. Tait BD, Susal C, Gebel HM, *et al.* Consensus guidelines on the testing and clinical management issues associated with

HLA and non-HLA antibodies in transplantation. *Transplantation* 2013; **95**: 19.
29. Kamar N, Del BA, Congy-Jolivet N, *et al.* Incidence of donor-specific antibodies in kidney transplant patients following conversion to an everolimus-based calcineurin inhibitor-free regimen. *Clin Transplant* 2013; **27**: 455.
30. Everly MJ, Rebellato LM, Haisch CE, *et al.* Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation* 2013; **95**: 410.
31. Stegall MD, Dean PG, Gloor J. Mechanisms of alloantibody production in sensitized renal allograft recipients. *Am J Transplant* 2009; **9**: 998.
32. Stegall MD, Raghavaiah S, Gloor JM. The (re)emergence of B cells in organ transplantation. *Curr Opin Organ Transplant* 2010; **15**: 451.
33. Liefeldt L, Brakemeier S, Glander P, *et al.* Donor-specific HLA antibodies in a cohort comparing everolimus with cyclosporine after kidney transplantation. *Am J Transplant* 2012; **12**: 1192.
34. Cai J, Terasaki PI, Mao Q, *et al.* Development of nondonor-specific HLA-DR antibodies in allograft recipients is associated with shared epitopes with mismatched donor DR antigens. *Am J Transplant* 2006; **6**: 2947.
35. Rebellato LM, Ozawa M, Verbanac KM, Catrou P, Haisch CE, Terasaki PI. Clinical and anti-HLA antibody profile of nine renal transplant recipients with failed grafts: donor-specific and non-donor-specific antibody development. *Clin Transpl* 2006; 241.
36. Loupy A, Hill GS, Suberbielle C, *et al.* Significance of C4d Banff scores in early protocol biopsies of kidney transplant recipients with preformed donor-specific antibodies (DSA). *Am J Transplant* 2011; **11**: 56.
37. Salama AD, Najafian N, Clarkson MR, Harmon WE, Sayegh MH. Regulatory $CD25^+$ T cells in human kidney transplant recipients. *J Am Soc Nephrol* 2003; **14**: 1643.