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Degree of HLA class II eplet mismatch load improves prediction of antibody-mediated rejection in living donor kidney transplantation

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A B S T R A C T

Background: HLA mismatching is a well known risk factor for worst outcomes in kidney transplantation.
Methods: In the present study, HLA antigen and eplet mismatches were determined in 151 living donor-recipient pairs transplanted between 2007 and 2014 and rejection episodes and graft survival were evaluated.
Results: We found that high HLA-II eplet mismatch load (EpMM ≥ 13, *versus* low EpMM ≤ 5), was an independent predictor of AMR (adjusted HR = 14.839; $P = 0.011$), while HLA-II AgMM was not. We also showed that HLA-II EpMM load was a significant better predictor of AMR than AgMM (*c*-statistic = 0.064; $P = 0.023$). After discriminating HLA-II into HLA-DR and HLA-DQ *loci* we demonstrated that high *versus* low eplet mismatch load for HLA-DR (T3 ≥ 6 *versus* T = 0–1, p = 0.013) and HLA-DQ (T3 ≥ 7 *versus* T = 0–1, p = 0.009) are independent predictors for AMR.

HLA-II EpMM increased discrimination performance of the classical HLA-II AgMM risk model (IDI, 0.061, 95%CI: 0.005–0.195) for AMR. Compared with AgMM, HLA-II eplet model adequately reclassified 13 of 17 patients (76.5%) with AMR and 92 of 134 patients (68.7%) without AMR (cfNRI, 0.785, 95%CI: 0.300–1.426).
Conclusions: Our study evidences that eplet-based matching is a refinement of the classical HLA antigen mismatch analysis in LDKT and is a potential biomarker for personalized assessment of alloimmune risk.

1. Introduction

Human leukocyte antigens (HLA) matching has been associated with better kidney graft survival for more than 30 years [1,2] and HLA-ABDR *loci* have been used in deceased donors (DD) kidneys allocation algorithms worldwide [3,4]. Moreover, HLA-DR antigen matching seems to be more beneficial in terms of long-term graft survival, when compared to HLA class I antigens, possibly as a result from matching at $DR\beta_{1/3/4/5}$ and $DQ\alpha_1/\beta_1$ haplotypes [5]. Notwithstanding, the strong linkage disequilibrium between HLA-DR and HLA-DQ antigens [6], and different HLA-$DR\beta_1$ alleles within an antigen group that may be associated with different $DQ\alpha_1/\beta_1$ antigens, result in different degrees of matching with subsequent distinct outcomes [7].

The outstanding importance of HLA matching in the field of transplantation led HLA typing to evolve greatly from serology-based methods to molecular typing techniques, which allowed the

Abbreviations: Å, Ångstroms; AAMM, aminoacid mismatch; Ag, antigen; AgMM, antigen mismatches; AMR, antibody-mediated rejection; AR, acute rejection; ATG, antithymocyte globulin; CDC, complement-dependent cytotoxicity; CI, confidence interval; CMR, cellular mediated rejection; cPRA, calculated PRA; CV, coefficient of variability; DD, deceased donors; DESA, donor epitope specific antibodies; DGF, delayed graft function; *dn*DSA, *de novo* DSA; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; EMS, electrostatic mismatch score; EpMM, eplet mismatches; GF, graft failure; HLA, human leukocyte antigens; HLA-I, HLA class I; HLA-II, HLA class II; HMS, hydrophobicity mismatch score; HvG, host-*versus*-graft; IDI, integrated discrimination improvement; IQR, interquartile range; IVIg, intravenous immunoglobulin; KPD, kidney paired donation; LDKT, living donor kidney transplantation; MFI, mean fluorescence intensity; MM, mismatches; NIH, National Institute of Health; NMDP, National Marrow Donor Program; NRI, net reclassification index; PRA, panel reactive antibodies; RT, room temperature; SAB, single-antigen bead; sCr, serum creatinine; SD, standard deviation; SPA, solid phase assays; TAC, tacrolimus; vXM, virtual crossmatching

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identification of more than 23,000 alleles so far (release 3.37.0 of the IPD-IMGT/HLA Database, 2019-07). This exponential increase of HLA alleles makes impracticable to perform allelic matching in kidney transplantation, as well as donor-specific antibodies (DSA) definition and accurate virtual crossmatching (vXM), as single-antigen bead (SAB) assay platforms usually only support 100 HLA antigen coated microparticles [8]. For this reason, kidney allocation is usually done considering HLA broad antigens (Ag) mismatches (MM) in host-*versus*-graft (H*v*G) direction. Recent published studies have shown that this limited strategy, that defines two HLA antigens as matched or unmatched for each HLA *locus*, may be rendered more precise using HLA epitope matching described by Rene Duquesnoy [9–12].

This *in-silico* theoretical approach considers each HLA antigen as a string of polymorphic aminoacid residues within 3.0–3.5 Ångstroms (Å) at the molecule surface, capable of ensuing alloantibody recognition and reactivity, termed functional epitopes or eplets. Patients HLA antigens represent the self-repertoire of eplets against which no antibodies are developed. HLA eplets can be private, if restricted to a single HLA antigen, or public if shared by multiple HLA antigens. Such public epitopes result in both intra- and inter-*locus* cross-reactions, identified many years ago as HLA cross-reactive groups [13], explaining the development of non-DSA, albeit donor epitope specific antibodies (DESA).

HLA matchmaker has been used worldwide by several groups that demonstrated that HLA eplet mismatch load (EpMM) is associated with the emergence of *de novo* DSA (*dn*DSA), acute rejection (AR), transplant glomerulopathy and graft failure (GF) [14–18]. This strategy for matching assessment is also important in serum analysis and identification of acceptable mismatches for highly sensitized patients awaiting kidney transplantation [19,20] and may also be a precious tool for immunosuppression minimization, safely reducing its adverse effects [21].

Although HLA matching importance has been mainly studied in deceased donation, improvements in matching strategies within living donor kidney transplantation (LDKT) are fundamental since the majority of transplants are performed with unrelated donors [22]. Besides, in patients with poorly matched living donors, kidney paired donation (KPD) programs should be considered, to allow for a better HLA matched transplant [23]. The possibility that HLA matching at epitope level would improve alloimmune risk prediction in LDKT is an attractive one and merits further investigation.

Hence, this study aimed to explore if HLA eplet mismatch load, defined by HLAmatchmaker algorithm, could significantly improve the prediction of acute rejection (T-cell [24] and antibody-mediated) in comparison with the 'classical' approach at antigen level, in a cohort LDKT recipients.

2. Materials and methods

2.1. Study population

We retrospectively analyzed 157 AB0-compatible consecutive LDKT between January 1, 2007 and December 31, 2014 performed in Hospital Santo António – Centro Hospitalar Universitário do Porto. Patients with early graft loss within the first 30 days post-transplant (n = 4, all losses were deemed technical) or without DNA based HLA typing (n = 2) were excluded, defining the remaining 151 LDKT recipients as the studied cohort. Median follow-up after transplant was 70.1 (56.2–104.2) months.

2.2. HLA typing and mismatch analysis

HLA intermediate resolution typing for HLA class I and class II (HLA-I + II) was performed for all pairs using reverse sequence-specific oligonucleotide (LABType® rSSO typing kits, One Lambda, Canoga Park, CA, USA). HLA allelic typing for HLA-A, -B, -C, -$DR_{\beta1/3/4/5}$ and -$DQ_{\alpha1/\beta1}$ *loci* was assigned based on the observed National Marrow Donor Program (NMDP) code, linkage disequilibrium and Caucasian population frequencies using HaploStats (available via http://www.haplostats.org/), a web-based application provided by the NMDP Bioinformatics Group for imputation of high resolution HLA genotypes [25,26].

Classical HLA-I + II broad antigen mismatches (AgMM) were determined by counting HLA-I + II AgMM for HLA-A, -B, C, –$DR\beta_1$ and –$DQ\beta_1$, in H*v*G direction. On the other hand, HLA eplet mismatch load was defined with allelic typing, allowing the quantification of intra- and interlocus mismatched eplets between donor and recipient alleles. To assess this, HLAMatchmaker software HLA-ABC matching version v02 and HLA DRDQDP matching version v02.1, available at http://www.epitopes.net/downloads.html, was used. Antigen level mismatch for HLA class I (HLA-I) and HLA class II (HLA-II) were analyzed as a continuous variable. EpMM for HLA-I and HLA-II were analyzed both as continuous variables and as categories defined by their terciles: T1 (EpMM ≤ 5) as low, T2 (5 < EpMM < 10) as moderate and T3 (EpMM ≥ 10) as high HLA-I EpMM load and T1 (EpMM ≤ 5) as low, T2 (5 < EpMM < 13) as moderate and T3 (EpMM ≥ 13) as high HLA-II EpMM load. HLA-DR and HLA-DQ were also analyzed separately as terciles: T1 (EpMM ≤ 1) as low, T2 (2 ≤ EpMM ≤ 5) as moderate and T3 (EpMM ≥ 6) for HLA-DR and T1 (EpMM ≤ 1) as low, T2 (2 ≤ EpMM ≤ 6) as moderate and T3 (EpMM ≥ 7) for HLA-DQ.

2.3. Anti-HLA antibodies assays

Patients in active waiting list are studied periodically to assess their HLA alloimmunization status with cellular and solid-phase assays (SPA). The cellular assay consists in standard complement-dependent cytotoxicity (CDC) National Institute of Health (NIH) crossmatch, using a home-made cell panel composed by 45–50 donors with known HLA typing, to test Dithiothreitol -treated and untreated patient's sera. This assay allows the determination of cytotoxic PRA, considered positive if higher than 5%, and identification of complement-fixing HLA antibodies.

SPA were carried out using coded-colour microbeads coated with purified class I or class II HLA antigens based on Luminex Xmap® Technology (LABScreen® Mixed kit, OneLambda, Canoga Park, CA, USA). Briefly, anti-HLA antibodies present in patient sera will bind to HLA antigens on the beads after 30 min incubation at room temperature (RT). After three washes antibody-antigen complexes are labeled with 100 µL of 1:100 R-Phycoerythrin-conjugated goat anti-human IgG (One Lambda Inc.) during a second 30 min at RT. incubation. After two final washes, mean fluorescence intensity (MFI) of each bead was measured using a LABScan™ 100 flow analyzer (Luminex, Austin, TX). Patients with a pretransplant positive screening for anti-HLA antibodies were tested with SAB assays using 6% ethylenediaminetetraacetic acid treated sera (LabScreen Single Antigen Beads®, OneLambda, Canoga Park, CA). The analysis was performed using HLAfusion™ software, version 3.4, and MFIs higher than 1,000 are considered positive, as widely reported. MFImax refers to the highest MFI level of all detected DSA. Calculated PRA (cPRA) was assessed with the online calculator at Eurotransplant website (http://www.etrl.org/Virtual%20PRA/Default.aspx), considering all antibody specification results available for each patient. Evaluation of post-transplant DSA status was only performed as clinically-driven, at time of graft dysfunction or proteinuria appearance or increase. All patients with acute rejection episodes had DSA status checked at time of rejection diagnosis.

All patients were tested negative for CDC crossmatch, using T and B lymphocytes separated with magnetic beads (Dynabeads™ HLA class I and class II, Invitrogen™ Carlsbad, CA, USA), directed from donor peripheral blood.

2.4. Clinical data

Data regarding recipient and donor characteristics, and pre- and

Table 1
Demographics, clinical and immunological characteristics of the studied cohort.

	All cohort N = 151	No AR N = 118	AR N = 33	*P*
Follow-up (months), median (IQR)	70.1 (56.2–104.2)	81.6 (54.7–107.2)	77.4 (62.0–89.9)	0.712
Recipient age (years), mean ± SD	38.2 ± 13.2	38.4 ± 13.7	37.6 ± 11.4	0.762
Donor age (years), mean ± SD	46.9 ± 10.2	47.6 ± 10.6	44.3 ± 7.9	0.108
Female recipient, n (%)	50 (33)	36 (31)	14 (42)	0.199
Female donor, n (%)	110 (73)	86 (73)	24 (73)	0.986
Living unrelated donor, n (%)	54 (36)	38 (32)	16 (49)	0.085
HLA share haplotype, n (%)*				0.321
0	13 (13)	11 (14)	2 (12)	
1	75 (77)	60 (75)	15 (88)	
2	9 (9)	9 (11)	0 (0)	
Dialysis vintage (months), median (IQR)	**11 (0–27)**	**9 (0–25)**	**20 (3–57)**	**0.030**
Preemptive KT, n (%)	38 (25)	33 (28)	5 (15)	0.134
Retransplant, n (%)	17 (11)	11 (9)	6 (18)	0.209
Previous blood transfusion, n (%)	41 (27)	28 (24)	13 (39)	0.074
Previous pregnancy, n (%)#	25 (50)	19 (53)	6 (43)	0.529
Cytotoxic PRA (%), median (IQR)	0 (0–0) [0–80]	0 (0–0) [0–20]	0 (0–0) [0–80]	0.447
Cytotoxic PRA ≥ 5%, n (%)	**13 (9)**	**7 (6)**	**6 (18)**	**0.038**
Calculated PRA (%), median (IQR)	**0 (0–0)**	**0 (0–0)**	**0 (0–31)**	**0.009**
Induction, n (%)				0.090
No	13 (9)	11 (9)	2 (6)	
Basiliximab	127 (84)	101 (86)	26 (79)	
ATG	11 (7)	6 (5)	5 (15)	
Calcineurin inhibitor, n (%)				0.296
Cyclosporine	12 (8)	8 (7)	4 (12)	
Tacrolimus	139 (92)	110 (93)	29 (88)	
Anti-HLA antibodies, n (%)	**26 (17)**	**15 (13)**	**11 (33)**	**0.006**
DSA, n (%)	**13 (9)**	**6 (5)**	**7 (21)**	**0.008**
Desensitized, n (%)§	6 (46)	3 (50)	3 (43)	1
HLA-I + II AgMM, mean ± SD	**4.79 ± 2.53**	**4.55 ± 2.58**	**5.67 ± 2.19**	**0.015**
HLA-I AgMM, mean ± SD	3.15 ± 1.64	3.03 ± 1.68	3.58 ± 1.44	0.107
HLA-II Ag MM, mean ± SD	**1.64 ± 1.16**	**1.52 ± 1.16**	**2.09 ± 1.04**	**0.008**
HLA-DR antigen MM, mean ± SD	0.93 ± 0.66	0.85 ± 0.65	1.21 ± 0.65	0.006
HLA-DQ antigen MM, mean ± SD	0.72 ± 0.62	0.67 ± 0.63	0.88 ± 0.55	0.061
HLA-I + II EpMM, mean ± SD	**16.8 ± 10.7**	**15.5 ± 10.7**	**21.2 ± 9.4**	**0.003**
HLA-I + II EpMM terciles, n (%)				**0.008**
T1: 0–10	**54 (36)**	**49 (42)**	**5 (15)**	
T2: 11–20	**48 (32)**	**37 (31)**	**11 (33)**	
T3: ≥21	**49 (33)**	**32 (27)**	**17 (52)**	
HLA-I EpMM, mean ± SD	7.5 ± 4.6	7.1 ± 4.8	8.6 ± 3.5	0.101
HLA-I EpMM terciles, n (%)				0.259
T1: 0–5	50 (33)	43 (36)	7 (21)	
T2: 6–9	51 (34)	38 (32)	13 (39)	
T3: ≥10	50 (33)	37 (31)	13 (39)	
HLA-II EpMM, mean ± SD	**9.3 ± 7.7**	**8.4 ± 7.7**	**12.7 ± 7.2**	**0.001**
HLA_DR epitope MM, mean ± SD	3.9 ± 3.8	3.6 ± 3.7	5.1 ± 4.1	0.035
HLA_DQ epitope MM, mean ± SD	5.4 ± 5.1	4.8 ± 5.0	7.6 ± 5.0	0.005
HLA-II EpMM terciles, n (%)				**0.008**
T1: 0–5	**54 (36)**	**49 (42)**	**5 (15)**	
T2: 6–12	**48 (32)**	**37 (31)**	**11 (33)**	
T3: ≥13	**49 (33)**	**32 (27)**	**17 (52)**	
HLA_DR epitope MM terciles, n (%)				**0.020**
T1: 0–1	**54 (36)**	**49 (42)**	**5 (15)**	
T2: 2–5	**50 (33)**	**36 (31)**	**14 (42)**	
T3: ≥6	**47 (31)**	**33 (28)**	**14 (42)**	
HLA_DR epitope MM terciles, n (%)				**0.041**
T1: 0–1	**49 (32)**	**44 (37)**	**5 (15)**	
T2: 2–6	**46 (30)**	**35 (30)**	**11 (33)**	
T3 ≥ 7	**56 (37)**	**39 (33)**	**17 (52)**	
Last SCr (mg/dl)¶, median (IQR)	**1.31 (1.10–1.53)**	**1.29 (1.07–1.49)**	**1.38 (1.19–2.29)**	**0.029**
Last eGFR (ml/min)¶, median (IQR)	**59.8 (49.2–72.5)**	**61.0 (51.9–74.9)**	**50.0 (31.1–68.5)**	**0.003**
Last proteinuria (g/g)¶, median (IQR)	**0.1 (0.1–0.3)**	**0.1 (0.1–0.3)**	**0.2 (0.1–1.0)**	**0.001**
Graft failure, n (%)	**9 (6)**	**2 (2)**	**7 (21)**	**< 0.001**

HLA, human leukocyte antigen; HLA-I, HLA class I; HLA-II, HLA class II; NDSA, non-donor-specific antibodies; DSA, donor-specific antibodies; Ep, eplet; Ag, antigen; MM, mismatches; SD, standard deviation; IQR, interquartile range; PRA, panel reactive antibodies, Anti-IL2R-Ab, anti-interleukin-2 receptor antibody; ATG, anti-thymocyte globulin; CsA, cyclosporine; sCr, serum creatinine; eGFR, estimated glomerular filtration rate.

Analysis considering only women (n = 50);

* Analysis considering only LRD (n = 97).

§ Analysis considering only patients with preformed DSA (n = 13);

¶ Analysis considering only patients with functioning graft at the end of follow-up (n = 140).

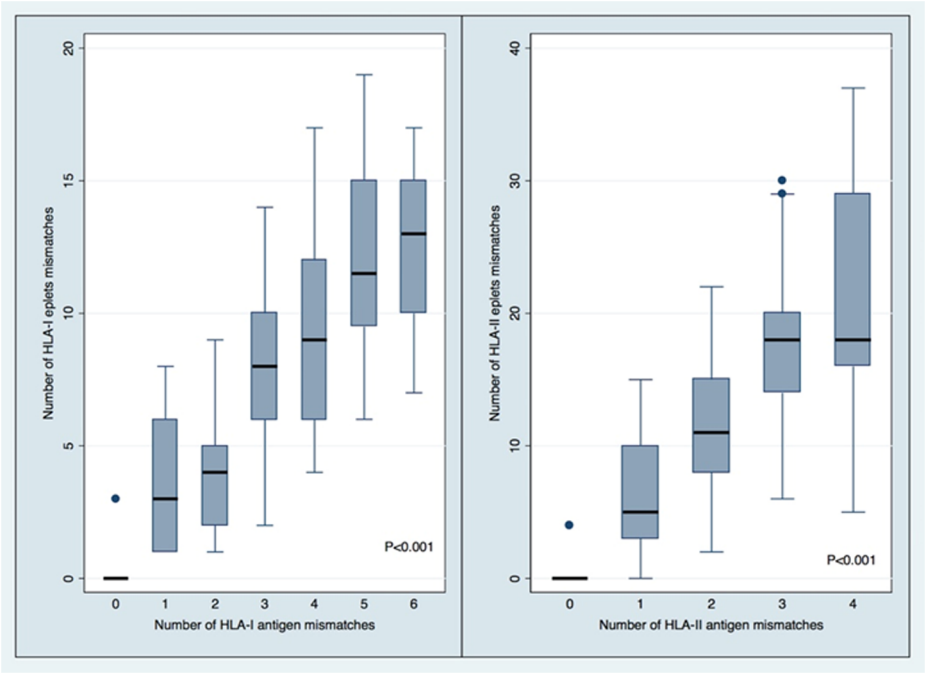

Fig. 1. Association between the number of HLA class I and class II antigen mismatches with the eplet mismatch load.

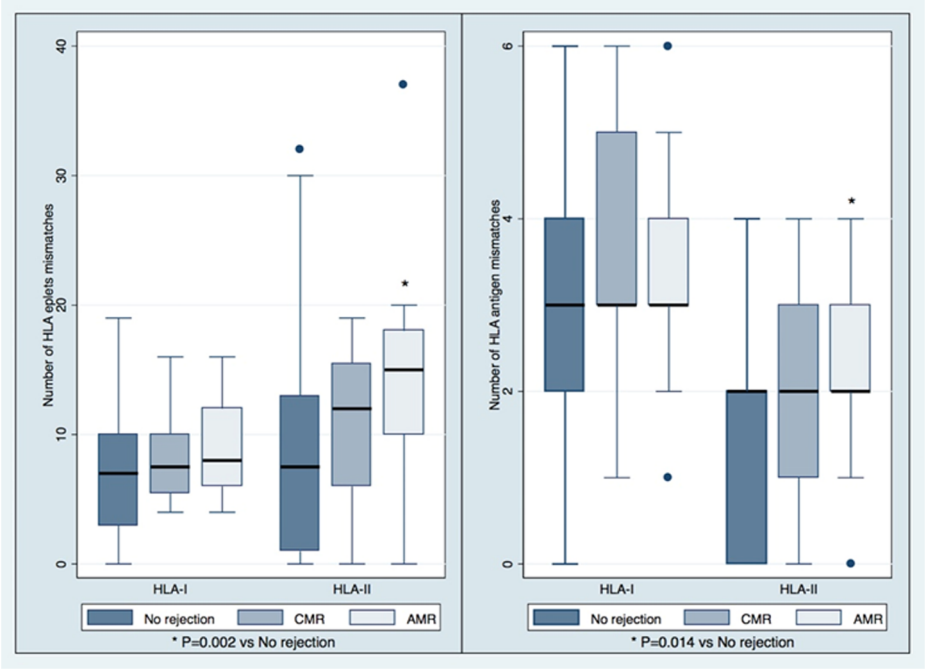

Fig. 2. Association between the number of HLA class I and class II eplet and antigen mismatches with rejection episodes considering: no rejection, CMR and AMR.

post-transplantation variables were collected retrospectively. Graft biopsies were performed for cause only, when in the presence of prolonged delayed graft function (DGF), a rise in serum creatinine (sCr, mg/dl) by more than 20% compared with previous measurements and/or increased levels of proteinuria (g/g). All patients were followed-up from time of transplant until death, GF defined as return to dialysis or retransplant or June 30, 2018. Graft survival was analyzed considering GF censored for death with a functioning graft. For patients with a functioning graft at the end of follow-up, the last value of sCr, estimated glomerular filtration rate (eGFR, ml/min) and proteinuria were registered. eGFR was evaluated using the 2006 Modification of Diet in Renal Disease equation [27].

2.5. Induction protocol and maintenance immunosuppression

Induction therapy was used in a majority of patients (91%), with an anti-IL-2 receptor antibody (Basiliximab Novartis®, 20 mg twice at day 0 and 4) or a polyclonal antithymocyte globulin (ATG Fresenius®, 3 mg/kg for 5–7 days). ATG was primarily used in highly sensitized retransplants patients (7%). All patients had similar triple maintenance immunosuppression, consisting of a calcineurin inhibitor, tacrolimus (TAC) or cyclosporine, mycophenolate mofetil or azathioprine, and prednisolone. No immunosuppression minimization strategy was implemented in these patients.

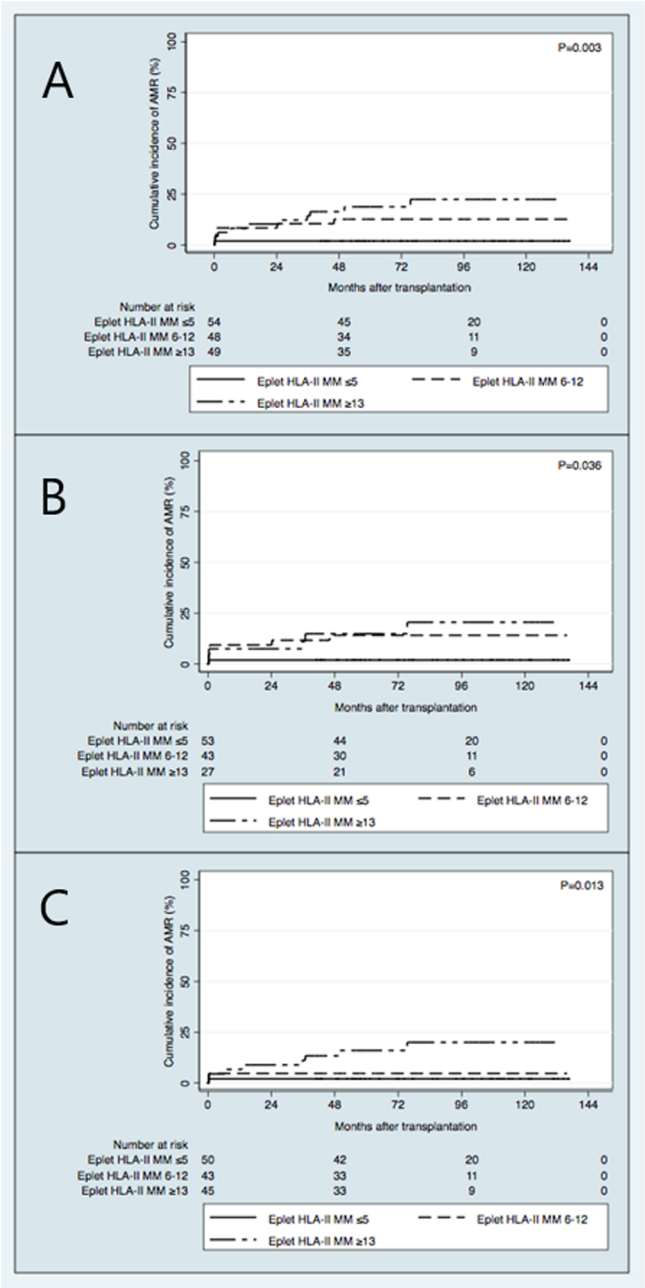

Fig. 3. Cumulative incidence curves at 96 month for antibody-mediated rejection considering HLA class II EpMM load in terciles: a) overall cohort (T1 = 2%, T2 = 13%, T3 = 22%, $P = 0.003$); b) patients with 0–2 HLA class II antigen MM (T1 = 2%, T2 = 14%, T3 = 20%, $P = 0.036$); c) Patients without preformed DSA (T1 = 2%, T2 = 5%, T3 = 20%, $P = 0.013$). MM – mismatches.

2.6. Rejection diagnosis and treatment

Graft rejection was defined as biopsy proven rejection (specimens were evaluated by light microscopy and immunofluorescence staining for C4d) and classified according to Banff classification updated in 2017 [28]. Mild acute cellular mediated rejection (CMR Banff grade I) was treated with pulse steroids (500 mg methylprednisolone for 3 days) and increased maintenance immunosuppression. All other acute CMR were treated with ATG. All patients with antibody-mediated rejection (AMR) were treated with plasmapheresis every other day and intravenous immunoglobulin (IVIg) 100 mg/kg after each session; per protocol, the number of plasmapheresis sessions was 4. After the last plasmapheresis session, every patient received high-dose IVIg (2 g/kg) divided in four daily doses and one dose of rituximab (375 mg/m^2); a similar dose of IVIg (2 g/kg) was repeated 1 month later.

2.7. Statistical analysis

Continuous data were described using mean (standard deviation, SD) or median (interquartile range, IQR) and categorical data were expressed as numbers (frequencies). The distributions of continuous variables were analyzed using Kolmogorov–Smirnov test. Categorical data including demographic, clinical and immunological features were compared using Pearson $\chi 2$ test or Fisher's exact test, as appropriate. Continuous variables were compared with Student *t*-test or Mann–Whitney *U* test, as appropriate. Graft survival curves were visualized using Kaplan–Meier method, with comparison between patients' groups being done by log-rank test.

Independent predictors of acute CMR and AMR were explored by univariate and multivariable Cox regression. The model used for the multivariable analyses included only those variables presenting a univariate P-value < 0.1. Then, we assessed the difference in the predictive capacity for TCMR and AMR of HLA antigen mismatch (as a continuous variable) and EpMM load (categorized in terciles) separately, considering two multivariable models adjusted for the same covariates. Afterwards, a detailed analysis was performed for HLA-II discriminating HLA-DR and HLA-DQ *loci* according to rejection *status*. Harrell c statistic was estimated for each model; c-statistic estimations were repeated 1000 times using bootstrap samples to derive 95% confidence intervals (CIs) and assess the difference in the c statistic between the models with its 95%CI. We used category-free net reclassification index (NRI) and integrated discrimination improvement (IDI) to assess the improvement of EpMM in comparison with antigen mismatch based models for the prediction of CMR and AMR. Models calibration and goodness of fit were assessed by visual examination of a calibration plot.

A two-sided P-value of < 0.05 was considered as statistically significant. Statistical calculations were performed using SPSS, version 23.0 (SPSS Inc., Chicago, IL, USA) and Stata/MP, version 14.1 (Stata Corp, College Station, TX).

3. Results

3.1. Cohort baseline characteristics

The studied cohort included 151 recipients of LDKT between January 1, 2007 and December 31, 2014. Thirty-three patients experienced that least one AR episode (21.9%) during median follow-up time after transplantation of 70.1 (IQR, 56.2–104.2) months. AR episodes were classified according to last Banff classification as CMR (n = 16) and AMR (n = 17). The median time until CMR was 1.4 months (IQR: 0.2–51.4) [range: 0.1–118.1] and until AMR 6.3 months (IQR: 0.3–36.3) [range: 0.2–75.4].

Baseline clinical and immunological characteristics are presented in Table 1. At transplant, patients that came to experience AR were more sensitized, with higher cytotoxic and calculated PRA values ($P = 0.038$ and $P = 0.009$), with longer dialysis vintage time ($P = 0.030$) and, as expected, more preformed DSA ($P = 0.008$).

Merely 9% of related LDKT were a HLA full identical match, that is, 94% of the patients were transplanted with HLA mismatches. The median number of HLA-I + II AgMM was 4.79 ± 2.53 (range 0–10), being significantly higher within AR sub-cohort (5.67 ± 2.19, $P = 0.015$). The median number of HLA-I + II EpMM was 16.8 ± 10.7 (range 0–53), which was significantly higher on AR patients (21.2 ± 9.4, $P = 0.003$). The mean number of HLA-II AgMM and EpMM were higher in AR patient group (2.09 ± 1.04 *versus* 1.52 ± 1.16, $P = 0.008$ and 12.7 ± 7.2*versus* 8.4 ± 7.7, $P = 0.001$), while the mean number of HLA-I AgMM and EpMM was similar between both groups. HLA-II EpMM analyzed as terciles groups with low,

Table 2
Univariate and multivariate analysis for each predictor for cellular-mediated rejection (n = 16).

	Univariate analysis			Multivariate analysis*		
	HR	95% CI	P	HR	95% CI	P
Recipient age, per 1-year increase	0.991	0.955–1.029	0.647			
Donor age, per 1-year increase	0.981	0.934–1.030	0.432			
Female (vs male) recipient	2.110	0.791–5.631	0.136			
Female (vs male) donor	0.855	0.297–2.465	0.772			
Living unrelated (vs related) donor	2.296	0.830–6.355	0.109			
Dialysis vintage, per 1-month increase	1.003	0.995–1.010	0.493			
Retransplant	1.917	0.546–6.731	0.310			
Cytotoxic PRA ≥ 5%	1.362	0.309–6.001	0.683			
ATG induction	**3.039**	**0.861–10.731**	**0.084**			
Tacrolimus (vs. cyclosporine) use	0.441	0.122–1.597	0.212			
Anti-HLA antibodies	1.768	0.568–5.502	0.325			
DSA, n (%)	0.762	0.100–5.782	0.792			
HLA-I antigen MM, per unit increase	1.282	0.934–1.758	0.124	1.267	0.919–1.747	0.149
HLA-II antigen MM, per unit increase	1.385	0.898–2.137	0.141	1.310	0.840–2.044	0.223
HLA-I epitope MM						
T1: 0–5	Ref.			Ref.		
T2: 6–9	1.426	0.402–5.058	0.583	1.777	0.480–6.575	0.389
T3: ≥10	1.697	0.475–6.059	0.415	1.988	0.541–7.304	0.301
HLA-II epitope MM						
T1: 0–5	Ref.			Ref.		
T2: 6–12	1.499	0.402–5.582	0.547	1.401	0.373–5.259	0.617
T3: ≥13	2.158	0.629–7.397	0.221	1.746	0.476–6.403	0.400

HLA, human leukocyte antigen; PRA, panel reactive antibodies; DSA, donor-specific antibodies; HLA-I, HLA class I; HLA-II, HLA class II; MM, mismatches; Ep, eplet; Ag, antigen; ATG, anti-thymocyte globulin; HR, hazard ratio.

* Adjusted for ATG induction; HLA-I/II mismatches were analyzed individually as predictors of CMR.

Table 3
Univariate analysis for each predictor for antibody-mediated rejection (n = 17).

	HR	95% CI	*P*
Recipient age, per 1-year increase	1.005	0.970–1.041	0.789
Donor age, per 1-year increase	0.972	0.928–1.018	0.233
Female (vs male) recipient	1.130	0.418–3.056	0.810
Female (vs male) donor	1.273	0.415–3.904	0.673
Living unrelated (vs related) donor	1.662	0.640–4.314	0.296
Dialysis vintage, per 1-month increase	1.004	0.998–1.011	0.170
Retransplant	1.829	0.525–6.367	0.343
Cytotoxic PRA ≥ 5%	**3.564**	**1.161–10.944**	**0.026**
ATG induction	1.790	0.409–7.830	0.439
Tacrolimus (vs cyclosporine) use	1.388	0.184–10.466	0.751
Anti-HLA antibodies	**3.879**	**1.469–10.244**	**0.006**
DSA, n (%)	**7.113**	**2.615–19.344**	**< 0.001**
HLA-I AgMM, per unit increase	1.135	0.847–1.522	0.396
HLA-II AgMM, per unit increase	**1.510**	**1.004–2.268**	**0.048**
HLA-I EpMM			
T1: 0–5	Ref.		
T2: 6–9	2.317	0.599–8.963	0.224
T3: ≥10	2.474	0.639–9.575	0.190
HLA-II EpMM			
T1: 0–5	**Ref.**		
T2: 6–12	**7.200**	**0.867–59.816**	**0.068**
T3: ≥13	**11.809**	**1.511–92.271**	**0.019**

HLA, human leukocyte antigen; PRA, panel reactive antibodies; DSA, donor-specific antibodies; HLA-I, HLA class I; HLA-II, HLA class II;
AgMM, number of antigen mismatches; EpMM, number of eplet mismatches; Ag, antigen; ATG, anti-thymocyte globulin; HR, hazard ratio.

moderate and high EpMM load, also showed significant differences between patients with or without AR ($P = 0.008$).

One hundred and forty (92.7%) patients remained with a functioning graft at the end of follow-up. In this group, those in whom AR occurred had higher SCr ($P = 0.029$), eGFR ($P = 0.003$) and proteinuria ($P = 0.001$).

3.2. Associations between HLA broad antigen, eplet mismatches and rejection episodes

As expected, there was a close correlation between the number of broad antigens and the number of eplet mismatch load for HLA-I and HLA-II, with Pearson's r-values of 0.775 ($P < 0.001$) and 0.799 ($P < 0.001$), respectively (Fig. 1).

HLA antigen and EpMM association with rejection episodes, considering no rejection, CMR and AMR episodes are shown in Fig. 2. Only HLA-II antigen and EpMM were correlated with AMR, when compared to no rejection group (HLA-II antigen with AMR, 2 (2–3), $P = 0.014$ *vs.* no rejection, 2 (1–2), HLA-II EpMM with AMR, 15 (10–18), $P = 0.002$ *vs.* no rejection, 8 (1–14)).

3.3. Incidence curves of antibody-mediated rejection by HLA-II eplet mismatch

The adjusted cumulative incidence curve for AMR is shown in Fig. 3. The incidence of AMR in patients transplanted with low, moderate and high HLA-II EpMM load were respectively 2%, 13% and 22%, at 96 months ($P = 0.003$) (Fig. 3A). Considering only patients with no more than two antigen mismatches in HLA-II (n = 123) (Fig. 3B), incidence of AMR in patients transplanted with low, moderate and high HLA-II EpMM load were respectively 2%, 14% and 13%, at 96 months ($P = 0.036$). Finally, considering only patients with no preformed DSA (n = 138) (Fig. 3C), AMR incidence for patients transplanted with low, moderate and high HLA-II EpMM load tercile were respectively 2%, 5% and 20% at 96 months ($P = 0.013$).

3.4. Independent predictors of cellular and antibody-mediated rejection

In univariate analysis, no variable was significantly associated with CMR. Multivariate analysis adjusted to ATG induction (the single

Table 4
Multivariate analysis of each predictor of antibody-mediated rejection separately, all adjusted for the variables with a p-value < 0.1 in the univariate analysis.

	HR	95% CI	*P*	AIC	BIC	c-statistics (95% CI)	Mean difference* (95% CI)
HLA-I Ep MM							
T1: 0–5	Ref.			163.4	178.5	0.707	0.046
T2: 6–9	2.196	0.565–8.530	0.256			(0.572–0.842)	(−0.079 to 0.171)
T3: ≥10	2.106	0.541–8.197	0.283				
HLA-I Ag MM, *per unit increase*	1.120	0.830–1.511	0.457	162.5	174.6	0.661 (0.507–0.815)	*P* = 0.472
HLA-II Ep MM							
T1: 0–5	**Ref.**			153.1	168.2	0.785	**0.064**
T2: 6–12	**7.753**	**0.929–64.724**	**0.059**			(0.675–0.895)	**(0.009–0.119)**
T3: ≥13	**14.839**	**1.846–119.282**	**0.011**				
HLA-II Ag MM, *per unit increase*	1.377	0.913–2.076	0.127	160.7	172.8	0.721 (0.596–0.847)	***P* = 0.023**

HLA, human leukocyte antigen; HLA-I, HLA class I; HLA-II, HLA class II; MM, mismatches; Ep, eplet; Ag, antigen; HR, hazard Ratio; AIC, area under the curve; BIC, bayesian information criterion.

* Percentile 95% CIs for c statistics were derived using 1000 bootstrap samples. The differences in c statistics were replicated 1000 times using bootstrap samples to derive 95% CIs.

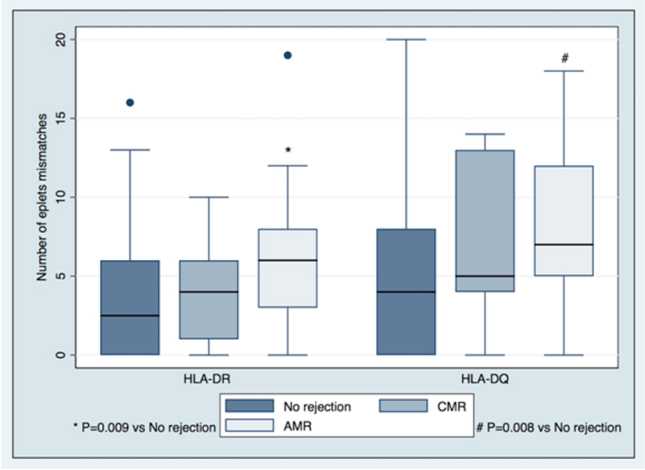

Fig. 4. Comparison of HLA-DR and HLA-DQ eplet mismatches according to rejection status (significant differences are shown).

variable with the defined threshold of p-value < 0.1) showed that neither antigen nor eplet mismatch load at HLA-I or HLA-II (Table 2) were independent predictors of CMR.

In univariate analyses, AMR predictors were: positive cytotoxic PRA (HR = 3.564; P = 0.026), preformed anti-HLA antibodies (HR = 3.879; P = 0.006), preformed DSA (HR = 7.113; P < 0.001), HLA-II Ag MM (HR *per unit increase* = 1.510; *P* = 0.048), HLA-II EpMM moderate load (*versus* patients with low HLA-II EpMM load, HR = 7.200; *P* = 0.068) and patients with high HLA-II EpMM load (*versus* patients with low HLA-II EpMM load, HR = 11.809; *P* = 0.019) (Table 3).

In the multivariate analysis neither EpMM nor antigen mismatch for HLA-I was associated with AMR. Differently, high (EpMM ≥ 13) *versus* low (EpMM ≤ 5) HLA-II eplet mismatch load, was an independent predictor of AMR (adjusted HR = 14.839; *P* = 0.011), while HLA-II antigen mismatch was not. The mean difference in the *c* statistic between EpMM load and antigen mismatch for HLA-II based risk models was 0.064 (*P* = 0.023), showing that the former was a significant better predictor of AMR than the latter (Table 4).

3.5. Multivariate analysis of each predictor for CMR and AMR occurrence

As we demonstrated in the multivariated analysis only HLA-II EpMM is an independent predictor for AMR. As such, we performed a more detailed analysis to understand if there was a different contribution of HLA-DR and HLA-DQ *loci*. Fig. 4 shows the number of eplet mismatches *per* HLA-II *loci*, considering no rejection, CMR and AMR. In the unadjusted model, patients with higher eplet mismatch load for HLA-DR and HLA-DQ *loci* expericenced more AMR episodes (*versus* no rejection, *P* = 0.009 and *P* = 0.008 respectively). The multivariated analysis of HLA-DR and HLA-DQ *loci* for CMR and AMR occurrence, adjusted for variables with a p < 0.1 in the univariate analysis as shown in Tables 2 and 3, is reported in Table 5. Neither HLA-DR nor HLA-DQ are independent predictors for CMR. On the other hand, high *versus* low eplet mismatch load for HLA-DR (T3 ≥ 6 *versus* T = 0–1, *P* = 0.013) and HLA-DQ (T3 ≥ 7 *versus* T = 0–1, *P* = 0.009) are independent predictors for AMR.

Table 5
Multivariate analysis of each predictor for CMR and AMR occurrence (adjusted for variables with a p-value < 0.1 in the univariate analysis as shown in Tables 2 and 3).

	HR	95% CI	*P*
Cellular-mediated rejection			
HLA-DR EpMM			0.802
T1: 0–1	Ref.		
T2: 2–5	2.107	0.630–7.049	0.226
T3 ≥ 6	0.763	0.165–3.521	0.729
HLA-DQ EpMM			0.564
T1: 0–1	Ref.		
T2: 2–6	2.357	0.598–9.293	0.221
T3 ≥ 7	1.678	0.409–6.874	0.472
Antibody-mediated rejection			
HLA-DR EpMM			0.013
T1: 0–1	Ref.		
T2: 2–5	6.188	0.734–51.899	0.093
T3 ≥ 6	10.079	1.273–79.808	0.029
HLA-DQ EpMM			0.009
T1: 0–1	Ref.		
T2: 2–6	1.559	0.281–8.655	0.611
T3 ≥ 7	5.943	1.272–27.760	0.023

CMR, cellular-mediated rejection; AMR, antibody-mediated rejection; HLA, human leukocyte antigen; EpMM, number of eplet mismatches; HR, hazard Ratio; CI, Confidence interval.

3.6. Improvement in risk prediction models for AMR

Improvement in calculated risk for AMR was assessed by IDI and NRI. The mean predicted probability of AMR increased among patients

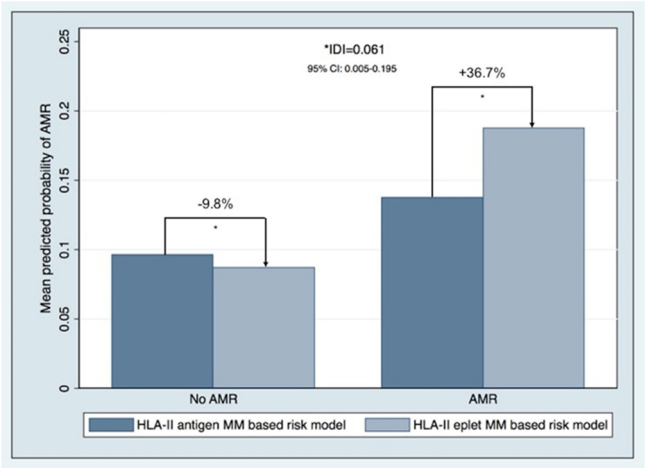

Fig. 5. Improvement in calculated risk of AMR considering HLA-II eplet mismatch in addition to classic HLA-II broad antigen mismatch.

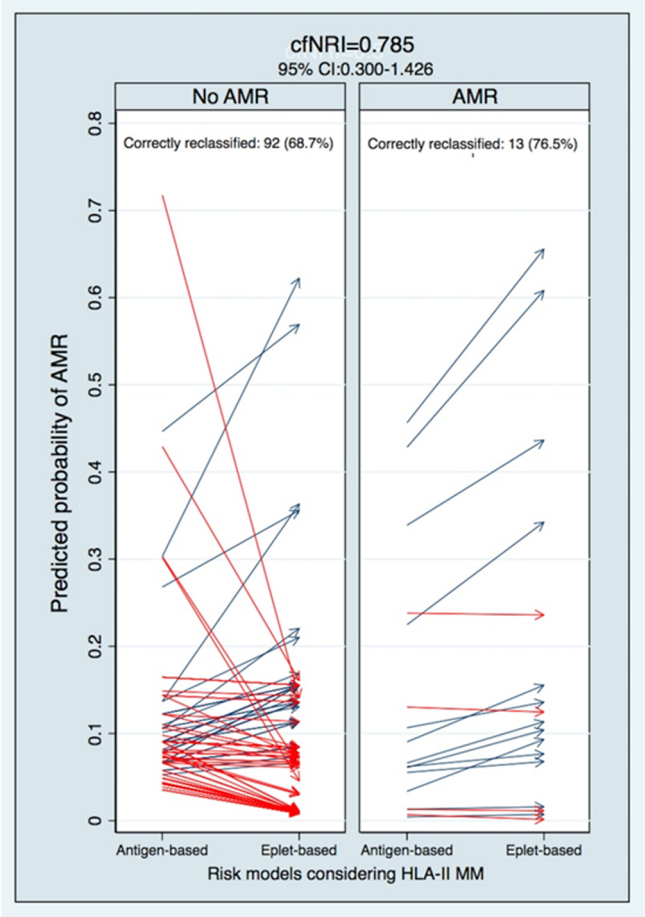

Fig. 6. Improvement in calculated risk of AMR considering HLA-II eplet mismatch in addition to classic.

with AMR (36.7%) and decreased in patients without AMR (9.8%), when comparing HLA-II eplet mismatch based to the classic HLA-II antigen mismatch risk models. The IDI was 0.061 (95%CI 0.005–0.195) (Fig. 5). Again, when HLA-II eplet based model was used comparatively to the antigen mismatch model, it reclassified correctly 92 of 134 patients (68.7%), among patients without AMR, and 13 of 17 patients (76.5%) within those with AMR. The category free net reclassification index (cfNRI) was 0.785 (95%CI 0.300–1.426) (Fig. 6).

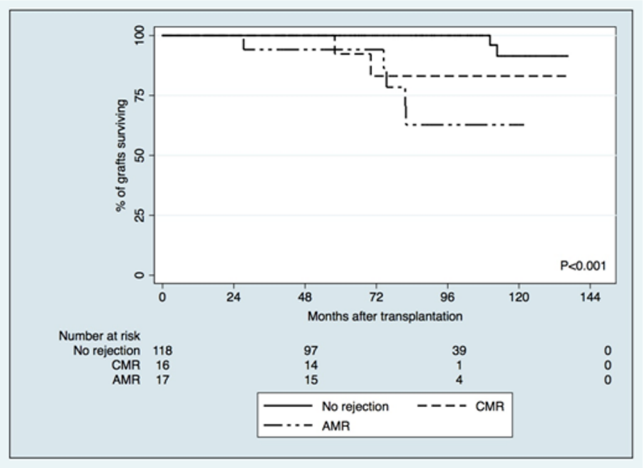

Fig. 7. Graft survival Graft at 120 months for patients with no rejection episodes, patients with CMR and patients with AMR.

3.7. Graft survival

GF occurred in 9 (6%) patients during the overall follow-up time of 70.1 (56.2–104.2) months. No association was found between graft failure, final sCr or eGFR with EpMM (data not shown). Differently, graft survival at 120 months (Fig. 7) was 91% for patients with no rejection episodes, 83% within patients with CMR and only 63% for patients with AMR ($P < 0.001$).

4. Discussion

This study shows that molecular matching based on eplet mismatch load is a more accurate strategy to assess risk of AMR, when compared to the conventional HLA broad antigen mismatch assessment currently used in clinical practice. We demonstrated that HLA class II eplet mismatch load was a strong predictor of AMR in a LDKT cohort.

Alongside with end-stage renal disease prevention and early recognition programs, in order to reduce demand, several strategies such as expanded criteria donor, hepatitis C virus-positive donors and donation after cardiac death have been implemented to maximize deceased donation [29]. However, deceased donation does not provide sufficient kidney grafts to ensure the increasing demand and living donation has been a successful strategy in order to increase organ donor pool. Furthermore, several programs to boost living donation have also been implemented such as AB0 incompatible donation and paired kidney exchange programs (KEP) [30–32]. However, this expansion in living donation contribution to organ supply arises chiefly from unrelated donation, which leads to higher degree of HLA mismatching, an unquestionable cause of poorer graft survival [5]. As such, strategies to improve HLA matching are of major importance and have been studied for more than 25 years [33–35].

Since then, several studies have been describing the impact of eplet mismatch and kidney transplantation. Duquesnoy et al. showed, in two different cohorts of kidney transplanted patients (United Network for Organ Sharing and Eurotransplant registries), almost identical survival rates between HLA-A,-B antigen mismatched grafts, but compatible at triplet level (continuous amino acid sequences), and HLA-A,-B antigen matched grafts [36]. Also, using the triplet HLAmatchmaker version, Dankers et al. described a strong correlation between the number of HLA class I triplet mismatches and the proportion of patients developing *dn*DSA in two different cohorts, one sensitized patients after allograft failure and the other of post-delivery pregnant women [37].

After HLAmatchmaker upgrade to include eplets, three-dimensional polymorphic patches in discontinuous sequence [38], Wiebe et al. showed that HLA-II eplet mismatches were an independent risk factor

for HLA-II *dn*DSA development, in a immunological low risk cohort [14], identifying optimal thresholds of 10 and 17 eplet mismatch load for HLA-DR and HLA-DQ, respectively.

In another approach to molecular matching, Kosmoliaptsis et al. showed that differences in aminoacid mismatch (AAMM), hydrophobicity mismatch score (HMS), and electrostatic mismatch score (EMS) between HLA specificities enabled prediction of HLA specific antibody responses [39,40]. Comparative analysis between classical HLA antigen mismatch analysis and molecular mismatch algorithms available showed that assessment of donor HLA immunogenicity based on EpMM, AAMM and ESM offered additional value to conventional HLA antigen mismatch for predicting HLA sensitization after kidney transplantation [18,41]. More recently, Snanoudj et al. showed that *dn*DSA were more strongly associated with the number of antibody-verified eplet mismatches than with the total eplet mismatch or antigenic mismatch number [42]. In this French study the HLA-II antibody-verified eplet load was 9.4 ± 6.8, being 12.1 ± 5.4 (*versus* 6.8 ± 6.1) for DSA positive group of patients ($P < 0.005$). In our cohort the HLA-II antibody-verified eplet mismatch load was very similar (9.3 ± 7.7), being 12.7 ± 7.2 (*versus* 8.4 ± 7.7) in the acute rejection sub-cohort ($P = 0.001$).

Besides the inherent biological risk due to HLA differences between donor and recipient pair, underexposure and/or non-adherence to immunosuppressive drugs is a risk factor for development of *dn*DSA, AMR and GF [43]. Importantly, Wiebe et al. also showed that this detrimental impact of non-adherence was strongly and synergistically modulated by higher HLA-II EpMM load [16].

A limitation of our study was the absence, at the present time, of adequate *dn*DSA longitudinal surveillance for this analysis. However, as *dn*DSAs are surrogate markers of AMR [44–47], our data confirms that high HLA-II EpMM load was associated with increased risk of humoral alloresponses in LDKT. On the other hand, the major strength of our study is the considered uniform cohort of living donors, younger patients under uniform immunosuppression therapy and with low DGF.

Beyond our study limitations, HLA epitope matching is currently still in progress as it is necessary to identify all antibody-verified epitopes in order to understand their immunogenicity.

In conclusion, our study evidences that eplet-based matching is a refinement of the classical HLA antigen mismatch analysis in LDKT, with clear improvement in risk assessment at transplant for downstream alloimmune responses. Its application in the clinical setting can be of particular importance in pediatric recipients but also as a boost for KEP programs, in which compatible pairs with high eplet mismatch load may enter, in order to find a more compatible donor [23,48,49]. Finally, eplet-based matching may be a biomarker for personalized assessment of alloimmune risk, allowing for the immunosuppression therapy fine-tuning with a more balanced cost-benefit.

5. Authorship

Sandra Tafulo – Participated in research design, in the performance of the research, in data analysis and in the writing of the paper

Jorge Malheiro – Participated in research design, in data analysis and in the writing of the paper

Sofia Santos – Participated in data analysis

Leonídio Dias – Participated in research design

Manuela Almeida – Participated in research design

La Salete – Participated in research design

Sofia Pedroso – Participated in research design

Cecília Mendes – Participated in the performance of the research

Luísa Lobato – Participated in research design

António Castro-Henriques – Participated in research design

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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