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HLA epitope matching for allocation improvement in kidney transplantation

Sandra Cristina Tafulo

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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HLA EPITOPE MATCHING FOR ALLOCATION IMPROVEMENT IN KIDNEY TRANSPLANTATION

Tese de Candidatura ao Grau de Doutor em Ciências Biomédicas, submetida ao
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Sandra Cristina Ribeiro Rafaelo

The greatest enemy of knowledge is not ignorance; it is the illusion of knowledge.

Stephen Hawking

PREFACE

The idea for replacing ill organs has been a dream for centuries, since Cosmos and Damien envisioned the transplantation of an entire leg. The major drawback in order to accomplish this goal successfully has been organ rejection.

As such, I started this journey five years ago with the primary objective of improving knowledge in order to provide a better HLA matching and more fairness in health service to all patients waiting for a compatible organ, many of them for a long time.

Although my PhD process represented a long process of never-ending days, and despite thinking of giving up several times with this last year being particularly challenging with the unexpected Covid-19 pandemic, I never lost focus and I can say that the time invested was deeply worth it.

This thesis resumes this five-years of work conducted in the HLA allosensitization laboratory within Centro de Sangue e da Transplantação do Porto, Instituto Português de Sangue e da Transplantação, in close collaboration with the nephrology department of Hospital Santo António at Centro Hospitalar Universitário do Porto.

I hope you will enjoy reading it.

Sandra Tafulo

December 2020

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This long road toward the PhD would not be possible without the support and encouragement of the Centro Hospitalar Universitário do Porto team but also of my family and friends.

None of this would be possible without the support of Doutora Luísa Lobato, Dr. Leonídio Dias e Dr. António Martinho, that supervised this project. I am truly grateful to work with excellent professionals from both clinical and laboratory perspectives. Thank you for believing in me.

This thesis was a sweet journey of scientific knowledge but also of personal maturing and growth and this wouldn't be possible without the support and confidence of all the nephrology service team of Centro Hospitalar Universitário do Porto. A special thank you to Doutora Luísa Lobato, my supervisor and always present tutor and advisor, and to Dr. António Castro-Henriques and Dr. Leonídio Dias, not just great nephrologists but very special human beings. Thank you for your unconditional support that made me a better professional but also a better person.

Also from nephrology department of Centro Hospitalar Universitário do Porto, Prof. Jorge Malheiro, an outstanding professional, was more than a supervisor, a mentor, an advisor and a true friend. I owe this thesis to Jorge and I am forever truly thankful.

To IPST that allowed me to accomplish this task, with a special gratitude to Dr. António Martinho, technical-scientific responsible of the Histocompatibility Laboratory in Centro de Sangue e da Transplantação de Coimbra, that has been my counselor, friend and sometimes my guardian angel for the last 20 years of my journey in the HLA world.

I thank deeply my family that has always inspired me to chase my dreams and for being always present, even when my mind and attention was only focused in work.

Finally, I thank all the patients that participated in this work, truly hoping that this work will improve the life of the transplanted patients and their overall quality of life.

ABSTRACT

Introduction

Since the first successful kidney transplant between monozygotic twins that HLA mismatches have been correlated with alloimmune risk and immunosuppression recognized as inevitable to avoid allograft rejection.

Advances in histocompatibility laboratory assays for HLA genotyping and HLA antibodies assessment, unveiled the vast extent of HLA polymorphism and allowed a deep insight of HLA antibodies specificities analysis. Despite the enormous evolution from serology to molecular methods, HLA mismatch analysis in kidney transplantation is still determined by HLA antigen.

With the understanding that each HLA molecule mismatch can represent a wide range of polymorphic amino acid mismatches, the improvement opportunity from molecular analysis refinement became clear and imperative.

Aims

For the development of this thesis, we defined 4 aims: 1) determine the degree of allosensitization in candidates waitlisted for KT and evaluate its impact on access to KT; 2) determine virtual PRA, classical and eplet-based, in candidates waitlisted for KT and investigate its impact on transplantability; 3) assess the impact of including compatible pairs in kidney exchange program (KEP) on transplantation rate and HLA eplet mismatch load; 4) evaluate HLA eplet mismatch load impact on *dn*DSA development and 5) determine if HLA eplet mismatch load improves prediction of antibody-mediated rejection (ABMR) development.

Materials and Methods

This thesis comprise 5 research studies, conducted in order to address specific aims under the hypotheses defined: 1) HLA Allosensitization has a major impact in transplantability and the Portuguese allocation system lacks efficiency to transplant highly sensitized patients in a timely manner; 2) HLA eplet analysis would improve greatly solid-phase immunoassays analysis and increase transplantability among hyper sensitized patients in waiting list for KT; 3) The inclusion of compatible pairs in KEP enables HLA epitope mismatch load minimization, increasing transplantation rate within the program; 4) HLA eplet mismatch load is a superior biomarker, when compared to HLA antigen mismatch analysis, for *dn*DSA development and 5) HLA eplet mismatch

load is a superior biomarker, when compared to HLA antigen mismatch analysis, for KT outcomes.

Results

Under hypothesis 1, we demonstrated that CDC-PRA profoundly underestimates patient's true HLA allosensitization status defined by vPRA. O blood type patients are in disadvantaged to find a compatible donor, when compared to the remaining groups. Furthermore, HS candidates as defined by vPRA were hugely disadvantaged in the access to KT, independently from AB0 blood groups.

Under hypothesis 2, eplet based vPRA granted the reclassification of 124 (79%) and 80 (51%) patients to a lower vPRA group (resulting in a greater access to KT) when considering total vPRA (vPRAt) and current vPRA (vPRAc) to vPRAe, respectively. Also, median percentage of change in median estimated number of match runs (eMR) from vPRAt to vPRAe was significantly less pronounced in candidates to retransplant with 100% of vPRA (P=0.010) and for patients with dialysis vintage ≥10 years (P=0.049 for all cohort, P=0.015 for vPRA=100% and P=0.005 for patients with vPRA between 97.50% and 97.99%). This observation reinforces the strength of vPRAe measure, which has an important decrease within first transplant candidates with lower cytotoxic PRA, and this impact is less pronounced in patients considered to be at highly immunological risk.

Under hypothesis 3, we observed that HLA allosensitization degree of Portuguese KEP is very high and blood groups frequencies within the Portuguese KEP are deeply imbalanced. The inclusion of fully mismatches compatible pairs in our national KEP cohort increased matched rate within ICP and the compatible pairs were also benefited by decreasing HLA eplet mismatch load, total and verified, when compared to the direct donor.

Under hypothesis 4, we demonstrated that the number of HLA class II, total and antibody-verified (AbVer), eplet mismatch load were greater in *dn*DSA group compared to no *dn*DSA, which is not verified when mismatches are determined traditionally considering the HLA class II broad or split molecule as a whole. Antibody-mediated rejection was significantly higher within HLA class II *dn*DSA positive sub-cohort and HLA class II total and AbVer eplet mismatch load were independent predictors for HLA class II *dn*DSA development. On the other hand, neither HLA class I broad and split antigen or HLA class I total or AbVer eplet mismatch load, had any predictive value for HLA class I *dn*DSA, in our cohort.

Under hypothesis 5, we found a close correlation between the number of broad antigens and the number of antibody-verified eplet mismatch load for HLA-I and HLA-II.

HLA class II antibody-verified eplet mismatch load was a strong predictor of ABMR, when compared to the conventional HLA broad antigen mismatch assessment.

Conclusions

Important measures need to be undertaken in order to mitigate the disadvantage that O blood type and, even more so, HS candidates have in accessing KT. In fact, patients degree of allosensitization is the major barrier to transplant and, as more stringent criteria to define unacceptable antigens is applied, more difficult it becomes to find a compatible donor. As such, histocompatibility laboratories have the responsibility to identify with precision HLA specificities to be considered forbidden to assure transplant success in a timely manner. HLA eplet analysis associated with SAB assays adds clinical relevance to the interpretation and enables a more accurate assignment of HLA antibodies of patients in waiting list for deceased donors.

Regarding living donor kidney transplantation, we realized that most of the compatible pairs are transplanted with high HLA antigen mismatches and, if introduced in the national Portuguese KEP, they would benefit by decreasing HLA eplet mismatch load when compared to the direct donor. Besides, and importantly, the matched rate within KEP would increase.

Finally, we could appreciate that HLA class II eplet mismatch load improved prediction of *dn*DSA development and ABMR, when compared to HLA classical antigen mismatch. To conclude, 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.

RESUMO

Introdução

Desde a realização do primeiro transplante renal (TR) com sucesso entre irmãos monozigóticos que os mismatches HLA têm sido correlacionados com risco alloimmune, sendo a imunossupressão inevitável para evitar a rejeição do aloenxerto.

Os enormes avanços nos laboratórios de histocompatibilidade, nomeadamente ensaios de genotipagem e identificação de anticorpos HLA, revelaram o extenso polimorfismo HLA e permitiram um conhecimento aprofundado na análise de especificidade de anticorpos HLA. Contudo, apesar da enorme evolução dos métodos serológicos para métodos moleculares, a análise de mismatches HLA em transplantação renal é ainda determinada a nível antigénico.

Com o reconhecimento que cada mismatch HLA pode representar um amplo número de aminoácidos polimórficos, a oportunidade de melhoria da análise de mismatches tornou-se claro e imperativo.

Objetivos

No desenvolvimento desta tese definimos quarto objetivos: 1) determinar o grau de alossensibilização dos candidatos em lista de espera para TR e avaliar o seu impacto no acesso ao TR; 2) determinar o PRA virtual, calculado de forma clássica e após análise de epletos, aos candidatos em lista de espera para TR e investigar o seu impacto na transplantabilidade; 3) estudar a possibilidade de aumentar a compatibilidade HLA se a doação renal cruzada (DRC) for considerada para pares compatíveis propostos a transplante renal com dador vivo, e verificar se esta estratégia aumentaria a taxa de transplantação no programa de DRC; 4) avaliar o impacto da carga de mismatches de epletos no desenvolvimento de anticorpos HLA *de novo* e específicos do dador; 5) determinar o impacto da carga de mismatches de epletos HLA no prognóstico do KT.

Materiais e Métodos

Esta tese compreende 5 estudos de investigação implementados para responder aos objetivos específicos sob as hipóteses definidas: a sensibilização HLA tem elevado impacto na transplantabilidade e o sistema de alocação renal Português carece de eficiência para transplantar doentes com elevado grau de sensibilização HLA; 2) a análise de epletos HLA permite melhorar a análise de imunoensaios de fase sólida, aumentando a transplantabilidade entre doentes hipersensibilizados em lista de espera para TR; 3) a carga de mismatch de epletos HLA pode ser usada para melhorar a

compatibilidade em transplantação renal com dador vivo usando o programa de doação renal cruzada, aumentado a taxa de transplantabilidade do programa; 4) a carga de mismatch de epletos HLA é um biomarcador superior, quando comparado com a análise de mismatches HLA, para o desenvolvimento de *dn*DSA; e 5) a carga de mismatch de epletos HLA é um biomarcador superior, quando comparado com a análise de mismatches HLA, para o prognóstico do TR.

Resultados

Na hipótese 1, demonstramos que o PRA determinado por ensaios de citotoxicidade subestima profundamente o verdadeiro grau de sensibilização HLA definido pelo vPRA. Os doentes com grupo sanguíneo O estão em desvantagem para encontrar um dador compatível, comparativamente aos restantes grupos. Para além disso, os candidatos hipersensibilizados, definidos por vPRA, estão em enorme desvantagem no acesso ao TR, independente do grupo sanguíneo.

Na hipótese 2, o vPRA determinado com base na análise de epletos HLA (vPRAe), permite a reclassificação de 124 (79%) e 80 (51%) doentes para um grupo menor, considerando o vPRA total (vPRAt) e vPRA corrente (vPRAc) para vPRAe, respetivamente. Ainda, a percentagem média da alteração do número médio estimado de matchings, de vPRAt para vPRAe, foi significativamente menos pronunciado em candidatos a retransplant com 100% de vPRA (P=0.010) e para doentes com tempo em diálise superior a 10 anos (P=0.049 para todo o cohort, P=0.015 para vPRA=100% e P=0.005 para doentes com vPRA entre 97.50% e 97.99%). Esta observação reforça o valor do vPRAe, que tem um decréscimo importante nos candidatos a primeiro transplante com valor baixo de PRA citotóxico, e o seu impacto é menos acentuado em doentes considerados em elevado risco imunológico.

Na hipótese 3, observamos que o grau de alossensibilização HLA no programa nacional de doação renal cruzada (PNDRC) é muito elevado e que as frequências de grupos sanguíneos são profundamente desequilibradas. Assim, a inclusão de pares compatíveis no PNDRC permite que estes beneficiem de uma diminuição da carga total de epletos HLA, aumentando a taxa de possíveis transplantes compatíveis no PNDRC.

Na hipótese 4, demonstramos que a carga de mismatches de epletos HLA classe II, totais e verificados, era superior no grupo de doentes com *dn*DSA, quando comparado com o grupo de doentes sem *dn*DSA, o que não foi verificado quando os mismatches HLA classe II foram determinados tradicionalmente considerando a molécula HLA como um todo.

A rejeição mediada por anticorpos foi significativamente superior no grupo com *dn*DSA para HLA classe II e a carga de mismatches de epletos HLA classe II, totais e verificados, foram preditores independentes para o desenvolvimento de *dn*DSA. Por outro lado, nem os mismatches antigénicos HLA classe I ou a carga de epletos HLA classe I, teve qualquer valor preditivo no desenvolvimento de dnDSA HLA classe I.
Na hipótese 5, encontramos uma estreita correlação entre o número de antigénios HLA e o número de epletos HLA classe I e classe II. A carga de mismatches de epletos HLA classe II é um forte preditor de rejeição mediada por anticorpos, quando comparada com a análise de mismatches clássica efetuada a nível antigénico.

Conclusões

As desvantagens conhecidas dos doentes em lista de espera para TR com grupo sanguíneo O e com elevada sensibilização HLA requerem medidas urgentes de forma a permitir o acesso destes doentes ao transplante. De facto, o grau de alossensibilização HLA é a maior barreira no acesso ao transplante e, quanto mais rigorosos forem os critérios de definição de antigénios inaceitáveis, mais difícil se torna encontrar um dador compatível.
Assim, os laboratórios de histocompatibilidade têm a responsabilidade de identificar com a maior precisão possível as especificidades HLA a considerar proibidas de forma a assegurar o sucesso do transplante. A análise de epletos HLA associados a ensaios de SAB permite a introdução de relevância clínica à interpretação, possibilitando a identificação de anticorpos HLA de forma mais precisa dos doentes em lista de espera para transplante com dador falecido.
Em transplantação com dador vivo, verificamos que maior parte dos transplantes são realizados com elevado número de mismatches HLA antigénicos e, se incluídos no PNDRC, beneficiariam de uma diminuição da carga de mismatches de epletos HLA comparativamente ao dador original. Para além disso, verificamos um aumento importante da taxa de soluções compatíveis no programa.
Finalmente, pudemos comprovar que a carga de mismatch de epletos HLA classe II melhora a predição do desenvolvimento de anticorpos *de novo* e específicos do dador, quando comparado com a avaliação de mismatches HLA antigénicos. Para concluir, a determinação da compatibilidade HLA a nível molecular é um potencial biomarcador de avaliação de risco alloimmune, permitindo o ajuste personalizado da terapia de imunossupressão.

SCIENTIFIC OUTPUTS

List of Original Publications included in the PhD Thesis

1. Tafulo S, Malheiro J, Dias L, Mendes C, Osorio E, Martins LS, Santos J, Pedroso S, Almeida M, & Castro-Henriques A. Low transplantability of 0 blood group and highly sensitized candidates in the Portuguese kidney allocation algorithm: quantifying an old problem in search of new solutions. HLA 2016 Nov; 88(5), 232-238.
doi: 10.1111/tan.12895
Candidate contributions: Sandra Tafulo participated in research design, laboratory assays and interpretation, data analysis and writing the paper.
Paper previously published as part of the second author PhD thesis: https://repositorio-aberto.up.pt/handle/10216/115823

2. Tafulo S, Malheiro J, Santos S, Dias L, Almeida M, Martins S, Pedroso S, Mendes C, Lobato L, Castro-Henriques A. Degree of HLA Class II Eplet Mismatch Load Improves Prediction of Antibody-Mediated Rejection in Living Donor Kidney Transplantation. Human Immunology 2019 Dec; 80(12), 966-975.
doi: 10.1016/j.humimm.2019.09.010
Candidate contributions: Sandra Tafulo participated in research design, in the performance of the laboratory research, in data analysis and in writing of the paper.

3. Tafulo S, Malheiro J, Dias L, Lobato L, Ramalhete L, Martinho A, Bolotinha C, Costa R, Ivo M. Improving HLA matching in living donor kidney transplantation using kidney paired exchange program. Transplant Immunology 2020 Oct; 62:101317.
doi: 10.1016/j.trim.2020.101317
Candidate contributions: Sandra Tafulo participated in research design, in the performance of the research, in data analysis and in writing of the paper.

4. Tafulo S, Malheiro J, Santos S, Dias L, Almeida M, Martins LS, Pedroso S, Mendes C, Lobato L, Castro-Henriques A. HLA class II eplet mismatch load improves prediction of *dn*DSA development after living donor kidney transplantation. International Journal of Immunogenetics. 2021 Feb; 48(1):1-7.
doi: 10.1111/iji.12519
Candidate contributions: Sandra Tafulo participated in research design, in the performance of the laboratory research, data analysis and in writing of the paper.

5. Tafulo S, Malheiro J, Dias L, Almeida M, Martins LS, Pedroso S, Osório E, Lobato L, Castro-Henriques A. Eplet based virtual PRA increases transplant probability in highly-sensitized patients. Transplant Immunology 2021 Jan9:101362.
doi: 10.1016/j.trim.2021.101362
Candidate contributions: Conceptualization; Data curation; Methodology; Writing - original draft; Writing - review & editing.

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KEYWORDS

Allograft failure

Allosensitization

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Anti-HLA antibodies

Crossmatch

Donor-specific antibodies

Epitopes

Eplets

Highly-sensitized

HLA Mismatches

Human leukocyte antigens

Kidney transplantation

Panel reactive antibodies

Solid-phase immunoassays

Waiting-list

ABBREVIATIONS

AB0i, AB0 Incompatible
ABMR, AntiBody-Mediated Rejection
AbVer, Antibody-Verified eplet
ACD, Acid-Citrate-Dextrose
AgMM, Antigen MisMatch
AHG, Anti-Human Globulin
AO, Acridine Orange
APC, Antigen-Presenting Cells
AR, Acute Rejection
AUC, Area Under the Curve
C4d, Complement split product 4d
CDC, Complement-Dependent Cytotoxicity
CDC-PRA, Complement-Dependent Cytotoxic Panel Reactive Antibodies
CDCXM, Complement-Dependent Cytotoxicity CROSSmatch
CHUP, Centro Hospitalar Universitário do Porto
CI, Confidence Interval
CKD, Chronic Kidney Disease
CMR, Cellular Mediated Rejection
CP, Compatible pairs
CREG, Cross REactive Group
CV, Coefficient of Variation
DC, Dendritic Cells
DD, Deceased Donor
DDKT, Deceased Donor Kidney Transplantation
DGF, Delayed Graft Function
DNA, Deoxyribonucleic Acid
***dn*DSA,** De Novo Donor-Specific Antibodies
DSA, Donor-Specific Antibodies
DTT, DiThioThreitol
EB, Ethidium Bromide
EDTA, EthyleneDiamineTetraacetic Acid
eGFR, estimated Glomerular Filtration Rate
ELISA, Enzyme-Linked Immunosorbent Assay
eMR, estimated Match Run
EpMM, Eplet MisMatch load
FCXM, Flow-Cytometry CROSSmatch
FITC, Fluorescein isothiocyanate
HLA, Human Leukocyte Antigen

HLAi, HLA Incompatible

HR, Hazard Ratio

HS, Highly-Sensitized

H*v*G, Host-versus-Graft

IHW, International Histocompatibility Workshop

IPST, Instituto Português do Sangue e da Transplantação

IQR, InterQuartile Range

IVIg, IntraVenous ImmunoGlobulin

KEP, kidney Exchange Program

KT, Kidney Transplantation

LR, Likelihood Ratio

Ly, Lymphocyte

MCS, Median Channel Shift

MFI, Mean Fluorescence Intensity

MHC, Major Histocompatibility Complex

MM, MisMatch

NGS, Next-Generation Sequencing

NIH, National Institutes of Health

OR, Odds Ratio

PCR, Polymerase Chain Reaction

PE, PhycoErythrin

PRA, Panel Reactive Antibodies

ROC, Receiver Operator Characteristic

RRT, Renal Replacement Therapy

rSSO, reverse Sequence-Specific Oligonucleotide

SAB, Single Antigen Bead

SD, Standard Deviation

SPI, Solid-Phase Immunoassays

SSP, Sequence-Specific-Primer

TBE, Tris-Borate EDTA

TCMR, T-cell Mediated Rejection

TerEps, Terasaki Epitopes

UA, Unacceptable Antigens

UV, UltraViolet light

vPRA, virtual Panel Reactive Antibodies

vXM, virtual CROSSmatch

XM, CROSSmatch

xMAP, Multi-Analyte Profiling® luminex® platform

CHAPTER I - INTRODUCTION

1. THESIS MOTIVATION

I have worked in Histocompatibility for the last twenty years and, during this time, I witnessed a huge evolution of the laboratory techniques and the Portuguese kidney transplantation (KT) allocation system. Initially, HLA-DR6 patients were prioritized in allocation, with HLA-DR6 donors, because they were considered high-responders (1). Since then, legislation changed greatly and now allocation is based in a score system that takes in consideration, not only HLA matching, but also time on renal replacement therapy (RRT), closest donor-recipient age and patient degree of allosensitization.

The techniques evolution was huge, since HLA serology typing to next-generation sequencing (NGS) and, alongside with these methodologies, the degree of HLA polymorphisms. In allosensitization assessment, the introduction of solid-phase immunoassays (SPI) was the major progress, allowing histocompatibility laboratories to have a true added value in immunological risk assessment.

However, I have been noticing that most of patients relisted for retransplantation have such high percentage of allosensitization that makes, in several cases, almost impossible to find them a compatible donor. These patients have prolonged waiting times and many of them die while waiting.

Although it's not feasible to think that we could perform kidney transplants without HLA mismatches, especially to the exponential growth of HLA alleles, I believe it's our duty to find these patients the better mismatch in order to prevent insurmountable allosensitization.

Additionally to the primary goal of preventing allosensitization, it's also our responsibility to understand the status of each patient already highly sensitized (HS), within the waiting list, and provide the best immunological assessment with the ultimate goal of finding them a matched donor.

This thesis was developed, with the encouragement of my tutors and the outstanding collaboration of the nephrology service of Centro Hospitalar Universitário do Porto (CHUP), to address these issues.

2. THESIS HYPOTHESIS

HLA matching fine-tuning would improve risk assessment with better kidney transplantation outcome. The deepest knowledge HLA antibodies pattern analysis would allow increase of transplants rate, lowering waiting times.

Hypothesis #1

HLA Allosensitization has a major impact in transplantability and Portuguese allocation system lacks efficiency to transplant highly sensitized patients in a timely manner.

Hypothesis #2

HLA eplet analysis would improve greatly solid-phase immunoassays (SPI) analysis and increase transplantability among hyper sensitized patients in waiting list for KT.

Hypothesis #3

The inclusion of compatible pairs in KEP enables HLA epitope mismatch load minimization, increasing transplantation rate within the program.

Hypothesis #4

HLA eplet mismatch load is a superior biomarker, when compared to HLA antigen mismatch analysis, for *dn*DSA development.

Hypothesis #5

HLA eplet mismatch load is a superior biomarker, when compared to HLA antigen mismatch analysis, for ABMR prediction.

3. THESIS AIMS

Our aims, regarding to each hypothesis proposed, are:

Aim #1

- Determine the degree of allosensitization in candidate's waitlisted for KT.
- Evaluate its impact on access to KT.

Aim #2

- Determine virtual PRA, classical and eplet-based, in candidate's waitlisted for KT.
- Investigate its impact on transplantability.

Aim #3

- Determine HLA eplet mismatch load within LDKT performed.
- Assess the impact of including compatible pairs in KEP on transplantation rate and HLA eplet mismatch load.

Aim #4

- Evaluate HLA eplet mismatch load impact on *dn*DSA development.

Aim #5

- Determine HLA eplet mismatch load impact on ABMR prediction.

4. THESIS OUTLINE

This thesis is divided in five chapters.

Chapter 1, Introduction

Motivation, hypotheses raised and specific aims of the research performed within this thesis with respect to HLA molecular matching in KT.

Chapter 2, Literature review

A state of art literature review of the theme in study is performed, emphasizing HLA matching importance in KT.

Chapter 3, Materials & Methods

Materials and methodology used within this thesis are described.

Chapter 4, Results

Description of the results obtained in the different studies carried out under the defined thesis hypotheses. All published articles are presented in appendixes. The reproduction of all published papers was authorized by the respective publisher.

Chapter 5, Discussion

The main findings of the performed studies articles are discussed. Also, future perspectives are outlined.

CHAPTER II - LITERATURE REVIEW

Transplantation as a treatment method to replace a failing organ started many centuries ago has a dream shaped into a legend or myth (2). However, it was only in 1954 that Joseph E. Murray performed the first successfully kidney transplant between identical twins (3). This surgical procedure in the context of allotransplantation is a significant bigger challenge due to allograft rejection and, despite several immunosuppressive drugs that have been developed, the fine balance between over and under immunosuppression after kidney transplantation can be very difficult to achieve. Moreover, alloimmune response continues to be the major cause of allograft failure after kidney transplantation (4).

The immune process of discrimination self from nonself tissues is called allorecognition and the major alloantigens recognized in transplantation are Human Histocompatibility Antigens (HLA).

1. HLA COMPLEX

It all started in 1958 when Jean Dausset described antibodies in sera from multitransfused patients that reacted with an antigen with leuco-agglutination property, named MAC after the three volunteer donors used in the experiment (5). Jon van Rood and Rose Payne pursued this work using sera from multiparous women (6, 7) and soon after many independent laboratories followed this goal identifying numerous HLA specificities with alloantibodies (8). To compare techniques, reagents and standardize nomenclature, International Histocompatibility Workshops (IHWSs) were established and the first was organized by Bernard Amos in 1964 at Duke University. The designation of Human Leucocyte locus A, HL-A, resulted from the work developed in the third IHWS, organized by Ruggero Ceppellini in 1967 at Turin University, where it was fully established that most of the specificities were encoded by closed linked genes at one chromosomal region. The nomenclature later changed to Human Leucocyte antigens (HLA) and, during the last sixty-two years, 29417 HLA alleles have been described by the HLA nomenclature and included in the IPD-IMGT/HLA Database (https://www.ebi.ac.uk/ipd/imgt/hla/), release 3.43.0, 2021-01-18 (Figure 1).

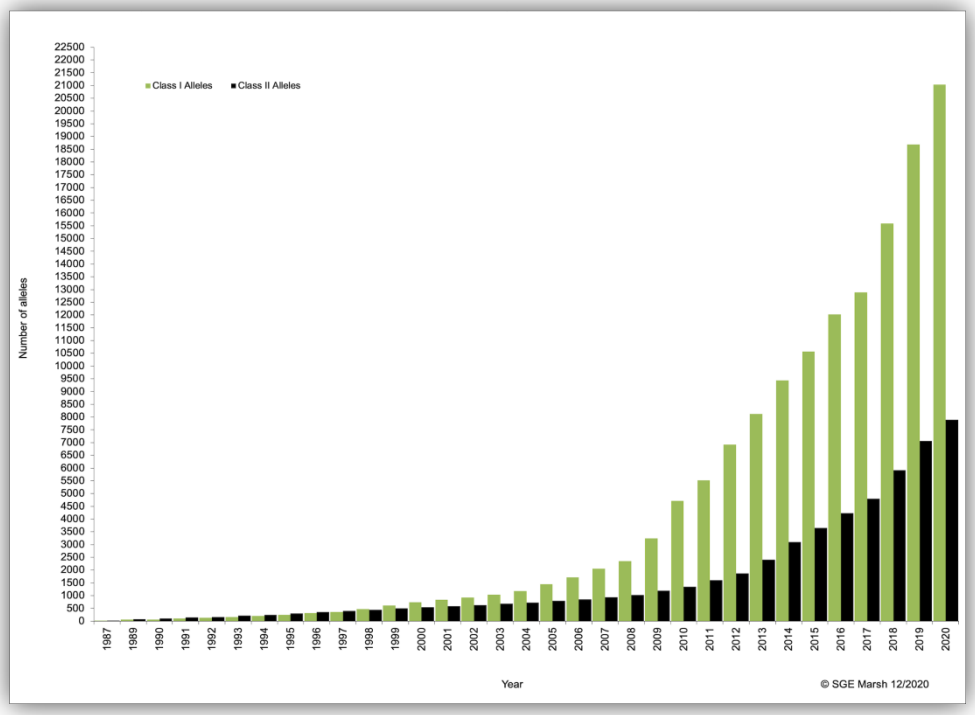


Figure 1 – Number of alleles named by year from 1987 to December2020. (http://hla.alleles.org/nomenclature/index.html)

1.1. Genome location and organization

The HLA complex is the human Major Histocompatibility Complex (MHC), located on the band 6p21.3 of the short arm of chromosome 6 (Figure 2).

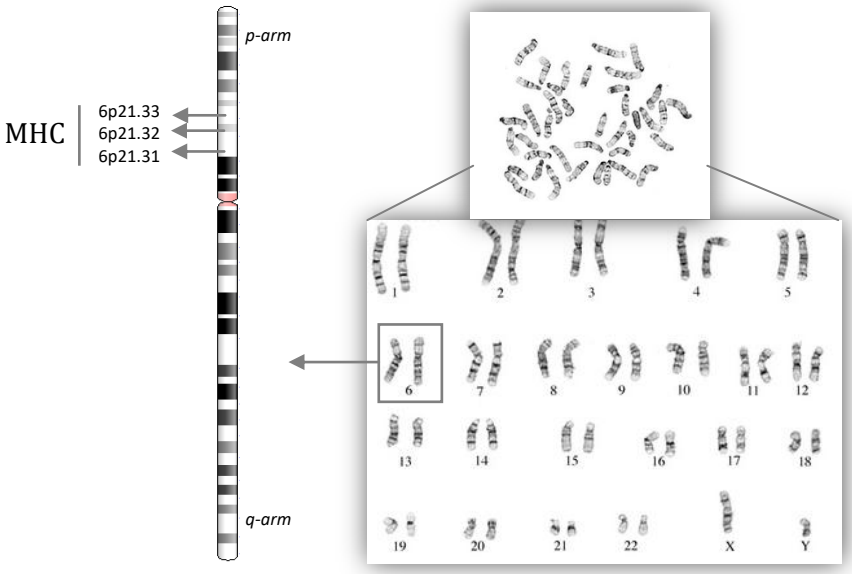


Figure 2 – Example of human karyotype emphasizing the two copies of chromosome 6 (right) and respectively G-banding ideogram, highlighting MHC locus location in 6p21.3 (left).

The classical MHC contains around 158 protein-coding genes and 86 pseudogenes in approximately 3,8Mbp (9), within the extended MHC region that spans about 7.6 Mb (Figure 3). The classical *loci* include three regions, HLA class I, II, and III, of highly polymorphic and co-dominant genes.

HLA class I comprises the classical genes HLA-A, HLA-B and HLA-C, and HLA class II includes the classical HLA-DR, HLA-DQ and HLA-DP genes. HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ genes, but not the HLA-DP gene, are in strong linkage disequilibrium. As such, each HLA haplotype, a particular allele combination of a gene *locus* on the same DNA strand, is inherited together more often than it would be expected by chance. HLA haplotype frequencies vary greatly between different regions and populations (10).

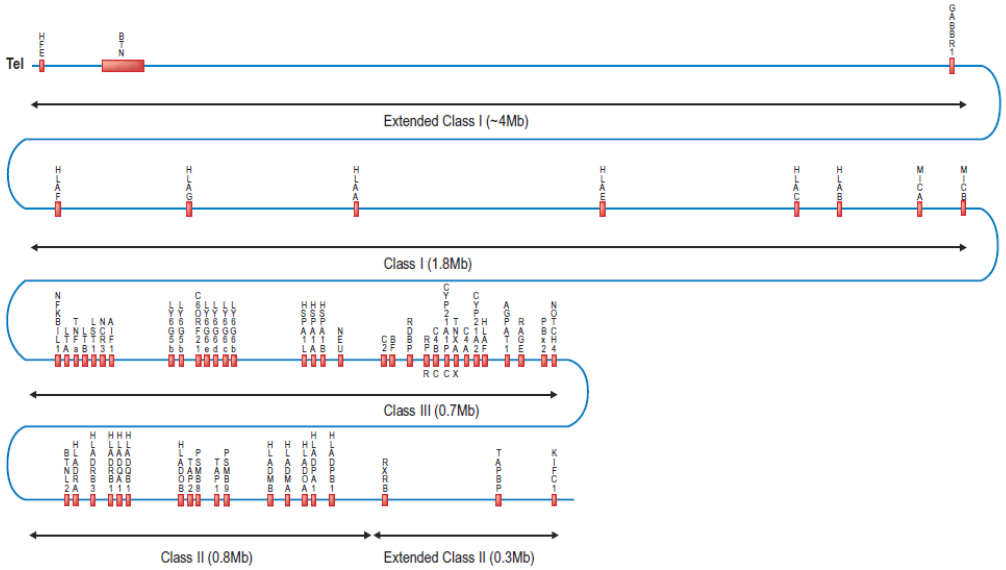


Figure 3 – Gene map of the extended Major Histocompatibility Complex (xMHC) from telomere (left) to centromere (right) on the short arm of the chromosome 6. Adapted from (11).

1.2. HLA molecules structure

The classic HLA class I molecules consist of two chains, α and β chains. The α chain is encoded by the respective HLA class I gene and the β chain, β2 microglobulin, is encoded by the B2M gene located in chromosome 15. The α chain has three extracellular domains encoded by exons 2, 3 and 4, a transmembrane segment encoded by exon 5 and a C-terminal cytoplasmic end encoded by exons 6 and 7. The

first two α domains, α1 and α2, constitute the peptide-binding groove (Figure 4a and 4d).

The HLA class II molecules are also heterodimers of two transmembrane glycoprotein α and β chains, both encoded by HLA genes (Figure 4e). DRA1, DQA1 and DPA1 include α1 and α2 domains that are encoded by exons 2 and 3 of α chain of HLA class II gene (Figure 4b) and DRB1, DQB1 and DPB1 include β1 and β2 domains encoded by exons 2 and 3 of β chain of HLA class II gene (Figure 4d). The α1 and β1 domains form the peptide-binding groove of the HLA class II molecule and are highly variable. The single exception is the α1 domain of DR, which is not polymorphic.

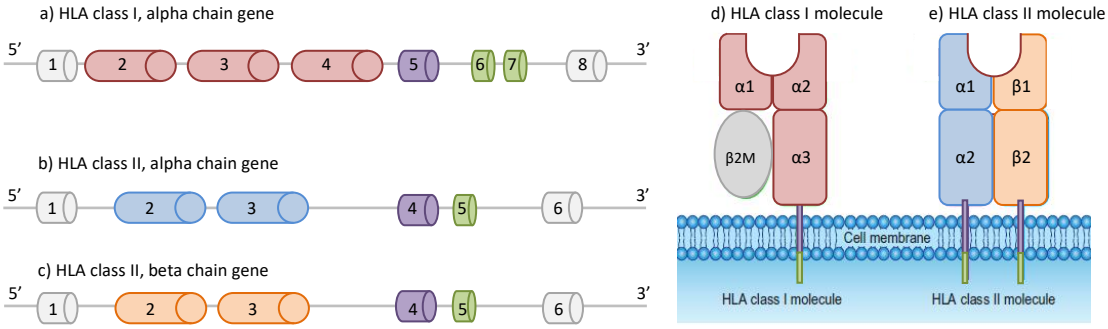


Figure 4 – Human Leukocyte Antigen (HLA) genes and domain organization: a) HLA class I alpha chain gene; b) HLA class II alpha chain gene; c) HLA class II beta chain gene. Exons numbers are represented in Arabic in a), b) and c). The colors used to represent the genes correspond to the domain in HLA molecule. Adapted from Rich R *et* al. Clinical Immunology, 5th edition (doi.org/10.1016/C2015-0-00344-6).

2. ALLORECOGNITION AND ALLOGRAFT REJECTION

The main function of HLA class I and class II molecules in adaptive immunity is to bind peptides, derived from self or nonself antigens, process and present them to the cell surface for recognition by the appropriate T cells. HLA class I molecules, expressed by all nucleated cells, present endogenous or intracellular peptides to CD8+ (cytotoxic) T cells. On the other hand, HLA class II molecules are expressed by antigen-presenting cells (APCs) such as dendritic cells (DC), macrophages and B cells, and present exogenous or extracellular peptides to CD4+ (helper) T cells.

The allograft recognition after organ transplantation is defined by the source of APCs. Allorecognition pathway is direct when is mediated by donor APC, mainly DC within the graft that migrate to lymph nodes, where they present alloantigens to reactive T cells. This mechanism occurs mostly in early posttransplant acute rejection (AR) since donor APC are cleared out after a short period of time (Figure 5a).

On the other hand, indirect allorecognition is mediated by recipients' APC that process and present different alloantigens to T cells, being the predominant mechanism in chronic rejection (Figure 5b). A semi-direct presentation was also described (Figure 5c) and occurs when donor intact HLA molecules are fused to recipient APC (12, 13).

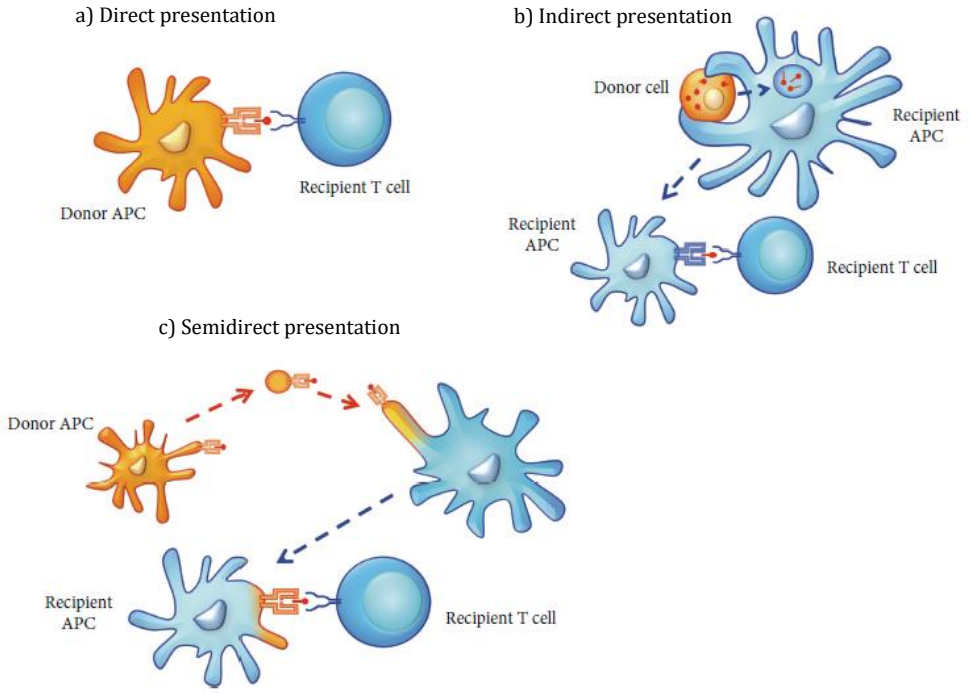


Figure 5 – Mechanisms of allorecognition: a) direct, b) indirect and c) semidirect. Adapted from (14).

Although several promising non-invasive molecular markers are being studied (15, 16), renal allograft biopsy is still the definitive method of diagnostic to assess allograft rejection type and degree (17). In most cases reveals morphologic injuries resulting from predominantly cellular or antibody-mediated mechanisms. Allograft rejection can be classified according to immunological characteristics as hyperacute, acute or chronic.

Hyperacute rejection occurs almost immediately after transplant and is due to preformed antibodies, chiefly against AB0 system or HLA. This type of rejection, especially due to markedly evolution of laboratory assays, is now very rare. However, preformed AB0 and HLA antibodies aren't an absolute contra-indication in kidney transplantation. In fact, AB0-incompatible (AB0i) kidney transplantation is performed, followed a desensitization protocol, in several transplant centers in order to increase transplantation rates (18). Centro Hospitalar Universitário do Porto started the first Portuguese AB0i kidney transplant program in 2015 accepting anti-A/B isoagglutinin titer of IgG class below 256. Also, HLA incompatibility (HLAi) can be surpassed, in same extent, using desensitization protocols (19, 20).

Acute rejection can occur within days to weeks after transplantation and chronic rejection usually develops months or years after transplant. Late kidney allograft failure remains the main cause of graft rejection (21) and, although the etiology is multifactorial, immune-mediated injury and non-compliance are major players (4).

Antibody-mediated rejection (ABMR), introduced as a distinct clinicopathological entity in 1997 International Banff classification (22), is frequently involved in early and late kidney allograft rejection (23). In 2013 Banff conference ABMR C4d negative diagnosis was introduced (24, 25) and the latest update in 2017 the criteria embraced the molecular diagnosis (26).

The presence of HLA donor-specific antibodies (DSA) is the key component of diagnosis of ABMR that can be present before transplantation or develop after transplantation. ABMR associated with *de novo* DSA often co-exists with T-cell-mediated rejection (TCMR) (27), while preformed DSA is memory-associated ABMR with a typically pure phenotype (28, 29).

A careful laboratory immunological risk assessment, interpreted within a multidisciplinary team of immunologists and nephrologists, are the key for preventing allograft rejection.

3. LABORATORY RISK ASSESSMENT

Immunological risk assessment is based on patients HLA allosensitization status and degree of HLA matching (Figure 6), alongside with patient anamnesis.

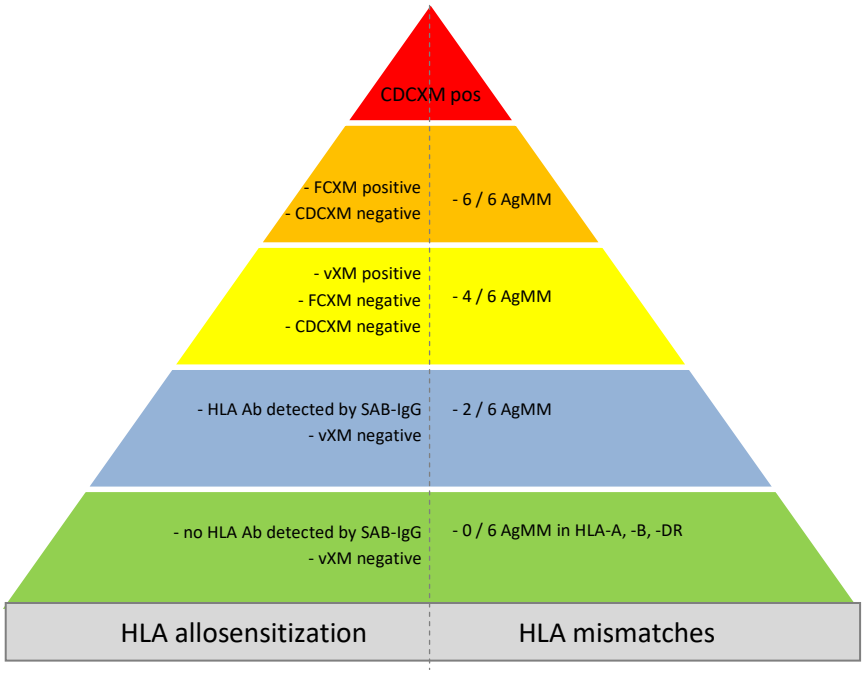


Figure 6 – Immunological risk assessment: HLA allosensitization & HLA mismatches.

3.1. HLA allosensitization

HLA allosensitization is defined as the development of HLA antibodies after exposure to a non-self HLA peptide which is recognized by a B lymphocyte. Sensitizing to non-self HLA can occur through pregnancy (paternal HLA), blood components transfusion and organ transplantation (donor HLA) (30). Other events, such as vaccination (31-33), viral/bacterial infections (34) and ventricular assist devices implementation (35), have also been implicated.

HLA antibodies developed after a sensitizing event can be against a specific allele or recognize an epitope that is shared among different HLA molecules resulting in several HLA antibodies development. The level of sensitization depends of the laboratory method used to detect HLA antibodies (36).

3.1.1. Cellular assays

3.1.1.1. Complement-Dependent Cytotoxicity crossmatch

The detection of HLA antibodies was performed historically by complement-dependent cytotoxicity (CDC), method described by Terasaki and McClelland in 1964 (37) and later accepted as the National Institutes of Health (NIH) standard procedure for histocompatibility testing. The assay consists in incubation of donor viable lymphocytes with the patient serum. If the serum contains antibodies against donors antigens an antibody-antigen complex will form at the cell surface. Rabbit complement is added as a source of complement and cell lysis occurs (Figure 7). The lysis is detected by nuclear staining dyes such as acridine orange (AO)/ethidium bromide (EB). AO is permeable to viable cells generating green fluorescence. EB enters dead cells with compromised membranes and stains all dead nucleated cells with red fluorescence. The percentages of dead cells are assessed using a fluorescence inverted microscope and the International Workshop scores from 1 to 8. The result is positive when the score is equal or greater than 2.

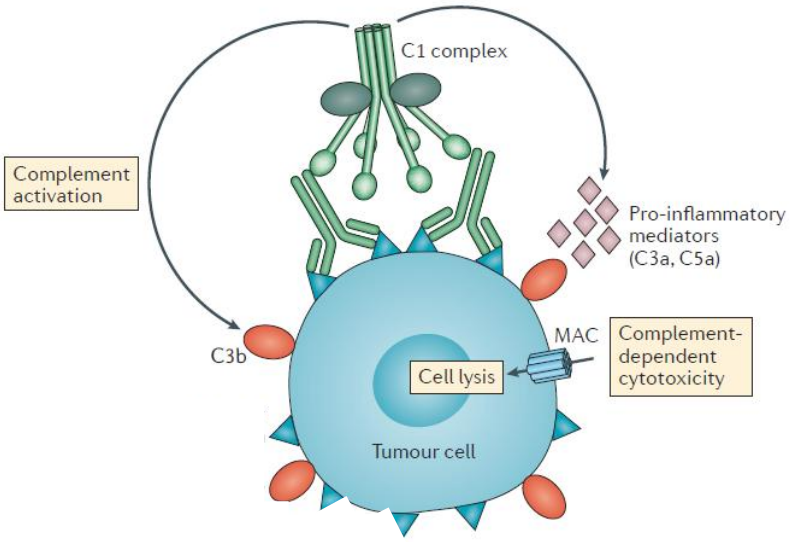


Figure 7 – Schematic illustration of complement-dependent cytotoxicity. Adapted from (38).

In 1969, Patel and Terasaki showed a strong association between a positive CDC crossmatch (CDCXM) and hyperacute rejection in kidney transplantation (39). Since then CDCXM has been improved with prolonged incubations times (40), the addiction of washing steps after the first incubation to remove unbound sera (41), or amplifying complement activation and cell lysis with addition of anti-human globulin (AHG) (42,

43), and became mandatory before transplant to assess if donor-specific antibodies (DSA) are present.

These assays are also associated with false positive results due to non-HLA antibodies and IgM antibodies. The latter are frequent in patients with autoimmune disorders and can be overcome by treating recipient's sera with dithiothreitol (DTT), reducing IgM disulfide bonds (44, 45). In these cases, to assist the interpretation of the allo-XM, an auto-XM is recommended (46).

This methodology is also performed with a panel of donors allowing the panel reactive antibodies (PRA) calculation, i.e., the percentage of donors that will yield a positive CDCXM (47). The use of this method for antibody analysis has been demonstrated to be inadequate for accurately identifying HLA antibodies specificities (48).

3.1.1.2. Flow cytometry crossmatch

Although CDCXM still remains the golden standard method in many histocompatibility laboratories (49), the assay lacks specificity and sensitivity. As such, in 1983 Garovoy *et* al developed a flow cytometry crossmatch (FCXM) assay in order to increase sensitivity (50). This complement activation independent assay was developed as a dual-color method in 1989 (51) and upgraded in 1996 to a three-color method in order to detect T and B cells simultaneously (52). Lobo *et* al showed that cell treatment with pronase increased sensitivity and specificity (53). This treatment has also the benefit of eliminating rituximab interference (54) used for desensitization in many transplant centers. More recently Liwski *et* al. optimized FCXM protocol and developed the Halifax and Halifaster protocols (55), augmenting the signal to noise ratio and interlaboratory concordance of the results.

This assay consists in an indirect immunostaining where antibody-antigen interactions are identified using fluorescein isothiocyanate (FITC) conjugated F(ab')2 fragment goat anti-human immunoglobulin (IgG) as a secondary antibody. T and B cells are identified using anti-CD3 and anti-CD19, respectively. Positive FCXM cutoffs are determined by median channel shift (MCS).

Several groups have showed that FCXM positivity is associated with increased risk of graft failure, even when CDCXM is negative (56-58).

Our laboratory performs FCXM to improve immunologic risk assessment in all living kidney donors and for deceased donors (DD), when preformed DSA are identified.

3.1.2. Solid-phase immunoassays

3.1.2.1. Immunoglobulin G (IgG) HLA antibodies

Solid-phase immunoassays (SPI) revolutionized histocompatibility laboratories, first with Enzyme-Linked Immunosorbent Assay (ELISA) (59) and, more recently, using polystyrene beads using multiple analyte profiling (xMAP®) technology (60). This methodology consists in a multiplex assay that uses a panel of fluorescently dyed micron-sized polystyrene microspheres, produced by the internal conjugation of variable amounts of two or three dyes, enabling the identification of 100 or 500 different beads, respectively (Figure 8a). HLA antigens are bound to these coded-color beads and, after incubation with patient's sera, any HLA alloantibodies present bind to the antigens on the beads. This reaction is detected, after a second incubation with R-Phycoerythrin (PE)-conjugated goat anti-human IgG, with a Luminex® flow analyzer that simultaneously detects the fluorescent emission of PE and the dye signature from each bead. The light signal produced by each bead reflects the relative fluorescence, expressed as the mean fluorescence intensity (MFI), and shouldn't be interpreted as a measure for quantity or concentration.

Three types of SPI kits using xMAP® technology have been developed: i) pooled antigen beads that carries a mixture of purified HLA class I and class II molecules from three or more donors; ii) phenotypic beads consisting of 30 or more beads where each bead carries an HLA class I or class II phenotype purified from a single donor (61); and iii) single antigen beads (SAB) where each bead carries a single recombinant HLA class I or class II allele (62) (Figure 8b).

The presence of preformed DSA identified by SAB assays are associated with increased risk of ABMR and graft failure (63-65). As such, this assay became the method of choice for HLA unacceptable antigens (UA) assignment for patients' waitlisted for deceased donor kidney transplantation (DDKT). However, this methodology revealed several technical limitations and interpretative challenges (66). Besides cut-off variability between laboratories, lot-to-lot variability and different amount of target HLA in the beads, false reactivities can also result from exposure of cryptic epitopes on denatured molecules. Several studies described the detection of these exposed cryptic epitopes in non-transfused males (67, 68) and it was demonstrated that they have no clinical impact in kidney transplantation (69, 70). Additionally, unspecific binding (71) and HLA antibodies present in medical products (72) can cause false positive results. On the other hand, false negative results have also been described due to shared epitopes between beads (73-75).

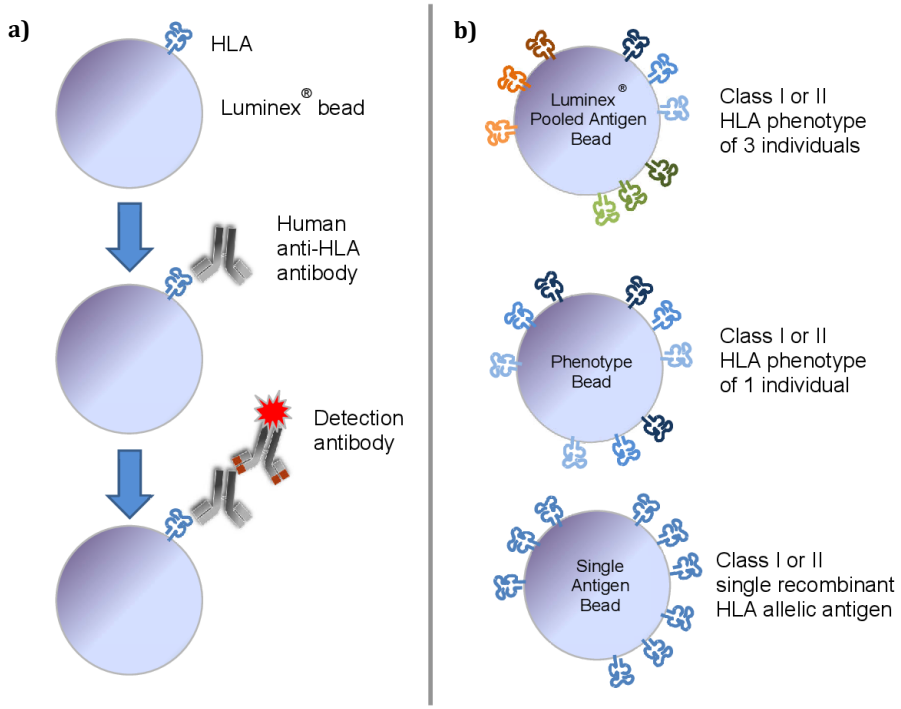


Figure 8 – Solid-phase immunoassays (SPI): a) scheme of the immunoassay reaction; b) types of polystyrene beads used in SPI.

3.1.2.2. Complement-fixing HLA antibodies

The classical pan-IgG SAB assay was modified in order to detect complement fixing HLA antibodies such as C1q (76) and C3d (77) assays.

In C1q-SAB assay, heat-inactivated sera are incubated with recombinant C1q and single-antigen beads. The addition of PE-labeled anti-C1q antibody enables binding detection of both antibody and C1q (Figure 9a). On the other hand, the C3d-SAB assay first incubation does not include a recombinant product and, after the incubation between beads and the sera, a standardized human serum is added as source of complement, followed by addition of a labeled anti-human C3d antibody (Figure 9b). Soon after the implementation of this novel assays, multiple studies showed that complement-fixing SAB results could be predicted by the MFI value of the classical IgG-SAB assay (78, 79). Our study showed that preformed C1q-binding DSA in comparison with DSA strength were a better predictor of ABMR and more strongly associated with allograft failure (80). Tambur *et* al showed that titration studies are the only method capable of providing the true value of antibody strength (81).

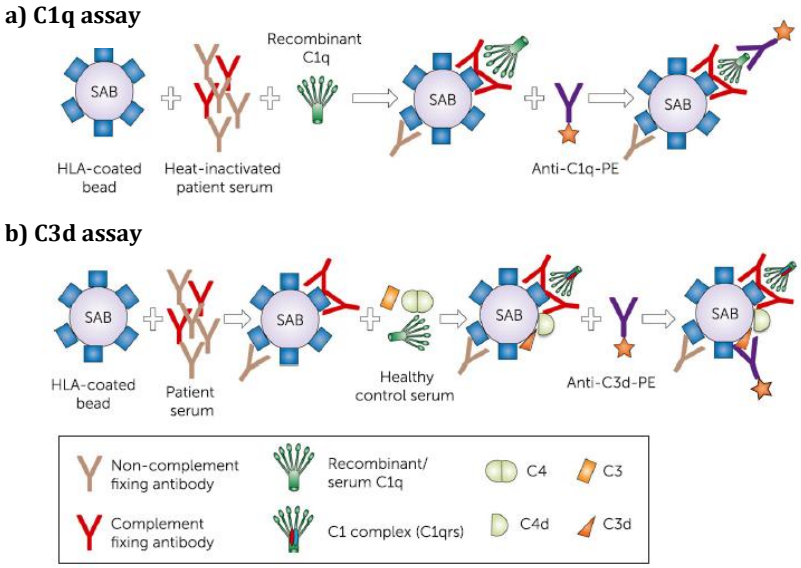


Figure 9 – Schematic principles of C1q assay (a) and C3d assay (b). Adapted from (82).

3.1.2.3. Immunoglobulin G (IgG) subclasses analysis

The four subclasses of IgG in humans are IgG1, IgG2, IgG3, and IgG4 that differ in quantity, structure and function. IgG1 and IgG3 are known to strongly activate complement, IgG2 are effective mainly at high epitope density and IgG4 are ineffective complement activators. While IgG1 is the most abundant subclass in serum and the more efficient in complement-mediated lysis, IgG3 has the highest affinity due to its long hinge region.

As such, several research groups have modified SAB assays in order to be able to analyze the IgG subclass distribution of HLA antibodies (83, 84). This modified assay revealed decreased sensitivity, when compared to classical pan-IgG method. Lefaucheur *et* al showed that circulating immunodominant DSA subtypes allows the identification of distinct patterns of antibody-mediated injury. The main finding is that IgG3 and IgG4 subclasses of the immunodominant DSA are associated with antibody-mediated damage but also correlated with its phenotypes, acute and subclinical ABMR, respectively. The presence of IgG3 DSA was associated with a greater risk of graft loss (85).

3.1.2.3. Virtual PRA

Complement-dependent cytotoxicity (CDC)–PRA assay became a routine test to measure waitlisted patients' degree of allosensitization. Several transplant centers introduced PRA value in allocation algorithms for DDKT, seeking fairness for highly

sensitized patients. However, cytotoxicity was a low sensitivity assay and CDC-PRA is extremely inconsistence. To overcome CDC-PRA limitations Cecka *et* al implemented virtual PRA (vPRA) (86). This calculation is based on SAB assay assigned unacceptable antigens, using a population of HLA phenotype donors.

The introduction of vPRA resulted in outstanding progress bringing more equity to sensitized patients (87-89) and contributed to shorter periods of cold ischemia time (90, 91).

3.1.3. Data interpretation

Histocompatibility laboratories use both solid-phase and cellular assays in immunological risk assessment. As described earlier each laboratory assay has different targets with variable levels of sensitivity and distinct limitations and interferences (Table 3). Therefore, to ensure the best possible evaluation and risk stratification, they must the interpreted together alongside with patient anamnesis.

Table 1 – Interpretation of cellular *vs.* solid-phase immunoassays.

CDC LyT	CDC LyB	CDC_{DTT} LyT	CDC_{DTT} LyB	FCXM LyT	FCXM LyB	SAB-IgG HLA-I	SAB-IgG HLA-II	Possible Interpretation
-	-	-	-	-	-	-	-	no HLA antibodies
+	+	-	-	-	-	-	-	IgM antibodies
+	+	+	+	+	+	-	-	non-HLA antibodies
-	-	-	-	-	-	+	-	false positivity in SAB assay or very low titer HLA-I, IgG Ab
-	-	-	-	-	-	-	+	false positivity in SAB assay or very low titer HLA-II, IgG Ab
-	-	-	-	-	+	-	+	non cytotoxic HLA-II, IgG Ab or low titer HLA-II, IgG Ab
-	+	-	+	-	+	-	+	cytotoxic HLA-II, IgG Ab
-	-	-	-	-	+	+	-	non cytotoxic HLA-I, IgG Ab or very low titer HLA-I, IgG Ab
-	-	-	-	+	+	+	-	non cytotoxic HLA-I, IgG Ab or low titer HLA-I, IgG Ab
-	-	-	-	+	-	+	-	low expression HLA-I, IgG Ab
+	+	+	+	+	+	+	-	cytotoxic HLA-I, IgG Ab
-	+	-	+	+	+	+	+	low titer HLA-I, IgG Ab and cytotoxic HLA-II, IgG Ab
+	+	+	+	+	+	+	+	cytotoxic HLA-I, IgG Ab and cytotoxic HLA-II, IgG Ab

CDC, Complement dependent cytotoxicity; FCXM, Flow cytometry crossmatch; SAB, Single antigen bead; Ly, Lymphocyte; Ab, antibody; HLA-I, HLA class I; HLA-II, HLA class II

SPI enable the introduction of virtual crossmatch (vXM), that is, the identification of DSAs by SAB and allowed the assignment of UA HLA antigens. This assessment depends on several variables, including the *cut-off* used to determine positivity, and a positive vXM should be interpreted alongside with cellular assays.

3.2. HLA Matching

The knowledge of HLA matching importance in kidney transplantation is almost 60 years old (92). However, due to advances in immunosuppression agents, alongside with concerns about disadvantages that HLA minorities were facing, the time on RRT became the main scoring factor in many programs. However, despite the overwhelming improvement in graft survival rates over the past thirty years (21), ABMR remains the major cause of late kidney allograft loss (23, 93) and HLA matching in kidney transplantation significance is now unquestionable (94-96).

3.2.1. Classical antigen matching

HLA mismatching was classically determined by counting HLA class I and class II antigen mismatches in host-versus-graft (H*v*G) direction and the number of mismatches, usually in HLA-A, -B, -DR *loci*, are used in allocation programs worldwide. Since this number is calculated using low-resolution typing, the real number of HLA mismatches may even be higher and HLA allelic difference may contribute to clinically relevant immune responses (97, 98).

HLA antigens mismatches can be assessed considered broad or split antigens (Table 1). Broad HLA antigens are defined by antigenic epitopes that are shared by two or more related antigens. Split antigens are distinguished from other antigens in the broad group by the presence of unique or private epitopes. Deceased donor allocation programs usually consider broad HLA antigen to assess compatibility, seeking equity to patients with low frequency split HLA antigens (99, 100).

3.2.2. Cross-reactive groups

Cross-reactive epitope groups (CREGs) are groups of similar HLA molecules that share public epitopes (Table 2). Several studies identified sera that exhibit cross-reactivity patterns with CREG of HLA antigens (101).

Allocation using CREGs was proposed due to extensive HLA polymorphism and, as described, to overcome inequality within racial minorities (102). This strategy has the advantage of equalizing the HLA effect, as rare HLA antigens are grouped with common HLA antigens sharing the same public epitopes and protection from sensitization to HLA (103). However, Laux *et* al showed that the outcome of this transplants are guided by the number of HLA antigen mismatches rather than CREG

mismatches (104). Also, Stobbe *et* al showed that CREG matching does not lead to better allocation and allograft survival (105). More recently, Nainani *et* al demonstrated that CREG antibodies were associated with acute ABMR in sensitized patients (106).

Table 2 – Original broad, splits and associated (*italic*) HLA antigens.

Original Broad Specificities	Splits and Associated HLA Antigens
A2	*A203, A210*
A9	A23, A24, *A2403*
A10	A25, A26, A34, A66
A19	A29, A30, A31, A32, A33, A74
A24	*A2403*
A28	A68, A69
B5	B51, B52, *B5102, B5103*
B7	*B703*
B12	B44, B45
B14	B64, B65
B15	B62, B63, B75, B76, B77
B16	B38, B39,*B3901,B3902*
B17	B57, B58
B21	B49, B50, *B4005*
B22	B54, B55, B56
B27	*B2708*
B39	*B3901, B3902*
B40	B60, B61
B51	*B5102, B5103*
B70	B71, B72
Cw3	Cw9, Cw10
DR1	*DR103*
DR2	DR15, DR16
DR3	DR17, DR18
DR5	DR11, DR12
DR6	DR13, DR14, *DR1403, DR1404*
DR14	*DR1403, DR1404*
DQ1	DQ5, DQ6
DQ3	DQ7, DQ8, DQ9

Table 3 – Definition of cross-reactive groups (CREGs), according to United Network for Organ Sharing, 1998.

CREG	Splits and Associated Antigens
A1C	A1, 36, A3, A11, A29, A30, A31, A80
A2C	A2, B57, B58
A9C	A2, A68, A69, A23, A24
A10C	A25, A26, A34, A66, A32, A33, A43, A74
A28C	A2, A68, A69
B5C	B51, B52, B35, B53, B18
B7C	B7, B8, B41, B42, B48, B60, B61
B8C	B8, B64, B65, B38, B39
B12C	B44, B45, B49, B50, B13, B60, B61, B37, B41, B47
B21C	B51, B52, B49, B50, B35, B53, B62, B63, B71, B72, B73
B22C	B7, B42, B54, B55, B56, B27, B46
B27C	B7, B27, B13, B60, B61, B47
Bw4C	B5, B13, B17, B27, B37, B38, B44, B47, B49, B51, B52, B53, B57, B58, B63, B77
Bw6C	B7, B8, B14, B18, B22, B35, B39, B40, B41, BB42, B45, B46, B48, B50, B54, B55, B56, B60, B61, B62, B64, B65, B67, B70, B71, B72, B73, B75, B76, B78

CREG, Cross Reactive Epitope Group.

3.3.3. HLA molecular matching

Although better HLA antigen matching is recognized as beneficial in kidney transplantation, it was soon realized that not every HLA mismatches are equally immunogenic or leads to DSA development (107). Therefore, the ability to predict if a HLA mismatch will elicit B-cell and T-cell mediated alloreactive responses has become of enormous importance.

HLA antigens are composed by a unique combination of epitopes and each individual epitope can shared between different HLA antigens (Figure 10). As such, an individual HLA mismatch can result in none, only a few or many foreign epitope mismatches, when compared to the epitopes present on the patient's own HLA alleles.

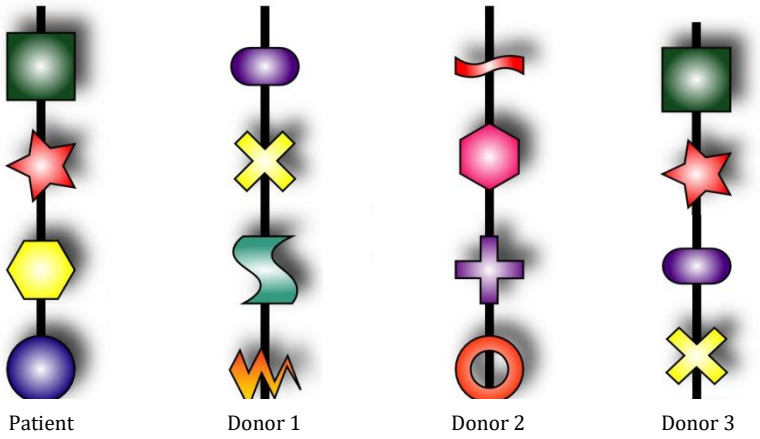


Figure 10 – Schematic HLA antigens that express a unique set of epitopes that are often shared among other HLA antigens. Adapted from (108).

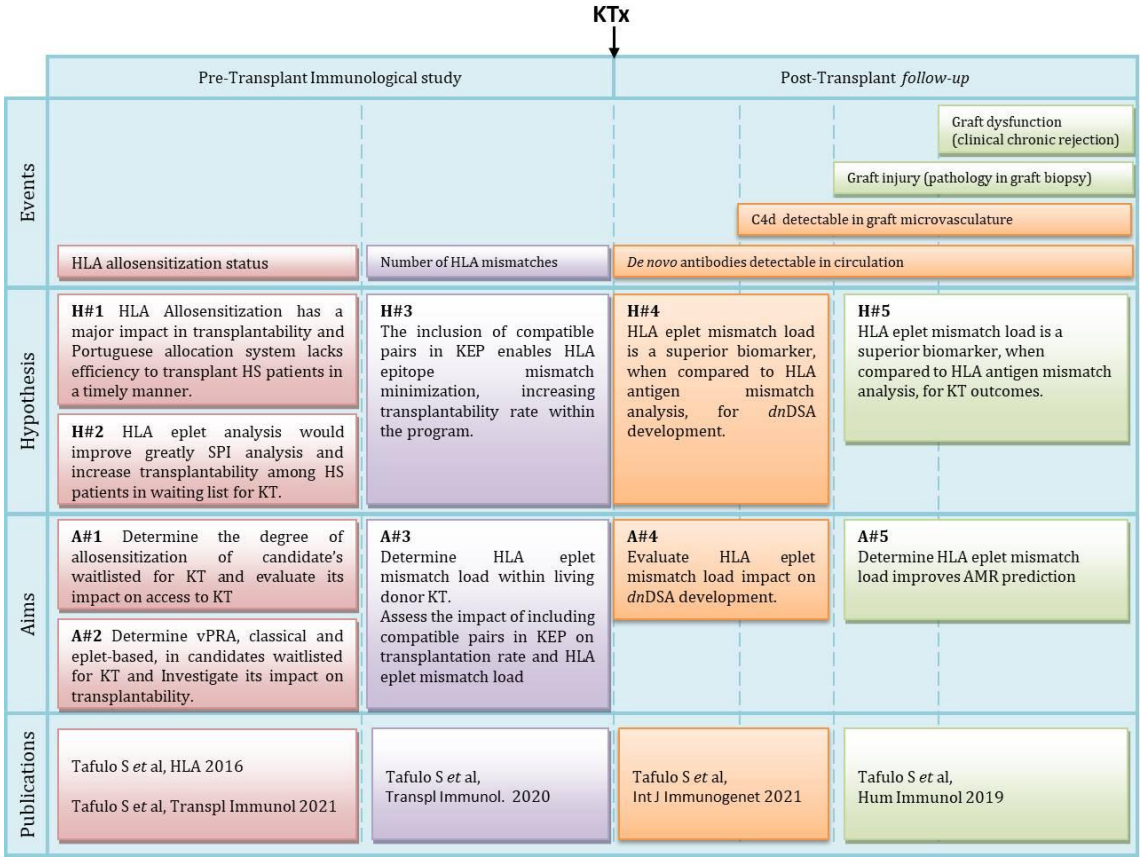
HLAMatchmaker has been the *in silico* theoretical algorithm most widely used. It was developed by Rene Duquesnoy to determine the differences in B-cell epitopes between donor and recipient by intralocus and interlocus comparisons of polymorphic triplets in sequence linear positions (109). In 2006 the program was updated to include structurally defined HLA epitope repertoire based on stereochemical modeling of crystallized HLA antigens, the eplet version (110). Each structural epitope has a functional epitope or eplet of 2-5 residues within 3.0-3.5 Å of a given sequence position on the molecular surface. While the structural epitope with 15-22 residues increases the stability of the antigen-antibody complex, the eplet defines strength and specificity of the antibody reactivity.

At the same time, El-Awar *et* al identified HLA epitopes by allosera adsorption with recombinant cells expressing a single HLA antigen, and testing the eluted antibody with single antigen coated beads (111). These epitopes were named Terasaki epitopes (TerEps). Duquesnoy and Marrari performed a comparative analysis between eplets and TerEps and showed correlation between 81 of 103 Terasaki's HLA class I epitopes are equivalent to individual eplets (n = 50) or pairs of eplets (n = 31), strengthening the concept that eplets are essential basic units of HLA epitopes (112).

HLA epitope registry (http://www.epregistry.com.br) lists all theoretical and known eplets within six databases: ABC, DRB, DQB+DQA, DPB+DPA, interlocus HLA class II and MICA. The registry provides information of antibody reactivity (antibody-verified eplets), structural epitope, associated luminex and all alleles and, more recently, the ElliPro score (113). The ElliPro is a web-tool available at http://tools.iedb.org/ellipro/ that predicts linear and discontinuous antibody epitopes based on a protein antigen's 3D structure. Duquesnoy *et* Marrari showed that ElliPro prediction score reflect eplets ability to induce specific antibody responses in HLA-ABC immunogenic eplets (114).

CHAPTER III - MATERIALS & METHODS

1. THESIS DESIGN



2. SUBJECTS

KT candidates and recipients from Nephrology & Kidney Transplantation Unit from the CHUP were enrolled in the studies undertaken. Kidney allografts from deceased donors were selected nationally according to Portuguese allocation system and living donors were studied and the allografts procured in CHUP.

In one single study, kidney transplanted pairs registered in the national paired kidney exchange program were considered (115).

The specific cohort for each study was defined accordingly and is described in detailed in the Results chapter.

3. CLINICAL DATA

Patients and donors demographic and clinical data were collected retrospectively from the clinical database of Nephrology & Kidney Transplantation Unit of CHUP. At transplant the variables usually considered were recipient age and gender, race, recipient height (cm) and weight (kg), chronic kidney disease etiology, diabetes and Hepatitis C history, induction immunosuppression, donor type, donor age and gender, donor height (cm) and weight (kg) and donor smoking history. After transplant, allograft and patient outcome data were collected such as, delayed allograft function, acute (cellular or antibody-mediated) rejection, renal allograft function, proteinuria, and allograft and patient survival.

Immunological data from patients and donors were collected from LusoTransplant database of CSTP, IPST and included HLA typing and number of HLA mismatches, AB0 phenotyping, previous allosensitizing events, PRA percentages, DSA presence and MFI values, time on the waiting-list, dialysis vintage time, number of deceased donor organ offers and transplantation date.

4. LABORATORY ASSAYS

4.1. HLA genotyping

HLA low and intermediate resolution genotyping was performed for all patients listed for KT and for all living and deceased potential donors. Routinely HLA genotyping was performed by reverse sequence-specific oligonucleotide (rSSO) and in emergency cases, such as deceased donors, HLA genotyping was performed by sequence-specific primer (SSP). Both methodologies are deoxyribonucleic acid (DNA) based requiring genomic DNA isolation from peripheral blood collected in Acid-Citrate-Dextrose (ACD, yellow top) or ethylenediaminetetraacetic acid (EDTA, lavender top).

4.1.1. Sequence-specific primer

HLA genotyping by PCR-SSP was originally described by Olle Olerup (116, 117). This methodology allows the discrimination between the different alleles during the PCR process. Genomic DNAs were isolated and purified using QIAamp blood kit (Qiagen Inc., Chatsworth, CA, USA), and the concentration was adjusted to 30 ng/μL. The purity for each sample was determined by 260:280 and 260:230 absorbance ratios using Nanodrop® ND-1000 spectrophotometer, with the accepted values being in the range of 1.5–1.9.

HLA-A, -B, -C, and –DRB1 genotyping was performed for all deceased donors using Olerup-SSP® HLA-ABC and HLA-DRDQ combi trays (Olerup SSP AB, Stockholm, Sweden), accordingly to the protocol and recommendations of the manufacturer using the GeneAmp® PCR System 9700 thermal cycler (Perkin-Elmer).

The amplified DNA fragments were sized separated by agarose 2% (w/v) gel electrophoresis in 0.5X Tris/borate/EDTA (TBE) buffer for 15-20 minutes (min.) at 8-10V/cm. The use of a dye electrophoresis marker of specific molecular weights (DNA ladder) enables determination of fragments sizes and visualization was achieved using GelRed®, a nucleic acid gel stain that allows amplicons visualization using ultraviolet (UV) light. HLA genotyping was determined using HELMBERG-SCORE™ software, updated with the latest version of IMGT/HLA database.

4.1.2. Sequence specific oligonucleotide

HLA-A, -B, -C, and –DRB1 intermediate resolution genotyping by rSSO was performed using LabType™ SSO typing kits (One Lambda, Canoga Park, CA) for all KT candidates and donors.

Target DNA is polymerase chain reaction (PCR) amplified using group-specific primers and then biotinylated, which allows the detection using R-PE conjugated streptavidin (SAPE). The PCR product is then denatured and allowed to hybridize to complementary DNA probes conjugated to coded-color microspheres. Fluorescent intensity of PE on each microsphere was determined using a LABScan™100 flow analyzer (Luminex®, Austin, TX, USA). The assignment of the HLA class I and class II typing is based on the reaction pattern, using HLA fusion™ software updated with the latest version of IMGT/HLA database.

4.1.3. HLA mismatches assessment

4.1.3.1. HLA antigen mismatches

HLA antigen mismatches (AgMM) were assessed by counting HLA class I (HLA-I) and HLA class II (HLA-II) broad and split HLA antigens, in host-*versus*-graft (H*v*G) direction.

4.1.3.2. HLA eplet mismatches

HLA eplet mismatches were defined using HLA-A, -B, -C, -DR$\beta_{1/3/4/5}$ and -DQα_1/β_1 allelic typing assigned based on the intermediate typing obtained by rSSO, linkage disequilibrium analysis 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 (118, 119).

HLA eplet mismatch load, total (EptMM) and antibody-verified (EpvMM), were assessed using HLAMatchmaker HLA-ABC and HLA-DRDQDP software, available at http://www.epitopes.net/downloads.html.

4.2. HLA antibodies assays

Patients in active waiting list are studied periodically to assess their HLA alloimmunization status with cellular and solid-phase immunoassays (SPA).

4.2.1. Cellular assays

4.2.1.1. Cytotoxic Panel Reactive Antibodies (PRA)

The CDC-PRA assay consists in standard CDCXM using a home-made cell panel composed by 45–50 donors, with known HLA typing, to test patient's sera. This assay allows the determination of CDC-PRA, considered positive if higher than 5%, and identification of complement-fixing HLA antibodies.

Donor's mononuclear cells were isolated using ficoll-paque gradient separation method that allows, after 35 min centrifugation, the differential migration of cells resulting in layers containing different cell types.

In a Terasaki plate, 1 μL of isolated cells incubated with 1 μL of patients sera at room temperature (RT) for 35 min. After incubation, 5 μL of rabbit complement (One Lambda, Canoga Park, CA) were added followed by 75min incubation at RT. Finally, a dye mixture of AO/EB (Fluoroquench™, One Lambda, Canoga Park, CA) was added and the reaction was scored accordingly with the International Workshop (0,1,2,4,6,8), using an inverted fluorescence microscope.

PRA percentage calculations were performed using Lambda Scan® Plus II Analysis software, version 5.9.

4.2.1.2. Complement-dependent cytotoxic crossmatch

All allograft recipients included in the different studies were transplanted with a negative T- and B-lymphocyte standard CDCXM, not enhanced with anti-human globulin, in current sera. The recipient sera were considered current when collected within the last three months. If a sensitizing event was reported during this period, CDCXM included a sample collected in the last 48 hours.

Patient's sera with known IgM antibodies, due to autoimmunity diseases, were tested after DTT treatment to prevent false positive results.

T and B cells were isolated using positive selection, from donor's whole peripheral blood collected in ACD, using Dynabeads™ CD8 (#11333D) and CD19 Pan B (#11143D) conjugated magnetic beads (Life Technologies Corporation, Carlsbad, CA). T and B cell suspensions were tested as described in section 4.2.1.1.

4.2.1.3. Flow cytometry crossmatch

All recipients with preformed DSAs included in the different studies were tested with FCXM. Donor mononuclear cells were separated as described in 4.2.1.1. After adjusting cell suspension concentration to $2x10^6$ cells /mL, using a hematology analyzer Sysmex XE-2100 (Sysmex Corporation, Kobe, Japan), 100 μL were added to a 96-well plate. After cell centrifugation and flicking, 20 μL of patient neat sera were added to each well in duplicate, as well as negative and positive controls.

The cell/serum mixture was incubated for 30 min at RT, followed by three washes with 150 mL of wash buffer and 5 min centrifugation at 250 rpm. Thereafter, the monoclonal mixture of anti-CD3 conjugated with PE (BD Bioscience, clone SK7, #347347), anti-CD19 conjugated with the tandem fluorochrome PerCP-Cyanine5.5 (BD Bioscience, clone SJ25C1, #340951) and a 1:100 fold diluted FITC-conjugated F(ab')2 goat anti-human IgG (Dako, #F0315) were added and incubated for 20 min at 4ºC. After two washes cells were transferred to the flow cytometry tubes and samples were acquired on a BD FACSCalibur™ flow cytometer. BD CellQuest™ Pro software was used to acquire and analyze data.

4.2.2. Solid-phase immunoassays

SPA were carried out using coded-colour microbeads coated with purified HLA class I or class II antigens based on Luminex Xmap® Technology (LABScreen® Mixed kit, OneLambda, Canoga Park, CA, USA). Briefly, 5 µL on the beads incubated in a round-well plate with 20 µL of patient's sera for 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, Canoga Park, CA) during a second 30 min at RT incubation. After two final washes, mean fluorescence intensity of each bead was measured using a LABScan™ 100 flow analyzer (Luminex®, Austin, TX, USA). Patients with a pre-transplant positive screening for HLA antibodies were tested with SAB assays using 6% EDTA-treated sera (LabScreen Single Antigen Beads®, OneLambda, Canoga Park, CA). The analysis was performed using HLAfusion™ software and MFIs higher than 1000 were considered positive.

4.2.2.1. Donor-specific antibodies assignment

Donor specific antibodies are determined by comparing the HLA antibodies determined by SAB assay with donor HLA-A, -B, -Cw, -DRB1/3/4/5 and DQA/B antigens. When SAB-IgG revealed allele-specific antibodies, HLA high-resolution typing of the donor was performed to determine with more accuracy if the HLA antibodies identified were DSA.

4.2.2.2. Virtual Panel Reactive Antibodies (PRA)

Virtual PRA was determined using the vPRA calculator from Eurotransplant Reference Laboratory, available at http://www.etrl.org/vPRA.aspx) and using a Portuguese deceased donor population (vPRApt).

Eurotransplant vPRA calculator version 2.0 was based on HLA phenotype of 6870 deceased donors, within the Eurotransplant service area, between the year 2010 and 2014. The Portuguese vPRA included 1100 deceased organ donors typed for HLA-A, -B, -C, -DRB1 and -DQB1 loci using PCR-SSP (Olerup® SSP HLA typing kits, Stockholm, Sweden). HLA antigen frequencies were performed on each of the five loci and no deviation from the Hardy- Weinberg equilibrium (HWE) was observed.

The vPRA percentage will depend on the cut-off used on SAB assay. As such, we calculated three vPRA values: a) total vPRA (vPRAt) using our routine the cut-off of 1000 MFI, considering the total sample tested; b) current vPRA (vPRAc) using the same cut-off, but considering only the last patient's sample; and c) eplet vPRA (vPRAe) that does not consider a rigid cut-off of 1000 MFI but uses HLA eplet analysis to determine UA. As such, allelic specific specificities found in the SAB that could not be explained with an antibody-verified eplet (AbVer), or high ElliPro score (HiElliPro), were not considered.

5. IMMUNOSUPRESSION

5.1. Induction protocol and maintenance immunosuppression

Induction therapy was used in a majority of patients 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 patients with high HLA mismatch, previous transplant and/or those with high PRA value (>20%). All patients had similar triple maintenance immunosuppression, consisting of a calcineurin inhibitor, tacrolimus (TAC) or cyclosporine, mycophenolate mofetil (MMF) or azathioprine, and prednisolone. No immunosuppression minimization strategy was implemented.

5.2. Rejection diagnosis and treatment

Kidney graft rejection was defined as biopsy-proven. 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). Specimens were evaluated by light microscopy and immunofluorescence staining for C4d and classified according to Banff classification updated in 2017 (13).

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. Antibody-mediated rejection was treated with plasmapheresis (at least 3–5 sessions) and intravenous immunoglobulin (IVIg) 100 mg/kg after each session. After the last plasmapheresis session, every patient received high-dose IVIg (2 g/kg, maximum 140 g) divided in four daily doses and one dose of rituximab (375 mg/m2); a similar dose of IVIg (2 g/kg) was repeated 1 month later.

Patients with *dn*DSA emergence but without signs of graft dysfunction received no specific treatment, besides optimization of TAC (trough level 8–10 ng/ml) and MMF dose.

6. STATISTICAL ANALYSIS

HLA class I and class II antigen and eplet mismatch load (EpMM) were analyzed as continuous variables. Additionally, EpMM class I and class II, and HLA-DR and HLA-DQ separately, were analyzed as categories defined by their terciles.

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. *De novo* DSA incidence and graft survival curves were visualized using Kaplan–Meier method, with comparison between patients' groups being done by log-rank test. In the case of death with a functioning graft, time was censored at the time of death.

All patients were followed-up from time of transplant until death and graft failure (GF) was defined as return to dialysis or retransplant or end of follow-up. 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 (120).

Independent predictors of acute CMR, ABMR and *dn*DSA were explored by univariate and multivariable Cox proportional hazards models. The model used for the multivariable analyses included only those variables presenting a univariate P-value < 0.1.

The Wilcoxon signed rank test was used to compare paired changes between vPRA different calculations and Spearman's rho correlation to examine the relationship between the three vPRA percentages. The strength of association between vPRA values was assessed by Goodman and Kruskal's Gamma rank correlation and Cohen's kappa for agreement.

Estimation of the number (n) of match runs needed for 95% probability of finding an acceptable donor was calculated as previously described (121). The percentage of change of eMR was calculated usind the formula %eMR = (eMRfinal – eMRinitial) / eMRfinal *100.

A two-sided P-value of < 0.01 was considered as statistically significant. Statistical calculations were performed using SPSS, version 23.0 (SPSS Inc., Chicago, IL) or STATA/MP, version 15.1 (Stata Corp, College Station, TX).

7. INSTITUTIONAL AND ETHICAL APPROVAL

The study was reviewed and authorized by the Departamento de Ensino, Formação e Investigação/ Gabinete Coordenador da Investigação and Comissão de Ética of CHUP [nº 215/231 (192-DEFI/175-CES)] and by the Conselho Directivo do IPST in 18-November-2015. The principles of the Declaration of Helsinki and the internal rules of the CHUP and IPST were observed. In all phases of the study the confidentiality of data collected and the identity of the participants were guaranteed. All databases were constructed and analyzed anonymously.

CHAPTER IV – RESULTS

1. PRE-TRANSPLANT HLA ALLOSENSITIZATION STATUS

Waitlisted patients for kidney transplant are routinely studied to assess their allosensitization status. Screening and identification of anti-HLA antibodies are performed to determine unacceptable antigens and virtual PRA.

1.1. HLA allosensitization degree and transplantability

Low transplantability of 0 blood group and highly sensitized candidates in the Portuguese kidney allocation algorithm: Quantifying an old problem in search of new solutions.

Tafulo *et* al. HLA. 2016 Nov; 88(5):232-238. doi: 10.1111/tan.12895.

Appendix 1

We performed a retrospective study to evaluate the difference of patient's access to DDKT, according to blood groups and HLA allosensitization status.

Prevalent and incident candidates to KT in Portuguese north waitlist, between January 2010 and December 2011, were included in the study (n=1020). Patient's sera were screened every three months by SPI to determine the presence of HLA class I and II antibodies and, if positive, a SAB assay was performed to identify the antibodies detected. HLA antibodies identified for HLA-A, -B and –DRB1 *loci,* considering 1000 median fluorescence intensity (MFI) as *cut-off*, were used to calculate vPRA in accordance to current Portuguese legislation for allocation algorithm.

Patients were followed until receiving a kidney allograft from a deceased (n=629) or a living donor (n=48), being removed from the waiting list by medical and/or patient choice (n=138), death (n=30), or until 31 December 2014.

Two hundred and forty (23.5%) patients were sensitized for HLA, 127 (12.5%) of them being HS with vPRA higher than 80%. It is noteworthy that only 14.2% of these patients (n=18) were considered HS considering CDC-PRA.

The DD organ offer rate according to blood type and vPRA groups is displayed in Table 4, considering only candidates listed in regular priority (n=987). Overall, there were 4257 organ offers that represented 1 offer every 6.7 months per candidate. The DD organ offer rate by blood type tended to decrease with the increase of vPRA for the candidates of A and B blood groups. For AB blood type patients, this difference was only seen in the extreme vPRA values perhaps due to the lower number of patients in these groups. Curiously, for blood type O patients, no difference was found between groups 1 and 2, emphasizing the long waiting time that even non-sensitized O blood group patients have to face. As such, DD organ offer rate was significantly different only in the comparisons between HS candidates and the remaining groups.

Table 4 - Deceased organ offer rate by blood group and vPRA.

		Mean months until 1 offer/candidate	95% CI	*P*
Overall (n=987)		6.7	6.3-7.1	-
Blood type A (n=434)	Global	6.0	5.7-6.2	A *vs.* B = 0.642 A *vs.* AB = 0.404 A *vs.* O ≤ 0.001
	1. vPRA 0% (n=312)	4.8	4.6-5.1	1 *vs.* 2 = 0.001 1 *vs.* 3 ≤ 0.001 2 *vs.* 3 ≤ 0.001
	2. vPRA 1-79% (n=52)	5.9	5.3-6.6	
	3. vPRA≥80% (n=70)	15.8	13.7-18.1	
Blood type B (n=40)	Global	5.8	5.0-6.6	B *vs.* AB = 0.675 B *vs.* O ≤ 0.001
	1. vPRA 0% (n=27)	4.0	3.5-4.7	1 *vs.* 2 = 0.024 1 *vs.* 3 ≤ 0.001 2 *vs.* 3 = 0.015
	2. vPRA 1-79% (n=3)	7.0	3.9-12.7	
	3. vPRA ≥80% (n=10)	28.7	16.3-50.5	
Blood type AB (n=20)	Global	5.5	4.5-6.6	AB *vs.* O ≤ 0.001
	1. vPRA 0% (n=14)	4.2	3.3-5.3	1 *vs.* 2 = 0.704 1 *vs.* 3 ≤ 0.001 2 *vs.* 3 = 0.133
	2. vPRA 1-79% (n=1)	3.5	1.6-7.8	
	3. vPRA ≥80% (n=5)	9.3	6.4-13.6	
Blood type O (n=493)	Global	10.6	10.1-11.1	-
	1. vPRA 0% (n=398)	9.9	9.4-10.4	1 *vs.* 2 = 0.320 1 *vs.* 3 ≤ 0.001 2 *vs.* 3 ≤ 0.001
	2. vPRA 1-79% (n=56)	10.6	9.3-12.1	
	3. vPRA≥80% (n=39)	29.8	20.9-38.9	

vPRA, virtual panel reactive antibodies; CI, confidence interval.

To determine the median waiting time for a kidney transplant from a DD by blood type and vPRA, we excluded patients who received a kidney graft from a living donor, those removed from the waiting list, or who died during follow-up. The remaining 776 patients were included in the longitudinal analysis that revealed that the median waiting time for transplant was greater for O blood type patients (65.3 months), when compared to the remaining blood types: A (35.1 months), B (22.8 months), and AB (14.5 months) (Table 5). The waiting time for A blood type patients increased with vPRA value (P<0.001) as the percentage of patients transplanted over time was significantly lower. For O blood group patients, this difference was only significant between non-sensitized patients and the remaining groups (P<0.001). For blood type B and AB patients, the difference was only significant between non-sensitized and HS candidates (P<0.001 and P=0.013, respectively).

A multivariable Cox regression model showed that younger [hazard ratio (HR)=1.020, P<0.001], vPRA≥80% (*versus* vPRA=0%, HR=0.090, P<0.001), and vPRA=0-79% (*versus* vPRA=0%, HR=0.380, P<0.001) candidates had a lower chance of been transplanted. Additionally, when compared to A blood type candidates, blood type B and AB patients had a higher chance of been transplanted (HR=1.574, P=0.019;

HR=2.582, *P*=0.001, respectively), while the opposite occurred in O blood type ones (HR=0.255, *P*<0.001).

Table 5 - Median waiting time for a kidney transplant by blood type and vPRA.

		Number of transplanted candidates (%)	Median waiting time	Log-rank *P*
Overall (n=776)		601 (77.4%)	47.5	-
Blood type A (n=344)	Global	290 (84.3%)	35.1	A *vs.* B = 0.366 A *vs.* AB = 0.117 **A *vs.* O ≤ 0.001**
	1. vPRA 0% (n=254)	239 (94.1%)	30.7	**1 *vs.* 2 ≤ 0.001**
	2. vPRA 1-79% (n=38)	29 (76.3%)	47.4	**1 *vs.* 3 ≤ 0.001**
	3. vPRA ≥80% (n=52)	22 (42.3%)	129.3	**2 *vs.* 3 ≤ 0.001**
Blood type B (n=35)	Global	31 (88.6%)	22.8	B *vs.* AB=0.638 **B *vs.* O=0.001**
	1. vPRA 0% (n=25)	25 (100%)	16.9	1 *vs.* 2 = 0.215
	2. vPRA 1-79% (n=3)	2 (66.7%)	27.4	**1 *vs.* 3 ≤ 0.001**
	3. vPRA ≥80% (n=7)	4 (57.1%)	144.0	2 *vs.* 3 = 0.156
Blood type AB (n=17)	Global	14 (82.4%)	14.5	**AB *vs.* O ≤ 0.001**
	1. vPRA 0% (n=13)	12 (92.3%)	13.5	1 *vs.* 2 = 0.867
	2. vPRA 1-79% (n=1)	1 (100%)	16.6	**1 *vs.* 3 = 0.013**
	3. vPRA ≥80% (n=3)	1 (33.3%)	70.5	2 *vs.* 3 = 0.083
Blood type O (n=380)	Global	266 (70.0%)	65.3	-
	1. vPRA 0% (n=308)	232 (75.3%)	62.0	**1 *vs.* 2 = 0.001**
	2. vPRA 1-79% (n=42)	21 (50.0%)	75.1	**1 *vs.* 3 < 0.001**
	3. vPRA ≥80% (n=30)	13 (43.3%)	91.1	2 *vs.* 3 = 0.176

vPRA, virtual panel reactive antibodies; CI, confidence interval.

Our study shows that median waiting time was significantly higher in O blood type patients, when compared to the remaining groups. However, a stronger impact on waiting time according to vPRA was observed, with HS patients having 368%, 632%, 486%, and 140% increases in blood groups A, B, AB, and 0, respectively, when compared to each blood group global median waiting time.

Major Conclusions:

- CDC-PRA profoundly underestimates patient's true HLA allosensitization status defined by vPRA.
- O blood type patients are in disadvantaged to find a compatible donor, when compared to the remaining groups.
- HS candidates as defined by vPRA were hugely disadvantaged in the access to KT, independently from AB0 blood groups.
- Important measures need to be undertaken in order to mitigate the disadvantage of O blood type and, even more so, of HS candidates have in accessing KT.

1.2. HLA allosensitization status by eplet-based analysis

Eplet based virtual PRA increases transplant probability in highly-sensitized patients. Tafulo *et* al. Transpl Immunol 2021 Jan;9:101362. doi: 10.1016/j.trim.2021.101362

Appendix 2

At 01-01-2020, 1973 patients were waitlisted for deceased donor kidney transplantation in Portugal. The Northern region includes 606 patients (30.71%) and 157 (25.9%) of these patients are highly-sensitized with vPRA higher than 98%, that represents 22.9% of the overall Portuguese HS population (n=683), and were included in this study.

All patients had at least one previous sensitizing event, such as transplant (n=120, 76%), blood component transfusions (n=125, 80%), and pregnancies (n=64 of 87 female patients, 74%). As expected patients with 100% of vPRA were predominantly candidates for retransplantation (p<0.001), had higher PRA-CDC (p<0.001), and longer dialysis vintage waiting time (p<0.001).

It is noteworthy that only 19 (12%) patients are classified as HS by the Portuguese legislation with PRA-CDC higher than 80%. In fact, only 47 (30%) patients had PRA-CDC higher than 50% and would be granted extra-points in Portuguese allocation process. It is also important to note that the median dialysis vintage time in our cohort is 106.9 (66.9-161.8). This is significantly increased in the vPRA=100% group with a medium dialysis vintage time of 134.9 (90.3-180.4). As expected, spearman rank-order coefficient showed strong correlation between vPRA calculations, vPRAt *vs*. vPRAc (ρ=0.715, p<0.001), vPRAt *vs*. vPRAe (ρ=0.531, p<0.001) and vPRAc *vs.* vPRAe (ρ=0.738, p<0.001).

Inter-group reclassification analysis is outlined in an overlay histogram (Figure 11). Reclassification between vPRAt and vPRAc (Table 6) showed that 87 (55%) patients remained in the same interval group. Reclassification from vPRAt to one-degree lower group by vPRAc occurred in 53 (34%) patients and 17 (11%) were reclassified from vPRAt to more than one-degree lower group by vPRAc. Also, 28 (18%) patients were reclassified to non-HS when considering only the current sera in vPRA calculation. Kappa and gamma correlation values between vPRAt *vs.* vPRAc were 0.383 (P<0.001) and 0.831 (P<0.001), respectively. When comparing vPRAt and vPRAe, as expected, the inter-group movement is higher remaining only 33 (21%) patients in the same interval group (Table 7).

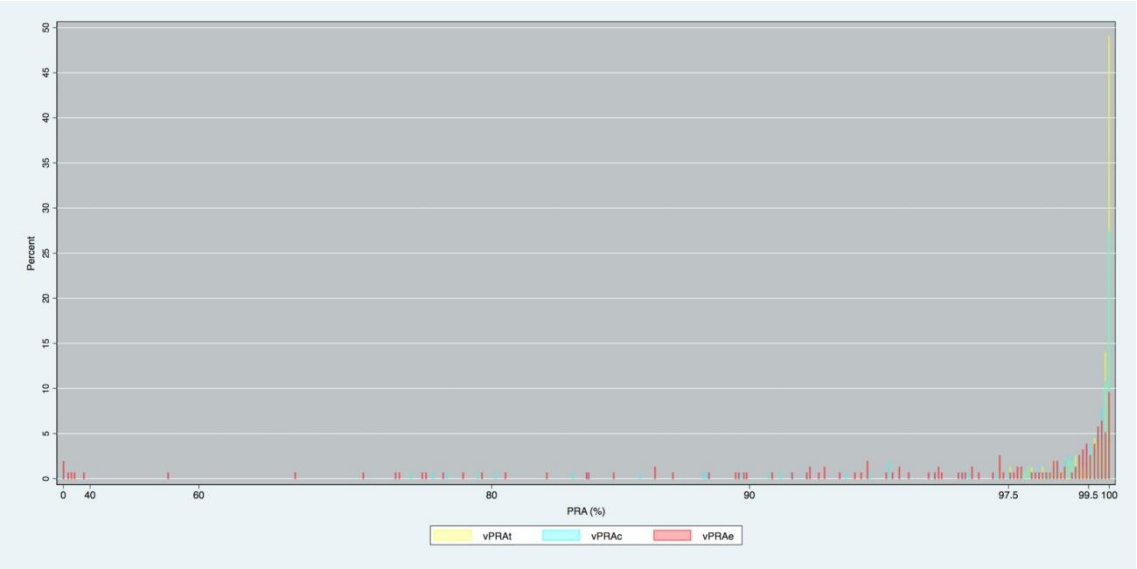


Figure 11 – Histogram of Intergroup reclassification between vPRA calculations.

Table 6 - Intergroup movement between total and current virtual panel reactive antibodies calculations.

			vPRAt		
			[97.50%-99.50%[N=34 (22%)	[99.50%-99.91%[N=46 (29%)	100% N=77 (49%)
		Estimated number of match runs needed for 95% probability of finding an acceptable donor	[150-600]	[600-3330]	~30000
vPRAc	[0%-97.50%[N=28 (18%)	[1-150]	17 *61%* 50%	6 *21%* 13%	5 *18%* 6%
	[97.50%-99.50%[N=36 (23%)	[150-600]	17 *47%* 50%	13 *36%* 28%	6 *17%* 8%
	[99.50%-99.91%[N=50 (32%)	[600-3330]	0	27 *54%* 59%	23 *46%* 30%
	100% N=43 (27%)	~30000	0	0	43 *100%* 56%
		Agreement test Rank correlation test	Kappa: 0.383 P<0.001 Gamma: 0.831 P<0.001		

Italic: row (current virtual PRA, vPRAc) percentages; Underline: column (total virtual PRA, vPRAt) percentages.
Same group: 87 (55%)
Reclassified from vPRAt to 1-degree lower group by vPRAc: 53 (34%)
Reclassified from vPRAt to >1-degree lower group by vPRAc: 17 (11%)
Reclassified as HS from vPRAt to non-HS by vPRAc (<97.50%): 28 (18%)

Table 7 – Intergroup movement between total and eplet virtual panel reactive antibodies calculations.

			vPRAt		
			[97.50%-99.50%[N=34 (22%)	[99.50%-99.91%[N=46 (29%)	100% N=77 (49%)
		Estimated number of match runs needed for 95% probability of finding an acceptable donor	[150-600]	[600-3330]	~30000
vPRAe	[0%-97.50%[N=66 (42%)	[1-150]	30 *45%* 88%	16 *24%* 35%	20 *30%* 26%
	[97.50%-99.50%[N=39 (25%)	[150-600]	4 *10%* 12%	16 *41%* 35%	19 *49%* 25%
	[99.50%-99.91%[N=37 (24%)	[600-3330]	0	14 *38%* 30%	23 *62%* 30%
	100% N=15 (10%)	~30000	0	0	15 *100%* 20%
		Agreement test Rank correlation test	Kappa: 0.049 P=0.116 Gamma: 0.637 P<0.001		

Italic: row (eplet virtual PRA, vPRAe) percentages; Underline: column (total virtual PRA, vPRAt) percentages.
Same group: 33 (21%)
Reclassified from vPRAt to 1-degree lower group by vPRAe: 69 (44%)
Reclassified from vPRAt to >1-degree lower group by vPRAe: 55 (35%)
Reclassified as HS from vPRAt to non-HS by vPRAe (<97.50%): 66 (42%)

Reclassification occurred for 124 patients, 69 (44%) from vPRAt to one-degree lower group by vPRAe and 55 (35%) from vPRAt to more than one-degree lower group by vPRAe. In fact, with eplet based vPRAe, 66 (42%) would be reclassified as non-HS. Kappa and gamma correlation values between vPRAt *vs.* vPRAe were 0.049 (P=0.116) and 0.637 (P<0.001), respectively.

Inter-group movement between current allosensitization calculated with vPRAc and vPRAe is lower with 75 (48%) patients remaining in the same interval group (Table 8). Reclassification occurs for 56 (36%) from vPRAc to one-degree lower group by vPRAe and 24 (15%) from vPRAc to more than one-degree lower group by vPRAe. Only 25% of the patients (n=39) were reclassified to non-HS. It is noteworthy that 2 (1%) patients were reclassified from vPRAc to one degree higher by vPRAe. Kappa and gamma correlation values between vPRAc *vs.* vPRAe were 0.319 (P<0.001) and 0.809 (P<0.001), respectively.

Median and median change between current and eplet vPRA calculations, considering vPRAt interval groups, were significantly different (P<0.001) (Table 9).

Table 8 - Intergroup movement between current and eplet virtual panel reactive antibodies calculations.

			vPRAc			
			[0%-97.50%[N=28 (18%)	[97.50%-99.50%[N=36 (23%)	[99.50%-99.91%[N=50 (32%)	100% N=43 (27%)
		Estimated number of match runs needed for 95% probability of finding an acceptable donor	[1-150]	[150-600]	[600-3330]	~30000
vPRAe	[0%-97.50%[N=66 (42%)	[1-150]	27 *41%* 96%	25 *38%* 69%	9 *14%* 18%	5 *8%* 12%
	[97.50%-99.50%[N=39 (25%)	[150-600]	1 *3%* 4%	11 *28%* 31%	17 *44%* 34%	10 *26%* 23%
	[99.50%-99.91%[N=37 (24%)	[600-3330]	0	0	23 *62%* 46%	14 *38%* 33%
	100% N=15 (10%)	~30000	0	0	1 *7%* 2%	14 *93%* 33%
		Agreement test Rank correlation test	Kappa: 0.319 P<0.001 Gamma: 0.809 P<0.001			

Italic: row (current virtual PRA, vPRAc) percentages; Underline: column (total virtual PRA, vPRAt) percentages.
Same group: 75 (48%)
Reclassified from vPRAc to 1-degree lower group by vPRAe: 56 (36%)
Reclassified from vPRAc to >1-degree lower group by vPRAe: 24 (15%)
Reclassified from vPRAc to 1-degree higher group by vPRAe: 2 (1%)
Reclassified as HS from vPRAc to non-HS by vPRAe (<97.50%): 39 (25%)

Table 9 - Comparisons of vPRA calculations median and median change, estimated number and percentage of change in estimated number of match runs needed for 95% probability of finding an acceptable donor (eMR).

	Total N=157	**1.[97.50%-99.50%[N=34 (22%)**	**2.[99.50%-99.91%[N=46 (29%)**	**3.100% N=77 (49%)**	**P**
vPRA (%), median (IQR)					
vPRAt	99.91 (99.64-100)	98.95 (98.36-99.18)	99.82 (99.73-99.91)	100 (100-100)	-
vPRAc	99.73 (98.64-100)	97.45 (90.64-98.73)	99.64 (99.00-99.82)	100 (99.73-100)	**<0.001***#¶
vPRAe	98.64 (93.27-99.73)	90.59 (76.73-95.64)	98.73 (94.55-99.64)	99.46 (97.36-99.91)	**<0.001***#¶
vPRA change (%), median (IQR)					
vPRAt to vPRAc	-0.09 (-0.82-0) Signed-rank P<0.001	-1.32 (-8.55-0)	-0.09 (-0.82-0)	0 (-0.27-0)	**<0.001***#¶
vPRAt to vPRAe	-1.27 [-6.36-(-0.18)] Signed-rank P<0.001	-8.41 [-22.18-(-3.00)]	-1.09 [-5.36-(-0.27)]	-0.55 [-2.64-(-0.09)]	**<0.001***#
vPRAc to vPRAe	-0.82 [-4.64-(-0.09)] Signed-rank P<0.001	-5.41 [-10.73-(-1.46)]	-0.50 [-4.45-(-0.09)]	-0.46 (-1.91-0)	**<0.001***#
eMR by vPRA, median (IQR)					
vPRAt	3290 (822-29949)	286 (182-365)	1645 (1096-3290)	29949 (29949-29949)	-
vPRAc	1096 (218-29949)	118 (30-234)	822 (298-1645)	29949 (1096-29949)	**<0.001***#¶
vPRAe	218 (43-1096)	31 (11-67)	234 (53-822)	548 (112-3290)	**<0.001***#¶
eMR change (%), median (IQR)					
vPRAt to vPRAc	-40.1 (-91.7-0) Signed-rank P<0.001	-60.0 (-87.3-0)	-50.0 (-81.9-0)	0 (-96.3-0)	0.899
vPRAt to vPRAe	-94.5 [-98.8-(-66.7)] Signed-rank P<0.001	-89.2 [-96.2-(-75.7)]	-82.9 [-97.7-(-50.0)]	-98.2 [-99.6-(-89.0)]	**<0.001**#¶
vPRAc to vPRAe	-66.8 [-92.9-(-16.2)] Signed-rank P<0.001	-55.6 [-84.2-(-14.0)]	-59.8 [-80.1-(-21.6)]	-79.5 (-97.8-0)	0.045

*1. *vs* 2. P<0.01; #1. *vs* 3. P<0.01; ¶2. *vs* 3. P<0.01; % of change: (final-initial)/final*100

The median estimated number of match runs (eMR) needed for 95% probability of finding an acceptable donor by vPRAt intervals are significantly different for vPRAc and vPRAe (P<0.001). Furthermore, also the percentage of change in eMR by vPRAt to vPRAe was significantly more pronounced by increasing vPRAt intervals (P<0.001). This percentage of change of eMR was not so pronounced between vPRAc to vPRAe (P=0.045) and was not observed for the percentage change of eMR from vPRAt to vPRAc (P=0.899) – Figure 12.

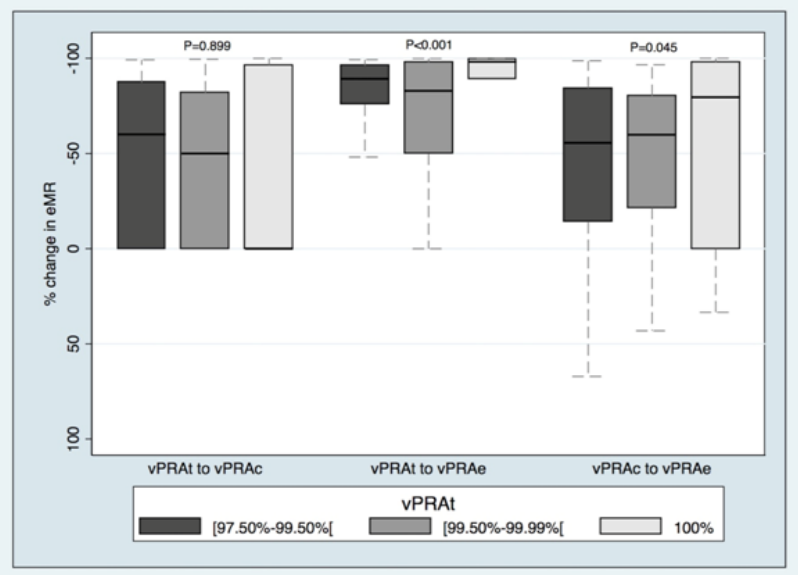


Figure 12 - Comparisons of percentage of change in estimated number of match runs (eMR) needed for 95% probability of finding an acceptable donor, from vPRAt to vPRAc or vPRAe and vPRAc to vPRAe.

Total vPRAt median change to current or eplet vPRA, vPRAc and vPRAe, according to previous sensitizing events such as transfusions, pregnancies and transplants, vintage dialysis and PRA-CDC is showed in Table 10. In the full cohort, median reduction from vPRAt to vPRAe was significantly less pronounced in candidates to retransplant (P<0.001) and those with dialysis vintage ≥10 years (P<0.001) or PRA-CDC ≥50% (P=0.002). The same effect was observed, when considering only vPRAt=100% patients, for retransplantation (P=0.010) and dialysis vintage ≥10 years (P=0.005), or, in the remaining cohort (vPRAt [97.50%-99.99%[), for dialysis vintage ≥10 years (P=0.008) and PRA-CDC ≥50% (P=0.019).

The comparison of percentage of change in eMR, needed for 95% probability of finding an acceptable donor, from vPRAt to vPRAc or vPRAe is showed in Table 11. Considering the whole cohort, the percentage of change in eMR was significantly less pronounced in candidates with dialysis vintage ≥10 years (P=0.049). This was also observed for patients with 100% of vPRA (P=0.005) and for the remaining cohort, with vPRA between 97.50% and 97.99% (P=0.015). For patients with vPRA of 100, also the percentage of change in eMR was less significant for patients with a previous transplant patients (P=0.010) (Figure 13).

Table 10 - Comparison between vPRAt change to vPRAc and vPRAe, according to sensitizing events and dialysis vintage over 10 years and cytotoxic PRA.

	Full cohort (n=157)			
	vPRAt to vPRAc change (%), median (IQR)	P	vPRAt to vPRAe change (%), median (IQR)	P
Previous transfusions		0.705		0.251
No	-0.09 (-1.32-0)		-3.27 [-7.82-(-0.27)]	
Yes	-0.09 (-0.73-0)		-1.09 [-6.09-(-0.18)]	
Previous pregnancy (n=87)		0.725		0.491
No	-0.09 (-1.64-0)		-0.73 [-7.82-(-0.09)]	
Yes	-0.09 (-1.14-0)		-1.77 [-6.36-(-0.23)]	
Previous transplant		0.044		**<0.001**
No	-0.09 (-2.55-0)		-4.91 [-16.82-(-1.18)]	
Yes	-0.05 (-0.73-0)		-0.68 [-4.64-(-0.18)]	
Dialysis vintage ≥10 years		0.121		**<0.001**
No	-0.09 (-1.64-0)		-2.95 [-9.18-(-0.45)]	
Yes	-0.01 (-0.46-0)		-0.27 [-1.55-(-0.09)]	
PRA-CDC ≥50%		**0.005**		**0.002**
No	-0.18 (-1.09-0)		-2.18 [-8.73-(-0.27)]	
Yes	0 (-0.18-0)		-0.55 [-1.36-(-0.09)]	
	vPRAt [97.50%-99.99%[			
	vPRAt to vPRAc change (%), median (IQR)	P	vPRAt to vPRAe change (%), median (IQR)	P
Previous transfusions		0.634		0.186
No	-0.27 [-2.55-(-0.01)]		-4.91 [-11.09-(-1.82)]	
Yes	-0.18 (-2.91-0)		-2.00 [-12.55-(-0.27)]	
Previous pregnancy (n=38)		0.398		0.657
No	-0.82 (-9.55-0)		-7.18 [-16.82-(-0.36)]	
Yes	-0.09 (-2.91-0)		-2.91 [-9.27-(-0.36)]	
Previous transplant		0.444		0.100
No	-0.27 [-4.09-(-0.01)]		-5.14 [-16.82-(-1.73)]	
Yes	-0.23 (-1.91-0)		-2.27 [-11.09-(-0.27)]	
Dialysis vintage ≥10 years		0.911		**0.008**
No	-0.23 (-3.91-0)		-4.86 [-14.18-(-1.14)]	
Yes	-0.27 (-1.59-0)		-0.50 [-7.59-(-0.09)]	
PRA-CDC ≥50%		0.097		0.019
No	-0.27 (-4.09-0)		-4.45 [-15.45-(-0.36)]	
Yes	-0.01 (-0.82-0)		-0.64 [-1.46-(-0.18)]	
	vPRAt=100%			
	vPRAt to vPRAc change (%), median (IQR)	P	vPRAt to vPRAe change (%), median (IQR)	P
Previous transfusions		0.283		0.310
No	0 (-0.09-0)		-0.36 (-2.91-0)	
Yes	0 (-0.27-0)		-0.55 [-2.64-(-0.18)]	
Previous pregnancy (n=49)		0.986		0.283
No	0 (-0.41-0)		-0.18 [-1.68-(-0.05)]	
Yes	0 (-0.27-0)		-0.64 [-3.46-(-0.09)]	
Previous transplant		0.255		0.010
No	-0.09 (-0.27-0)		-3.46 [-24.73-(-0.36)]	
Yes	0 (-0.18-0)		-0.46 [-1.73-(-0.09)]	
Dialysis vintage ≥10 years		0.348		**0.005**
No	-0.05 (-0.27-0)		-1.64 [-5.46-(-0.27)]	
Yes	0 (-0.18-0)		-0.27 [-0.91-(-0.09)]	
PRA-CDC ≥50%		0.283		0.523
No	0 (-0.36-0)		-0.55 [-2.91-(-0.18)]	
Yes	0 (-0.09-0)		-0.50 [-1.36-(-0.09)]	

Table 11 - Comparison % of change in estimated number of match runs (eMR) needed for 95% probability of finding an acceptable donor from vPRAt to vPRAc or vPRAe.

	Full cohort			
	vPRAt to vPRAc % of change in eMR (%), median (IQR)	P	vPRAt to vPRAe % of change in eMR (%), median (IQR)	P
Previous transplant		0.282		0.522
No	-50.2(-89.0-0)		-95.0 [-98.9-(-81.7)]	
Yes	-10.8 (-92.1-0)		-94.5 [-98.6-(-64.2)]	
Dialysis vintage ≥10 years		0.385		0.049
No	-53.9 (-92.6-0)		-96.3 [-99.2-(-76.6)]	
Yes	-9.0 (-91.6-0)		-94.5 [-98.4-(-50.0)]	
PRA-CDC ≥50%		0.049		0.734
No	-54.2(-93.8-0)		-94.9 [-98.7-(-75.0)]	
Yes	0 (-89.0-0)		-94.5 [-98.8-(-50.0)]	
	vPRAt [97.50%-99.99%[			
	vPRAt to vPRAc % of change in eMR (%), median (IQR)	P	vPRAt to vPRAe % of change in eMR (%), median (IQR)	P
Previous transplant		0.950		0.597
No	-50.0 [-72.0-(-0.2)]		-87.5 [-96.2-(-75.2)]	
Yes	-54.1 (-87.4-0)		-85.4 [-97.3-(-57.2)]	
Dialysis vintage ≥10 years		0.941		0.015
No	-53.9 (-86.4-0)		-89.4 [-97.9-(-75.0)]	
Yes	-50.0 [-84.2-(-4.5)]		-62.6 [-94.2-(-46.4)]	
PRA-CDC ≥50%		0.051		**0.008**
No	-58.1 (-90.9-0)		-90.2 [-97.7-(-66.7)]	
Yes	-9.0 (-57.7-0)		-58.4 [-85.8-(-50.0)]	
	vPRAt=100%			
	vPRAt to vPRAc % of change in eMR (%), median (IQR)	P	vPRAt to vPRAe % of change in eMR (%), median (IQR)	P
Previous transplant		0.255		0.010
No	-89.0 (-96.3-0)		-99.7[-99.96-(-97.3)]	
Yes	0 (-94.5-0)		-97.8 [-99.4-(-89.0)]	
Dialysis vintage ≥10 years		0.348		**0.005**
No	-44.5 (-96.3-0)		-99.4 [-99.8-(-96.3)]	
Yes	0 (-94.5-0)		-96.3 [-98.9-(-89.0)]	
PRA-CDC ≥50%		0.283		0.523
No	0 (-97.3-0)		-98.2 [-99.7-(-94.5)]	
Yes	0 (-89.0-0)		-98.0 [-99.3-(-89.0)]	

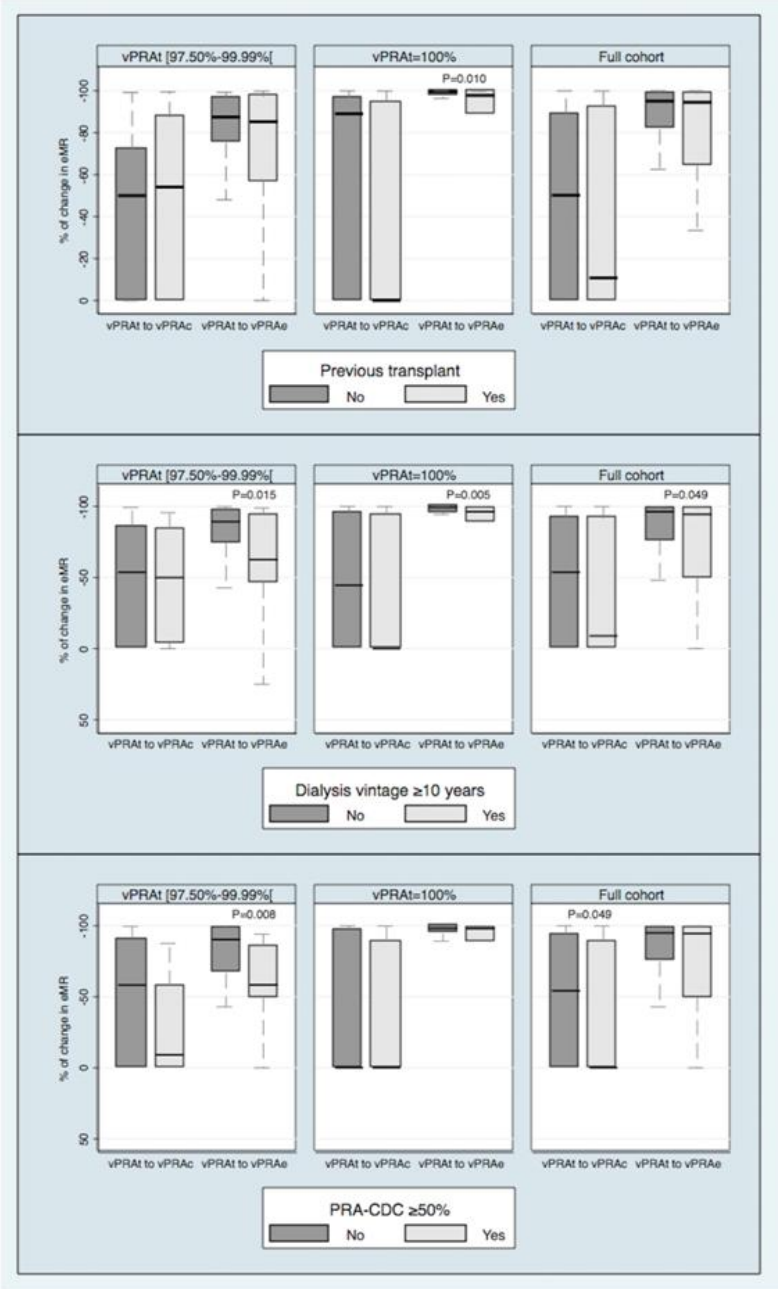


Figure 13 - Comparison % of change in estimated number of match runs (eMR) needed for 95% probability of finding an acceptable donor from vPRAt to vPRAc or vPRAe, considering previous transplant, vintage dialysis over 10 years and cytotoxic PRA over 50%.

Major Conclusions:

- vPRA can reflect variability depending on considered *loci,* serum dates and/or cut-off values.
- Almost half of the HS patients (45%, n=70) would be reclassified to a lower vPRA interval, when considering current vPRA, decreasing the number of eMR needed for 95% probability of finding a compatible donor, and hence increasing transplant probability.

- Eplet based vPRA granted the reclassification of 124 (79%) and 80 (51%) patients to a lower group when considering vPRAt and vPRAc to vPRAe, respectively. In fact, 66 (42%) and 39 (25%) patients would actually be reclassified as non-HS patients with vPRA lower than 98%.
- Our study showed also that median percentage of change in eMR from vPRAt to vPRAe was significantly less pronounced in candidates to retransplant with 100% of vPRA (P=0.010) and for patients with dialysis vintage ≥10 years (P=0.049 for all cohort, P=0.015 for vPRA=100% and P=0.005 for patients with vPRA between 97.50% and 97.99%). This observation reinforces the strength of vPRAe measure, which has an important decrease within first transplant candidates with lower cytotoxic PRA, and this impact is less pronounced in patients considered to be at highly immunological risk.

2. TRANSPLANT IMMUNOLOGICAL RISK ASSESSMENT

Improving HLA matching in living donor kidney transplantation using kidney paired exchange program.

Tafulo *et* al. Transpl Immunol 2020; Oct;62: 101317. doi: 10.10167j.trim.2020.101317.

Appendix 3

We performed a simulation study to evaluate if the introduction of compatible pairs (CP) in the Portuguese kidney paired exchange program (KEP) would result in a better matched transplant for these patients and, if this strategy would increase transplant rate within KEP.

We included 17 compatible pairs, with 6 antigen mismatches in HLA-A, -B, -DR, in kidney paired exchange pool of 35 incompatible pairs (ICP) (Table 12). The donors within CP group blood type was mainly O (64.7%) while recipient's were predominant A blood type (58.8%).

Table 12 - Characteristics of the compatible pairs included in KEP match simulation (n=17).

Pair-ID	Blood Group		Gender		Virtual	HLA class I EpMM		HLA class II EpMM	
	D	R	D	R	vPRA	AbVer	Other	AbVer	Other
CP1	O	O	F	M	0,00	17	29	23	27
CP2	O	A	F	M	0,00	12	24	15	3
CP3	O	O	F	M	0,00	20	35	12	15
CP4	O	A	F	M	0,00	15	40	21	51
CP5	A	A	F	M	0,00	16	27	10	24
CP6	O	O	F	F	15,79	22	52	6	13
CP7[a)]	A	O	F	M	0,00	10	31	22	32
CP8[b)]	O	O	M	F	21,12	16	41	15	26
CP9	O	A	M	F	0,00	13	15	11	14
CP10	O	O	M	F	0,00	21	31	18	25
CP11	A	A	F	M	0,00	17	32	15	19
CP12	A	A	F	M	78,15	13	29	6	12
CP13	A	A	F	M	6,870	14	22	12	20
CP14	O	A	F	M	0,00	14	25	11	19
CP15[b)]	A	A	F	M	55,66	16	33	12	21
CP16	O	O	F	M	0,00	10	23	11	23
CP17	O	A	M	F	11,63	9	17	12	20

a) AB0i; b)HLAi; CP, compatible pair; D, donor; R, recipient; F, feminine; M, masculine; vPRA, virtual panel reactive antibodies;
HLA, Human Leucocyte antigens; EpMM, eplet mismatches; AbVer, antibody-verified; Other, no antibody-verified eplets.

The KEP cohort included 17 AB0i, 17 HLAi and one pair with both types of incompatibility. It is characterized by blood group unbalance with 25.7% O, 60.0% A, 8.6% B and 5.7% of AB blood type. Besides disproportion of blood group, the extremely elevated degree of allosensitization worsens transplant probability among this group. The median vPRA was 59.29%, with 21 (60%) patients with vPRA higher than 50% and 16 (45.7%) with vPRA higher than 95%. Regarding gender the

differences are smoother with 21 (58.3%) female potential donors and 17 (47.2%) female recipients.

Table 13 - Characteristics of the incompatible pairs included in KEP match simulation (n=35).

Pair-ID	Blood Group		Gender		vPRA
	D	R	D	R	
ICP in KEP with at least one match possibility without CP (n=9)					
2-way match run					
ICP 1	A	O	F	M	56,16
ICP 2	O	A	F	F	99,26
3-way match run					
ICP 3	A	A	M	F	99,75
ICP 4	A	O	M	F	99,48
ICP 5	O	A	F	M	9,11
4-way match run					
ICP 6	O	A	F	F	49,16
ICP 7	O	O	M	F	89,77
ICP 8	A	A	F	F	52,79
ICP 9	A	O	F	M	4,92
Additional ICP with at least one match possibility with CP (n=7)					
HLA incompatibility					
ICP 10	O	B	F	M	99,88
ICP 11	A	AB	F	M	97,44
ICP 12	A	A	F	M	100,00
AB0 incompatibility					
ICP 13	A	O	M	M	22,99
ICP 14	A	O	F	M	0,00
ICP 15	A	O	F	M	0,00
ICP 16	A	O	F	M	0,00
ICP with no match possibility with CP simulation (n=19)					
HLA incompatibility					
ICP 17	AB	AB	M	F	99,56
ICP 18	O	A	M	F	100,00
ICP 19	A	A	F	M	100,00
ICP 20	O	O	F	M	84,82
ICP 21	A	A	M	F	99,97
ICP 22	O	O	F	F	99,99
ICP 23	O	O	M	M	99,55
ICP 24	B	B	F	F	98,39
AB0 incompatibility					
ICP 25	A	O	M	F	98,65
ICP 26	A	O	M	F	99,99
ICP 27	AB	B	F	M	1,79
ICP 28	A	B	M	F	99,35
ICP 29	A	O	M	F	10,74
ICP 30	B	A	F	M	0,00
ICP 31	A	O	F	M	0,00
ICP 32	B	O	M	F	30,99
ICP 33	A	O	F	M	7,39
ICP 34	A	O	F	M	6,61
AB0 and HLA incompatibility					
ICP 35	A	O	F	F	56,49

CP, compatible pair; ICP, incompatible pair; D, donor; R, recipient; F, feminine; M, masculine; vPRA, virtual panel reactive antibodies;

HLA eplet mismatch (EpMM) load was calculated for each patient considering the direct compatible pair and the best donor found in KEP match simulation, using HLA MatchMaker, version 3.0. Although all CP were equally mismatched, with six HLA antigen mismatches, the total number of HLA class I eplet mismatch (EptMM-I) from recipients of CP ranged from 26 to 74, and 19 to 85 for HLA class II eplet mismatch load (EptMM-II).

The match simulation performed without the inclusion of CP identified nine possible transplants between ICP in 4-way, 3-way and a 2-way loop. This match allowed the transplantation of seven (77.8%) patients with vPRA higher than 50%, three of them being higher than 95%. On the other hand, the match simulation cycle with the inclusion of CP, 16 out of 17 (94.1%) had at least one cross-over kidney transplant possibility and 13 out of 16 (81.25%) patients succeed to find a better HLA eplet matched donor (81.4±17.6 vs. 64.4±19.5; P=0.007). Considering the inclusion of the 13 CP that would benefit by entering this program we observed an increased transplantation rate with 7 additional patients with a matching possibility in the same ICP pool (Table 13).

The comparison of eplet mismatch load, total (EptMM) and antibody-verified (EpvMM), between cross-over kidney donors that would be considered (n=13) and CP original donor, are presented in Table 14. HLA class I and class II EptMM and EpvMM was significantly different between CP and KEP donors (83.9±16.9 *vs.* 59.8±12.2, *P*=0.002 and 30.1±5.5 *vs.* 21.2±3.0, *P*=0.003, respectively). This difference remained statistically significant if HLA class I (45.8±8.1 *vs.* 32.2±9.7, *P*=0.002 and 15.5±3.0 *vs.* 10.9±3.5, *P*=0.003, respectively) and class II (38.1±12.2 *vs.* 27.7±12.8, *P*=0.016 and 15.3±4.0 *vs.* 9.7±4.7, *P*=0.004, respectively) were analyzed independently.

Table 14 - Comparison of eplet mismatch load between compatible pair donor (CPD) and kidney paired donors (KPD), obtained after match in Portuguese kidney exchange program.

	CPD (N=13) Mean±SD	KPD (N=13) Mean±SD	Mean of difference (95% CI)	*P*	Pos ↑ Zero = Neg ↓
Ept_I+II	83.9±16.9	59.8±12.2	-24.1 (-32.1; -16.0)	**0.002**	0 0 13
Epv_I+II	30.1±5.5	21.2±3.0	-8.9 (-13.1; -4.7)	**0.003**	2 0 11
Ept_I	45.8±8.1	32.2±9.7	-13.7 (-19.1; -8.3)	**0.002**	0 1 12
Epv_I	15.5±3.0	10.9±3.5	-4.5 (-6.9; -2.2)	**0.003**	1 1 11
Ept_II	38.1±12.2	27.7±12.8	-10.4 (-17.9; -2.9)	**0.016**	2 0 11
Epv_II	15.3±4.0	9.7±4.7	-5.5 (-9.0; -2.1)	**0.004**	0 1 10
Donor age	49.8±9.9	48.5±7.8	-1.2 (-10.2; 7.7)	0.674	6 1 6
Female donor, n (%)	10 (77)	8 (62)	-	0.727	-

CPD, compatible pair donor; KPD, kidney exchange program donor; Ept, total eplet mismatch load; Epv, antibody-verified eplet mismatch load; I, HLA class I; II, HLA class II; I+II, HLA class I and class II; ↑, number of patients with increased value of Ep or age, considering KPD versus CPD; =, number of patients with equal value of Ep or age, considering KPD versus CPD; ↓, number of patients with decreased value of Ep or age, considering KPD versus CPD;

Major Conclusions:

- HLA allosensitization degree of Portuguese KEP is very high.
- Blood groups frequencies within the Portuguese KEP are deeply imbalanced.
- The inclusion of fully mismatches compatible pairs with national Portuguese KEP increased matched rate within ICP.
- Compatible pairs included in KEP benefit by decreasing HLA eplet mismatch load, total and verified, when compared to the direct donor.

3. POST-TRANSPLANT FOLLOW-UP

3.1. HLA class II eplet mismatch load improves *dn*DSA prediction

HLA class II antibody-verified eplet mismatch load improves prediction of *dn*DSA development after living donor kidney transplantation.

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Appendix 4

We retrospectively analyzed a cohort of 210 LDKT between January 1, 2008 and December 31, 2017 performed in CHUP. Patients presenting post-transplant anti-HLA antibodies assessment performed with SAB assays were considered, defining the 96 LDKT recipients as the studied cohort. Median follow-up after transplant was 52.4 (33.7–77.7) months.

Thirty two patients (33%) had preformed anti-HLA antibodies of which eight (8%) were donor specific. Thirteen patients (14%) experienced one rejection episode, eight cellular mediated and five antibody-mediated rejections. Six patients developed HLA class I *dn*DSA (Table 15).

Table 15 - Clinical and immunological characteristics considering *dn*DSA for HLA class I.

	No *dn*DSA-I N=90	***dn*DSA-I N=6**	**P**
HLA-I antigen broad MM, mean±SD	3.26±1.85	2.50±2.07	0.321
HLA-I antigen split MM, mean±SD	3.31±1.91	2.83±1.94	0.509
HLA-I eplet total MM, mean±SD	13.3±8.2	9.2±6.5	0.256
HLA-I eplet AbVer MM, mean±SD	7.8±4.9	4.7±2.26	0.101
Acute cellular rejection, n (%)	7 (8)	1 (17)	0.415
Antibody-mediated rejection, n (%)	5 (6)	0	1

HLA-I, HLA class I; MM – mismatches; AbVer, antibody verified; SD, standard deviation.

The incidence of *dn*DSA for HLA class I at 6-years was 7%. No significant difference were found for HLA broad and split antigen and total and AbVer eplet mismatches in *dn*DSA-I group compared to the no *dn*DSA-I group (2.50±2.07 *vs.* 3.26±1.85, p=0.321; 2.83±1.94 *vs.* 3.31±1.91, p=0.509; 9.2±6.5 *vs.* 13.3±8.2, p=0.256 and 4.7±2.6 *vs.* 7.8±4.9, p=0.101, respectively). In a multivariate analysis no predictors for *dn*DSA-I were identified (Table 16).

Table 16 - Multivariate models of *dn*DSA for HLA class I.

Model 1	**HR**	**95% CI**	**P**
No predictor detected			
Model 2	**HR**	**95% CI**	**P**
HLA-I eplet total MM	0.942	0.841-1.054	0.295
Model 3	**HR**	**95% CI**	**P**
HLA-I eplet AbVer MM	0.874	0.709-1.077	0.207
Model 4	**HR**	**95% CI**	**P**
HLA-I antigen broad MM	0.903	0.559-1.458	0.676
Model 5	**HR**	**95% CI**	**P**
HLA-I antigen split MM	0.975	0.617-1.543	0.915

Model 1: included the following variables: recipient age and gender, dialysis type and vintage, donor age and gender, unrelated living donor, induction IS, retransplant, virtual PRA and preformed DSA, but excluded HLA class II eplet and antigen mismatches. Final model with independent predictors was defined by stepwise backward selection (P<0.05 used for retention in the model). Model 2 to 5: univariate (unadjusted) analysis for HLA-I total eplet MM, HLA-I AbVer eplet MM, HLA-I antigen broad MM and HLA-I antigen split mismatch, respectively.

Regarding HLA class II, seven patients developed *dn*DSA during follow-up time (Table 17). HLA class II total and AbVer eplet mismatches were greater in *dn*DSA-II group compared to the no *dn*DSA-II (41.3±18.9 *vs.* 23.1±16.7, p=0.018 and 18.0±8.7 *vs.* 9.9±7.9, p=0.041), which is not observed for HLA class II antigen broad and split mismatches (2.29±0.49 *vs.* 1.56±1.22, p= 0.090 and 2.43±0.79 *vs.* 1.84±1.30, p=0.248). As expected ABMR was greater within *dn*DSA-II group (3.0 *vs.* 2.0; p=0.002).

Table 17 - Clinical and immunological characteristics considering *dn*DSA for HLA class II.

	No *dn*DSA-II N=89	***dn*DSA-II N=7**	**P**
HLA-II antigen broad MM, mean±SD	1.56±1.22	2.29±0.49	0.090
HLA-II antigen split MM, mean±SD	1.84±1.30	2.43±0.79	0.248
HLA-II eplet total MM, mean±SD	23.1±16.7	41.3±18.9	0.018
HLA-II eplet AbVer MM, mean±SD	9.9±7.9	18.0±8.7	0.041
Acute cellular rejection, n (%)	7 (8)	1 (14)	0.467
Antibody-mediated rejection, n (%)	2 (2)	3 (43)	0.002

HLA-II, HLA class II; MM - mismatches SD, standard deviation.

The incidence of *dn*DSA-II at 6-years was 17% (Figure 14). For HLA broad antigen mismatch considering 0-1, 2 and 3-4 mismatches were 0%, 24% and 33%, respectively (p=0.060) - Figure 14A. Regarding total eplet mismatches terciles intervals of less than 17, between 17 and 32, and higher than 32 were 0%, 13% and 41%, respectively (p=

0.028) – Figure 14B. Finally, AbVer eplet mismatch load terciles of less than 6, between 6 and 14, and higher than 14 were 0%, 4% and 42% (p=0.006) – Figure 14C.

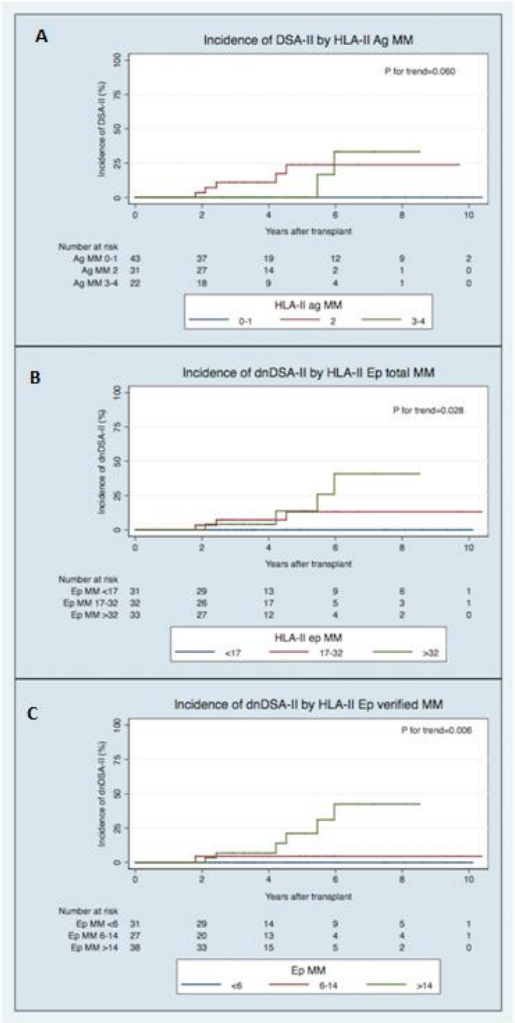

Figure 14 - Incidence of *dn*DSA for HLA class II considering: A) antigen mismatches, B) total eplet mismatches; C) antibody-verified eplet mismatch load.

In a multivariate analysis we found that preformed DSA (HR=7.983; 95IC:1.329-47.968; p=0.023), living unrelated donors (HR=8.052; 95IC:1.313-49.394; p=0.024) and retransplantation (HR=14.393; 95IC:1.946-106.441; p=0.009) were predictors for *dn*DSA-II (AUC = 0.801; 95%CI: 0.622-0.981) (Table 18, Model 1). HLA class II total and AbVer eplet mismatches (HR=1.042; 95IC:1.004-1.082; p=0.031; AUC = 0.852 and HR=1.105; 95IC:1.011-1.208; p=0.028; AUC = 0.856) showed to be superior predictors of *dn*DSA-II, when compared to broad or split antigen mismatches (HR=1.740; 95IC:0.877-3.452; P=0.113; AUC=0.783 and HR=1.677; 95IC:0.847-3.318; P=0.138; AUC=0.818), when adjusted for Model 1 (Table 18, Model 3, 5, 7 and 9 respectively).

Table 18 - Multivariate models for *dn*DSA for HLA class II.

Model 1	**HR**	**95% CI**	**P**	**Model C-statistics (95% CI)**
Preformed DSA	7.983	1.329-47.968	0.023	0.801 (0.622-0.981)
Living unrelated donor	8.052	1.313-49.394	0.024	
Retransplant	14.393	1.946-106.441	0.009	
Model 2	**HR**	**95% CI**	**P**	**P**
HLA-II eplet total MM, unadjusted	1.056	1.015-1.098	0.007	0.755 (0.630-0.919)
Model 3	**HR**	**95% CI**	**P**	**P**
HLA-II eplet total MM, adjusted to Model 1	1.042	1.004-1.082	0.031	0.852 (0.718-0.986)
Model 4	**HR**	**95% CI**	**P**	**P**
HLA-II eplet AbVer MM, unadjusted	1.141	1.042-1.249	0.004	0.738 (0.565-0.911)
Model 5	**HR**	**95% CI**	**P**	**P**
HLA-II eplet AbVer MM, adjusted to Model 1	1.105	1.011-1.208	0.028	0.856 (0.726-0.748)
Model 6	**HR**	**95% CI**	**P**	**P**
HLA-II antigen broad MM, unadjusted	1.645	0.883-3.063	0.117	0.659 (0.570-0.748)
Model 7	**HR**	**95% CI**	**P**	**P**
HLA-II antigen broad MM, adjusted to Model 1	1.740	0.877-3.452	0.113	0.783 (0.605-0.961)
Model 8	**HR**	**95% CI**	**P**	**P**
HLA-II antigen split MM, unadjusted	1.458	0.804-2.643	0.214	0.590 (0.498-0.683)
Model 9	**HR**	**95% CI**	**P**	**P**
HLA-II antigen split MM, adjusted to Model 1	1.677	0.847-3.318	0.138	0.818 (0.628-1)

Model 1: included the following variables: recipient age and gender, dialysis type and vintage, donor age and gender, unrelated living donor, induction IS, retransplant, virtual PRA, preformed DSA, excluding HLA class II eplet and antigen mismatches, were included. Final model with independent predictors was defined by stepwise backward selection (P<0.05 used for retention in the model); Model 2, 4, 6 and 8: univariate (unadjusted) analysis for each predictor; Model 3, 5, 7 and 9: Multivariate (adjusted for independent predictors detected in Model 1) analysis for each predictor.

Six patients (6,25%) experienced graft failure deemed as alloimmune-related. Overall graft survival was 79% at 9-years of follow-up (Figure 15). Graft survival was significantly lower within *dn*DSA-II patients group (36% vs. 88%, p < 0.001). No significant difference was observed for *dn*DSA-I (83% *vs.* 77%, p=0.926).

Finally, in a multivariable model adjusted for recipient age and gender, donor age and gender, living related *vs.* unrelated donor, retransplant, preformed DSA, virtual PRA (vPRA), *dn*DSA-I, we found that rejection episodes (HR=16.026; 95IC:1.420-180.87; p=0.025) and HLA class II *dn*DSA development (HR=20.447; 95IC:1.994-209.687; p=0.011) were independent predictors of allograft failure (Table 19).

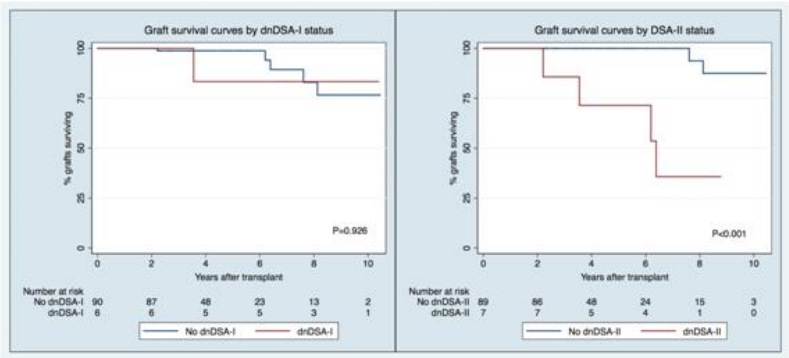

Figure 15 - Kaplan-Meier graft survival curves comparisons by *dn*DSA status.

Table 19 - Multivariate model for allograft failure.

	HR	95% CI	P
Rejection	16.026	1.420-180.887	0.025
*dn*DSA-II	20.447	1.994-209.687	0.011

Multivariable model adjusted for: recipient age and gender, donor age and gender, living related *vs.* unrelated donor, retransplant, preformed DSA, vPRA, *dn*DSA-I.

Major Conclusions:

- The number of HLA class II, total and particularly AbVer, eplet mismatch load were greater in *dn*DSA-II group compared to no *dn*DSA-II, which is not verified when mismatches are determined traditionally considering the HLA class II broad or split molecule as a whole.
- Antibody-mediated rejection was significantly higher within HLA class II *dn*DSA positive sub-cohort (3.0 *vs.* 2.0; p=0.002).
- HLA class II total and AbVer eplet mismatch load were independent predictors for HLA class II *dn*DSA development.
- Neither HLA class I broad and split antigen or HLA class I total or AbVer eplet mismatch load, had any predictive value for HLA class I dnDSA, in our cohort.

3.2. HLA eplet mismatch load improves prediction of ABMR

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

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doi: 10.1016/j.humimm.2019.09.010. PMID: 31604581.

Appendix 5

We retrospectively analyzed 157 AB0-compatible consecutive LDKT between January 1, 2007 and December 31, 2014 performed in CHUP.

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. All patients were followed-up from time of transplant until death and GF was defined as return to dialysis or retransplant or June 30, 2018. 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 ABMR (n=17). The median time until CMR was 1.4 months (IQR: 0.2–51.4) [range: 0.1–118.1] and until ABMR 6.3 months (IQR: 0.3–36.3) [range: 0.2–75.4]. 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).

At transplant, patients that came to experience AR were more sensitized, with higher cytotoxic and vPRA 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 class I and class 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 class I and class 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 class II Ag 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 class I AgMM and EpMM was similar between both groups.

Eplet mismatch load for HLA class I and class II were analyzed as 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. HLA-II EpMM analyzed as terciles groups also showed significant differences between patients with or without AR (*P*=0.008).

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 (Figure 16). HLA antigen and EpMM association with rejection episodes, considering no rejection, CMR and ABMR episodes are shown in Figure 17. Only HLA-II antigen and EpMM were correlated with ABMR, when compared to no rejection group (HLA-II antigen with ABMR, 2 (2–3), *P*=0.014 *vs.* no rejection, 2 (0–2), HLA-II EpMM with ABMR, 15 (10–18), *P*=0.002 *vs.* no rejection, 8 (1–14)).

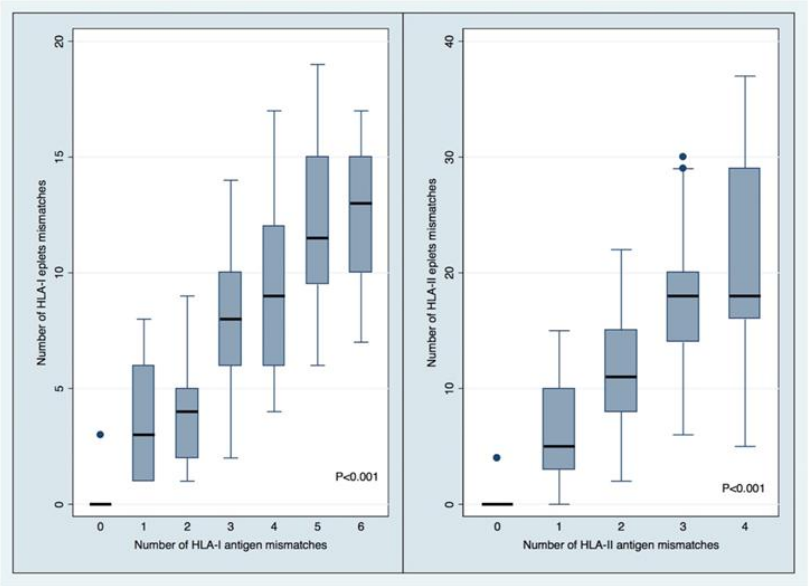

Figure 16 – Association between the number of HLA class I and class II antigen mismatches with the eplet mismatch load.

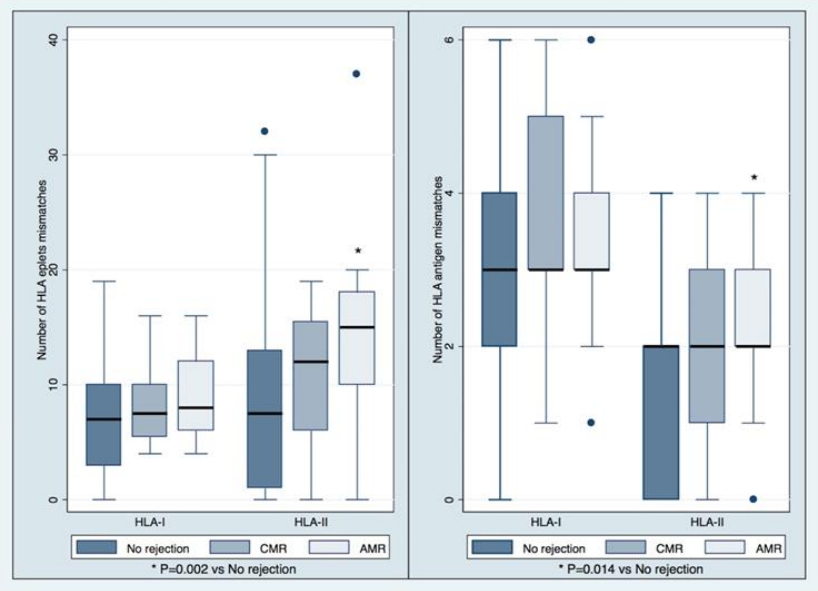

Figure 17 - Association between the number of HLA class I and class II eplet and antigen mismatches with rejection episodes considering: no rejection, CMR and ABMR.

The incidence of ABMR 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. 18A). Considering only patients with no more than two antigen mismatches in HLA-II (n=123) (Fig. 18B), incidence of ABMR 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. 18C), ABMR 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).

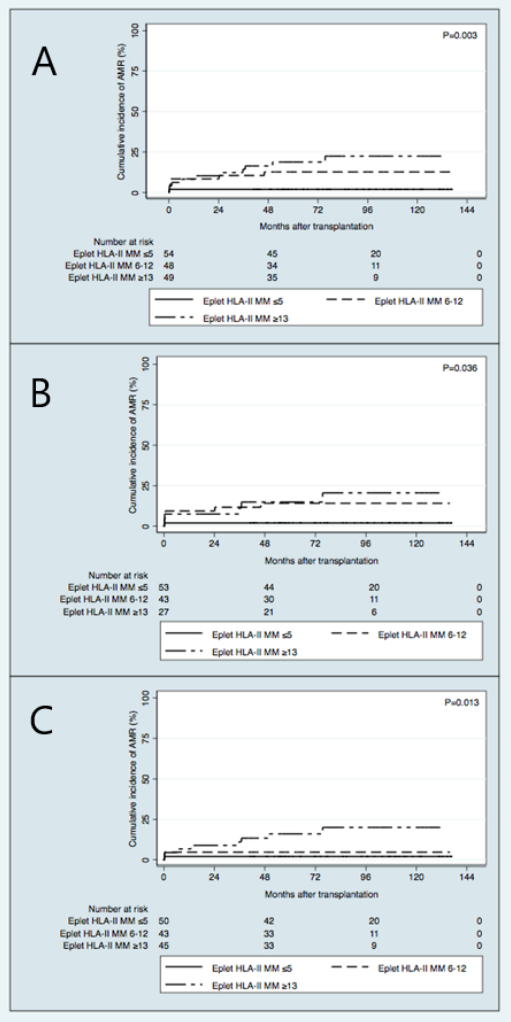

Figure 18 - Cumulative incidence curves at 96 month for antibody-mediated rejection considering HLA class II EpMM load in terciles: A) overall cohort (T1=2%, T2=13%, T3=22%, *P*=0.003); B) patients with 0–2 HLA class II antigen MM (T1=2%, T2=14%, T3=20%, *P*=0.036); C) Patients without preformed DSA (T1=2%, T2=5%, T3=20%, *P*=0.013). MM – mismatches.

In univariate analysis, no variable was significantly associated with CMR. Multivariate analysis adjusted to ATG induction (the single 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 20) were independent predictors of CMR. In univariate analyses, ABMR 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 21).

Table 20 - 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

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

Table 21 - 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
Preformed anti-HLA antibodies	**3.879**	**1.469-10.244**	**0.006**
Preformed DSA, n (%)	**7.113**	**2.615-19.344**	**<0.001**
HLA-I antigen MM, per unit increase	1.135	0.847-1.522	0.396
HLA-II antigen MM, per unit increase	**1.510**	**1.004-2.268**	**0.048**
HLA-I epitope MM			
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 epitope MM			
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;
MM, mismatches; Ep, eplet; Ag, antigen; ATG, anti-thymocyte globulin; HR, hazard ratio;

In the multivariate analysis neither HLA EpMM nor antigen mismatch for HLA-I was associated with ABMR. Differently, high (EpMM≥13) *versus* low (EpMM≤5) HLA-II eplet mismatch load, was an independent predictor of ABMR (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 ABMR than the latter (Table 22).

Table 22 - Multivariate analysis of each predictor of antibody-mediated rejection, 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 (0.572-0.842)	0.046 (-0.079-0.171)
T2: 6-9	2.196	0.565-8.530	0.256				
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 (0.675-0.895)	**0.064 (0.009-0.119)**
T2: 6-12	**7.753**	**0.929-64.724**	**0.059**				
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**

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

As we demonstrated in the multivariate analysis only HLA-II EpMM is an independent predictor for ABMR. As such, we performed a more detailed analysis to understand if there was a different contribution of HLA-DR and HLA-DQ *loci*.

Figure 19 shows the number of eplet mismatches *per* HLA-II *loci*, considering no rejection, CMR and ABMR. In the unadjusted model, patients with higher eplet mismatch load for HLA-DR and HLA-DQ *loci* experienced more ABMR episodes (*versus* no rejection, *P*=0.009 and *P*=0.008 respectively). The multivariate analysis of HLA-DR and HLA-DQ *loci* for CMR and ABMR occurrence, adjusted for variables with a p < 0.1 in the univariate analysis as shown in Tables 20 and 21, is reported in Table 23. Neither HLA-DR nor HLA-DQ were 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) were independent predictors for ABMR.

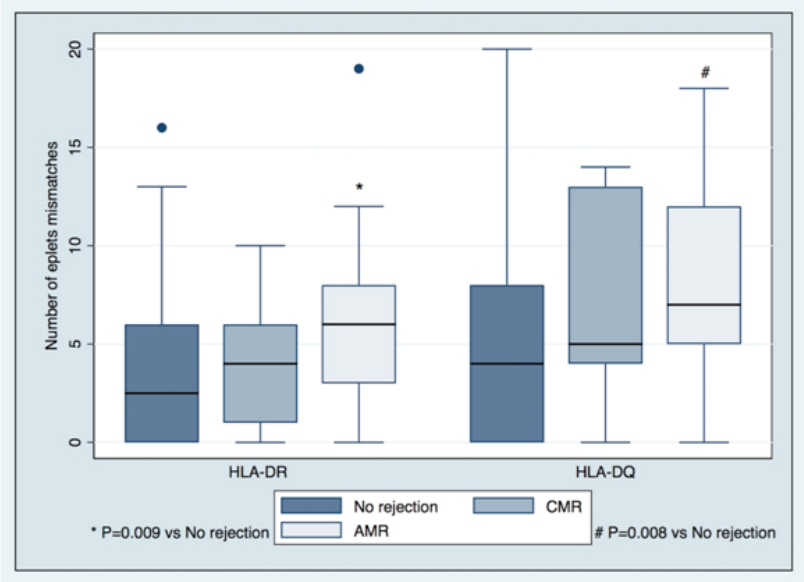

Figure 19 - Comparison of HLA-DR and HLA-DQ eplet mismatches according to rejection status (significant differences are shown).

Table 23 – Multivariate analysis of each predictor for CMR and ABMR occurrence (adjusted for variables with a p-value <0.1 in the univariate analysis as shown in table 20 and 21).

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

Improvement in calculated risk for ABMR was assessed by IDI and NRI. The mean predicted probability of ABMR increased among patients with ABMR (36.7%) and decreased in patients without ABMR (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) (Figure 20). 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 ABMR, and 13 of 17 patients (76.5%) within those with ABMR. The category free net reclassification index (cfNRI) was 0.785 (95%CI 0.300–1.426) (Figure 21).

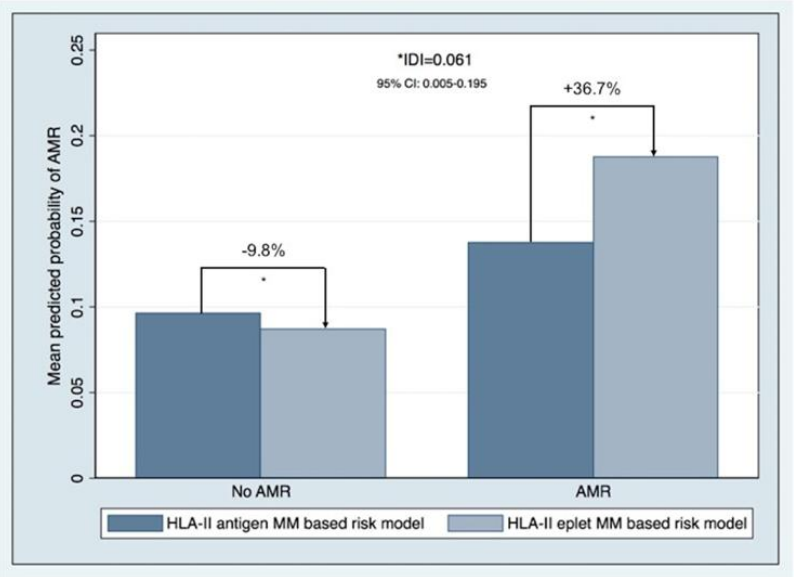

Figure 20 - Improvement in calculated risk of ABMR considering HLA-II eplet mismatch in addition to classic HLA-II broad antigen mismatch.

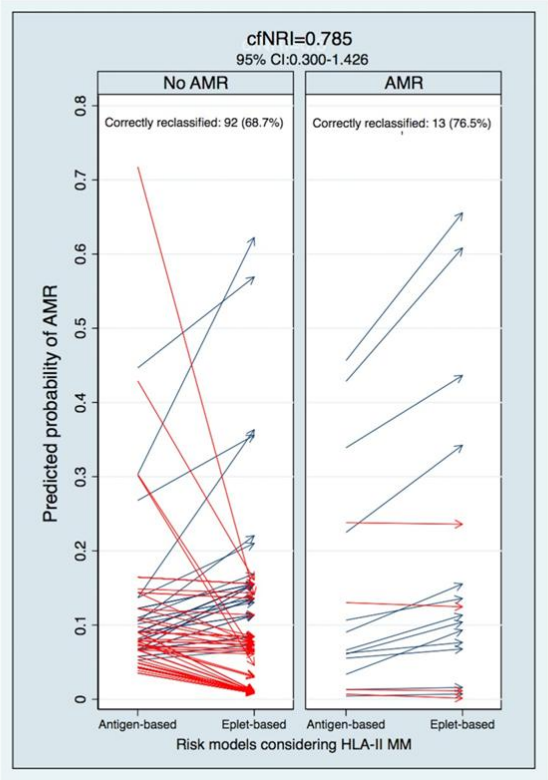

Figure 21 - Improvement in calculated risk of ABMR considering HLA-II eplet mismatch in addition to classic.

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 (Figure 22) was 91% for patients with no rejection episodes, 83% within patients with CMR and only 63% for patients with ABMR ($P < 0.001$).

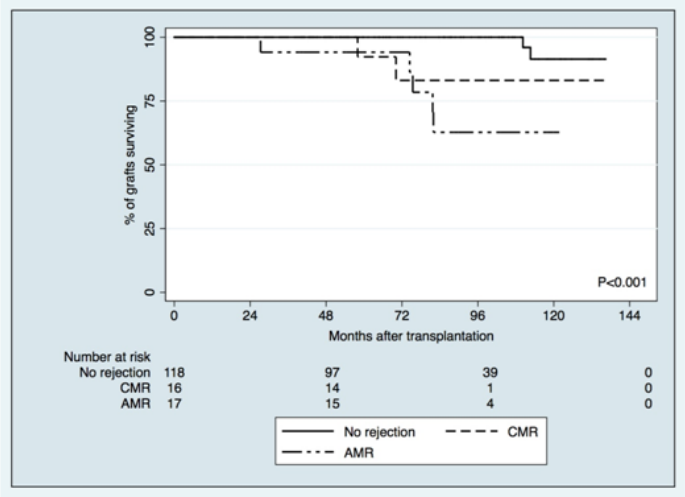

Figure 22 - Graft survival Graft at 120 months for patients with no rejection episodes, patients with CMR and patients with ABMR.

Major Conclusions:

- There was a close correlation between the number of broad antigens and the number of antibody-verified eplet mismatch load for HLA-I and HLA-II.
- HLA class II antibody-verified eplet mismatch load was a strong predictor of ABMR, when compared to the conventional HLA broad antigen mismatch assessment.

CHAPTER V – DISCUSSION AND CONCLUSIONS

1. DISCUSSION

Chronic kidney disease (CKD) has a tremendous impact on public health (122) and only a small percentage of these patients are eligible of kidney transplantation, the best renal replacement therapy available since it reduces morbidity and mortality (123). Notwithstanding the multiple strategies to narrow the gap between organs supply and demand, patients still suffer long waiting distress times while watching the degradation of their global health condition (124).

Access to kidney transplantation

In our study (125) we observed that patients' blood type and degree of HLA allosensitization are the major factors determining longer waiting times. In the Portuguese allocation system, we could verify that O blood type patients are in disadvantaged to find a compatible donor, when compared to the remaining groups, and HS candidates were hugely disadvantaged in the access to KT, independently from AB0 blood groups. As such, in an effort to bring more equity to allocation systems, it became essential to improve HLA antibodies immunoassays and the ability to assess immunologic risk.

The introduction of the sensitive solid-phase immunoassays, when compared to classic cytotoxic assays, enabled an outstanding improvement in the HLA allosensitization analysis (48). Beyond increased sensitivity, SPI are specific for immunoglobulin G (IgG) HLA antibodies, while CDC detects both IgG and IgM HLA and non-HLA complement fixing antibodies. Single-antigen bead assays were particularly relevant by allowing the introduction of virtual PRA (88), virtual crossmatch (126) and an accurate evaluation of sera with multiple HLA antibodies which was substantially difficult, or even impractical, using cell-based assays. These assays are a fundamental in allocation systems, chiefly within hyper sensitized patients, kidney paired kidney exchange programs, and immunological risk stratification protocols (127).

Despite the unquestionable advantages of these methods, as histocompatibility laboratories implemented these sensitive assays to assign HLA antibodies, patients´ HLA allosensitization increased greatly reducing transplant probability. However, soon after, several technical limitations were associated with these assays and it became clear that the accurate identification of clinically relevant HLA specificities is crucial to assure transplant success in a timely manner (128, 129).

Unacceptable HLA antigen assignment

SAB assays, alongside with HLA sequencing methods and the elucidation of the three-dimensional molecular structure, enable the confirmation that HLA antigens are strings of multiple eplets, determined by amino acid residues in polymorphic positions, shared between several molecules. As such, SAB assays interpreted with the awareness of the described technical limitations and using eplet analysis enables HLA antibody reactivity pattern definition in the context of patient's allosensitization history (130).

Our study revealed a concerning accumulation of hyper sensitized patients with 34.6 % (n= 683 of 1973) in the national Portuguese waitlist and 25.9% (n=157 of 606) in the north region, considering SAB to assign UA with a 1000 MFI *cut-off* and vPRAt equal or greater than 98% (131). The variability of HLA allosensitization status with the type of method used was indubitable, as already described (36). Within the HS in the north region only 12.1% (n=19 of 157) have PRA-CDC equal or greater than 80%, the historical definition for HS in Portugal and still in practice to attribute additional points in allocation system.

Besides the unquestionable value of SAB assays in risk assessment and UA assignment, when compared to old CDC, the use of SAB with a stringent cut-off for the last 15 years led to dramatically overestimation of HLA allosensitization, augmenting the difficulty to find a compatible donor. The conservative vPRAt criteria used in Portuguese allocation algorithm was already described to be inefficient for transplanting highly-sensitized patients that are accumulating in waitlist with extremely prolonged waiting times (125). In fact, in our study 120 (76%) of HS patients are re-transplant candidates and 126 (80%) patients have dialysis vintage time over five years.

Our study showed that almost half of the HS patients (45%, n=70) would be reclassified to a lower vPRA interval, when considering current vPRA, decreasing the number of eMR needed for 95% probability of finding a compatible donor, and hence increasing transplant probability. However, due to the SAB assays limitation already described, using the current vPRA with a fixed 1k MFI cutoff may also be misleading and must be interpreted with caution. The introduction of HLA eplet analysis in SAB analysis allowed us to introduce clinical relevance in the interpretation with eplet vPRA. With this measure, cut-off is determined based on patient's sensitization history and HLA eplet analysis assigns as unacceptable HLA antigens with explainable antibody-verified eplet or high ElliPro score. ElliPro is a prediction program based on three-dimensional structures of antigen-antibody complexes developed by Ponomarenko (113) that enables the characterization of clinically relevant eplet repertoires in HLA matching (114).

Eplet based vPRA granted the reclassification of 124 (79%) and 80 (51%) patients to a lower group when considering vPRAt and vPRAc to vPRAe, respectively. In fact, 66 (42%)

and 39 (25%) patients would be reclassified as non-HS, respectively. This was particularly significant when considering the third group of patients with 100% (P<0.001).

This represented a huge impact on the percentage of change of eMR needed for 95% probability of finding an acceptable donor for the whole cohort, when considering vPRAt to vPRAe (P<0.001) or vPRAc to vPRAe (P=0.045). This was not observed in the percentage of change in eMR from vPRAt to vPRAc (P=0.899). Our study showed also that median percentage of change in eMR from vPRAt to vPRAe was significantly less pronounced in candidates to retransplant with 100% of vPRA (P=0.010) and for patients with dialysis vintage ≥10 years (P=0.049 for all cohort, P=0.015 for vPRA=100% and P=0.005 for patients with vPRA between 97.50% and 97.99%). This observation reinforces the strength of vPRAe measure, which has an important decrease within first transplant candidates with lower cytotoxic PRA, and this impact is less pronounced in patients considered to be at highly immunological risk (80, 132, 133). Although our study did not include cellular assays to prove the efficacy of the strategy, and we must be aware that a percentage of cellular based crossmatches could be positive, we demonstrated that UA assignment based on eplet analysis increases transplant probability in a hyper sensitized cohort.

Compatible pairs in kidney paired donation

HLA allosensitization is a barrier to successful kidney transplantation also with living donation. Incompatible pairs have the option of kidney paired exchange programs but these programs are not able to present solutions for every ICP, and also struggle to find new solutions, such as participation in wider international programs (134), the use of desensitization programs within KEP (135) and the introduction of compatible pairs (CP) (136, 137).

Our study (115) shows that the inclusion of fully mismatches CP with national Portuguese KEP increased matched rate within ICP. This was expected as we observed an improved balance for ABO blood groups and vPRA. In fact, allosensitization degree of Portuguese patients is very high, aggravated by the lack of a national program for highly sensitized patients and, together with O blood type imbalance, a well-known problem (138), results in lower transplantability among these patients (125, 139).

Furthermore, both HLAi pairs that were desensitized to be transplanted directly and included in the CP sub-cohort, would find a compatible match without donor specific antibodies (DSA), sparing desensitization. On the other hand, the matching simulation did not find a matched pair for ABOi pair, CP7 desensitized and transplanted directly. This is not surprising as this patient was blood type O and these donors were in minority (25.7%) within ICP in KEP. In fact, ABOi barrier can be crossed with outcomes equivalent to ABO-

compatible transplantation. Several groups have described that acceptance of ABOi matching significantly enhances transplant rates in KEP (140).

Our study showed that, in the pool of 35 ICP, 16 CP (94.1%) obtained one or more transplants possibility within the program, of which 13 (81.25%) were able to be transplanted with a better matched donor considering HLA class I and class II eplet mismatch load. The introduction of CP within KEP to seek immunological benefit has been described by other groups (141, 142). Beyond that, also age, gender and size improved matching could be a reciprocal benefit offer, as these factors also affects transplant outcome (143, 144).

Although some studies raise some ethical issues and not all CP would approve their participation in KEP (145), we believe that the altruistic nature of living donors (146), allied to prove increasing benefit to the intended recipient, would also be a favorable factor in the decision of CP to participate in KEP (147, 148). We believe it is our duty to offer the best option for each patient and the inclusion of HLA mismatched CP within KEP allocation matching, in order to seek for alternative better-matched donors, would allow the clinical team to evaluated all possibilities and provide a better medical advice.

HLA eplet mismatch load

It is now unquestionable that *de novo* donor-specific HLA antibodies detected post-transplant are an independent risk factor for late allograft dysfunction (149, 150). As their development is a response to encountered mismatched donor HLA, an accurate evaluation of HLA mismatches is an important factor to consider in pre-transplant laboratory assessment of immunological risk.

However, Portuguese legislation was last updated in 2007, being completely obsolete providing great importance to time on dialysis in expense of HLA typing compatibility. This explains the dramatic accumulation of hyper sensitized patients in waiting list, most of them retransplant candidates.

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, with exponential increase of HLA alleles. The extremely high HLA polymorphism makes impracticable to perform allelic matching in kidney transplantation, as well as DSA definition and accurate vXM, as SAB assay platforms usually only support 100 HLA antigen coated microparticles (151). For this reason, kidney allocation is usually done considering HLA broad antigens mismatches in host-*versus*-graft (H*v*G) direction. However, epitope-specific HLA antibody reactivity unveils the flaws of this strategy (152).

Besides the demonstrated limitation of the HLA antigen mismatch analysis, another important drawback of the current allocation algorithms, such as Portuguese legislation,

usually only consider HLA-A, -B, -DR *loci*. The strong linkage disequilibrium between HLA class II (HLA-DR and HLA-DQ) doesn't seem to be enough with several studies pointing to the higher frequency of HLA-DQ *dn*DSA (153, 154). Another explanation for the high incidence of HLA-DQ *dn*DSA may also be related to the high number of polymorphic epitopes on both α and β chains of the HLA-DQ molecule (155).

Wiebe *et* al showed that eplet-based mismatch analysis was a better predictor of HLA class II *dn*DSA development, when compared to classical low-resolution or high-resolution HLA antigen mismatch (156). He demonstrated that, for the 134 patients with lower HLA-DR (<10) eplet mismatch, none developed HLA-DR *dn*DSA, and only 4 out of 145 patients with lower HLA-DQ (<17) eplet mismatch load, developed HLA-DQ *dn*DSA after a median follow-up of 6.9 years.

HLA eplet matching allowed HLA classical matching refinement with proved importance for acceptable mismatches identification and to avoid sensitization induced by an HLA mismatch. HLA antigen mismatch considers the whole HLA molecule and identifies a limited range of possible values (0, 1 or 2 per locus). As such, one antigen mismatch can represent a wide variety of eplet mismatches and, consequently, different immunological risk.

Our study (157) evidences that eplet-based matching is a refinement of the classical HLA antigen mismatch analysis. We showed that HLA class II eplet mismatch load was a strong predictor of ABMR in a LDKT cohort, when compared to the conventional HLA broad antigen mismatch assessment currently used in clinical practice.

Lastly we showed that the number of HLA class II, total and AbVer, eplet mismatch load were greater in *dn*DSA HLA class II group compared to no *dn*DSA HLA class II, which are not verified when mismatches are determined traditionally considering the HLA class II broad or split molecule as a whole (158). Although the mechanism for how epitope load increases the risk of *dn*DSA development is unknown, the probability of allorecognition by a specific B cell clone likely increases with an increasing number of mismatches, as would the likelihood of an immunodominant epitope being present. Also, ABMR was significantly higher with HLA class II *dn*DSA positive sub-cohort (3.0 *versus* 2.0; $p = 0.002$).

Limitations

The research work developed within this thesis has several limitations.

One of major limitation regards HLA eplet mismatch load analysis that was performed using PCR-rSSO intermediate resolution HLA typing and Caucasian population frequencies. However, this represents the resolution possible in the context of deceased donation due to limitations of cold ischemia time. Furthermore, Fidler *et* al have shown that, in a predominantly Caucasian cohort, two-digit alleles converted to four-digit alleles reliably calculate the number of eplet mismatches at both class I and II loci compared to four-digit molecular HLA typing method (159).

Another important limitation of our study is due to the sample size in particular the cohorts of living donor's kidney transplant pairs but, on the other hand, is a very uniform cohort of living donors, younger patients under uniform immunosuppression therapy.

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. Moreover, effective and user-friendly tools to HLA eplets analysis in deceased donation context are necessary to assure safe and prompt immunological risk analysis.

2. CONCLUSIONS AND FUTURE DIRECTIONS

Our study highlighted the assess inequity in organ allocation from decease donors. It revealed the paramount importance of updating allocation policies in order to mitigate the disadvantage of O blood type and, even more so, of HS candidates have in accessing KT. Despite the unquestionable value of SPI, our study showed that they need to be interpreted with caution and within patient's allosensitization history. HLA allosensitization urges to be re-assessed including HLA eplet analysis to SAB assays, so the known limitations can be in some extent surpassed. This strategy is crucial to understand and clarify HLA antibodies reactivity patterns and, consequently, improve UA assignment (160, 161). Despite this improvement in HLA antibodies assessment, other strategies are absolutely indispensable to increase transplantation such as the introduction of compatible pairs within Portuguese KEP.

We believe the major conclusion of our study is that, despite the evolution of immunosuppressive drugs, HLA matching is still relevant for graft outcome. As such, allosensitization prevention is of prime importance and minimization of HLA eplet load mismatches is imperative to reduce risk of *dn*DSA development. Moreover, HLA eplet mismatch load can also be used to assess risk following minimization of immunosuppression (162).

Finally the most important future research direction, because not all eplet mismatches are equally immunogenic, is the determination of HLA eplet immunogenicity. A few theories have been considered such us differences in structural or physiochemical disparities. In fact, hydrophobicity and electrostatic charge between mismatched amino acids have been reported to be able to induce an alloantibody response (163, 164). Kosmoliaptsis *et* al showed that this approach may allow selection of low immunogenicity donor-recipient HLA combinations with a beneficial effect on graft outcomes (165). Assessment of the surface electrostatic properties of HLA B-cell epitopes could be, alongside with HLAmatchmaker, an important tool to determine HLA immunogenicity and antigenicity (166, 167).

Other important factor to determine immunogenicity may be Predicted Indirectly ReCognizable HLA Epitopes presented by HLA-DRB1 (PIRCHE-II), that determines HLA T-helper epitopes differences between donor and recipient to estimate the risk of *de novo* antibody development and transplant outcome (168). T-helper epitopes are required for B-cell activation, necessary for production of HLA IgG antibodies, and antibody isotype switching (169). Lachmann *et* al showed that PIRCHE-II score had a major impact for prediction of donor HLA-DRB and HLA-DQB (170).

CHAPTER VII – REFERENCES

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CHAPTER VIII - APPENDIX

Appendix 1

Low transplantability of 0 blood group and highly sensitized candidates in the Portuguese kidney allocation algorithm: quantifying an old problem in search of new solutions.

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Low transplantability of 0 blood group and highly sensitized candidates in the Portuguese kidney allocation algorithm: quantifying an old problem in search of new solutions

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Abstract

The impact of patient's biological differences in waiting time for kidney transplantation is well known and has been a subject of extensive debate and struggle in transplantation community. Our purpose was to evaluate patient's access to kidney transplantation in Portugal, regarding their degree of allosensitization and blood type. A retrospective cohort study including 1020 candidates for kidney transplantation between 01 January 2010 and 31 December 2011 in transplant unit Centro Hospitalar do Porto was performed. The deceased donor organ offer by blood type decreased with the calculated panel reactive antibody (cPRA) increase for A and B blood groups candidates, while in 0 blood group candidates, a significant reduction in organ offer was only observable in hypersensitized (HS) ones. As a consequence, the median waiting time was also significantly higher in 0 blood group patients, when compared to the remaining groups. However, waiting time increased extensively with cPRA regardless blood type, especially HS patients with increases of 368%, 632%, 486%, and 140% for blood groups A, B, AB, and 0, respectively, when compared to each blood group global median waiting time. Our study shows that important measures need to be undertaken in order to mitigate the huge disadvantage that HS and 0 blood type patients naturally have.

Introduction

Kidney transplantation is undoubtedly the best replacement therapy for eligible patients with end-stage renal disease (ESRD) when compared to maintenance on dialysis (1–3), improving quality of life in a cost-effective manner. Unfortunately, the scarcity of kidney grafts procured from deceased donors (DD) does not respond to the increasing number of ESRD patients on the transplant waiting list. To offset this tendency, a number of strategies have been used worldwide to increase the number of kidney transplants, such as organ procurement in extended criteria (4) and nonheart beating donors (5), living donation (6), AB0 incompatible (AB0i) transplantation (7), kidney paired donation (KPD) programs (8, 9), and the more recent concept called longevity matching (10), which consists of preferential allocation of best quality organs to wait-list candidates with the longest predictive survival.

As a scarce good, kidney allocation policies vehemently seek to distribute grafts fairly and equitably. However, it is known that patients waiting time for a kidney transplant differs according to blood type (11), race (12, 13), gender (14), degree of human leukocyte antigen (HLA) allosensitization (15) and, in some countries, also socioeconomically conditions (16). To achieve balance in kidney grafts distribution, allocation programs have been evolving (17), minimizing the importance of HLA matching, benefiting rare HLA phenotypes and HLA homozygous patients, increasing the emphasis on dialysis waiting time, transplantation between identical AB0 blood type patients, extra points for HLA-sensitized patients, acceptable mismatch (AM) programs (18), and national allocation priority for hypersensitized (HS) patients.

In Portugal, DD kidney graft distribution is performed nationally for pediatric, high urgency, and multiorgans waiting patients. Besides these specific cases, allocation is regional with three independent lists: North, Center, and South regions, and it is done considering AB0 iso-groups distribution. The allocation matching software initially runs a virtual cross-match (vXM), excluding patients with donor-specific antibodies (DSA) to HLA-A, -B, and -DRB1 *loci*, considering 1000 as mean fluorescence intensity (MFI) cutoff. The remaining candidates are

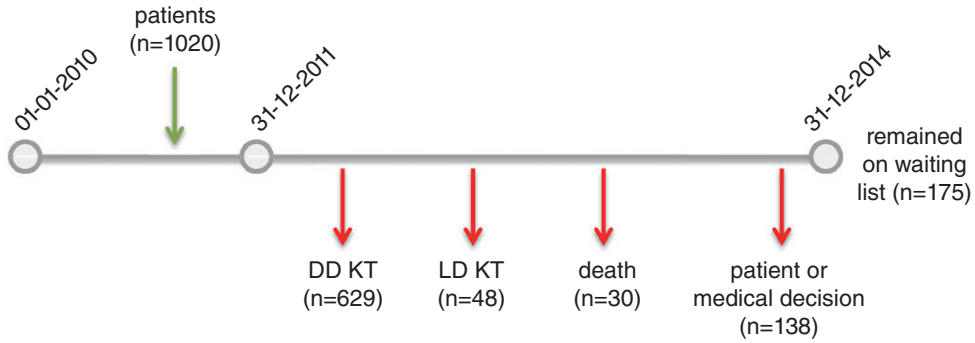

Figure 1 Retrospective study design timeline including 1020 prevalent and incident kidney transplantation candidates between 01 January 2010 and 31 December 2011, with follow-up until 31 December 2014. DD, deceased donor; KT, kidney transplant; LD, living donor.

listed based on a score algorithm attributing points to dialysis waiting time (0,1 point *per* month), HLA phenotypic compatibility (1–12 points), age proximity between patient–donor pair (4 points), and pediatric candidates receive additional points (4 points for candidates with 11–18 years old and 5 points for patients under 11 years). Moreover, the Portuguese allocation program assigns 4 and 8 extra points for patients with complement-dependent cytotoxicity (CDC) panel reactive antibody (PRA) greater than 50% and 80%, respectively, which has been proven to underestimate patient allosensitization when compared to calculated PRA (cPRA) (19, 20). In fact, considering only CDC-PRA in scoring allocation extra points, leaving aside cPRA determined with single antigen bead (SAB) assays, disadvantages HS patients as does the a priori exclusion from the matching list of candidates based on vXM accessed by SAB with a low MFI cutoff (≥1000) for determining unacceptable antigens.

As kidney allocation is done according to donor's blood type and cPRA percentage determines each candidate chance of having a negative vXM, a step necessary in order to proceed in matching process and be tested with cell-based cross-match, it is of extremely important to determine in what extent different blood groups and cPRA values impact waiting time for kidney transplantation.

Living donor (LD) kidney transplantation is gaining expression and in the last year represented 32.7% (33 of 101) of the total number of transplantations performed in Centro Hospitalar do Porto (CHP) transplantation unit (TRU). To enhance LD transplantation, a national KPD program was implemented in 2010 in order to enable transplantation of incompatible pairs (21, 22). Furthermore, in 2014, CHP TRU also launched an ABOi program for LD, performed only for nonsensitized patients, but for the purpose of improving 0 blood type patients transplant rate.

The aim of this study was to evaluate the difference of patient's access to kidney transplantation in Portugal analyzing data of CHP TRU, considering blood groups and HLA allosensitization status.

Material and methods

Patients

A retrospective cohort study was performed including 1020 prevalent and incident candidates on the waiting list for kidney transplantation between 01 January 2010 and 31 December 2011 in a singular Portuguese TRU CHP. Demographical, clinical, and immunological data at the entry of the waiting list were recorded in incident patients, while for prevalent patients, the earliest data entry within the study period was chosen. CHP has the second largest waiting list in the country and performed an average of 81 kidney transplants with deceased donation per year, over the past 5 years (2011–2015). Patients may register in two TRU and are encouraged to do so as this increases their transplant probability (22). In fact, 890 (87.3%) patients of our population cohort were registered in two TRU.

Patients were followed until receiving a kidney graft from a DD ($n = 629$) or a LD ($n = 48$), being removed from the waiting list by medical decision and/or patient choice ($n = 138$), dying ($n = 30$), or until 31 December 2014. At the end of follow-up, 175 patients remained on the waiting list. For the overall description of the cohort, all patients were included ($n = 1020$) (Figure 1). Then, we excluded patients with a higher prioritization on the allocation system (14 patients urgently enlisted and 23 pediatric patients), leaving 987 (4 pediatric patients were also urgently enlisted) patients for the analysis of deceased organ offer rate, with time being defined by the period between patient entry in study and occurrence of one of the outcomes enunciated or end of follow-up. An organ offer was considered every time a patient was tested negative for CDC cross-match assay and sent in the final list to TRU. Finally, to determine the median waiting time for a kidney transplant from a DD by blood type and cPRA, we also excluded patients who received a kidney graft from a living donor, those removed from the waiting list, or who died during follow-up, with 776 patients remaining for this analysis, with time being defined between date of patient admission on the waiting list and date of DD transplantation or end of follow-up.

Table 1 Characteristics of patient's cohort by cPRA groups[a]

N = 1020	cPRA = 0% (*n* = 780)	0 < cPRA < 80% (*n* = 113)	cPRA ≥ 80% (*n* = 127)	*P*
Age[b]	47.8 ± 13.8	47.8 ± 11.2	45.8 ± 10.4	0.271
Female candidate	225 (28.8%)	68 (60.2%)	79 (62.2%)	<0.001
Previous transplants	49 (6.3%)	49 (43.4%)	82 (64.6%)	<0.001
Previous transplant = 1	48 (6.2%)	44 (38.9%)	72 (56.7%)	
Previous transplants = 2	1 (0.1%)	5 (4.5%)	10 (7.9%)	
Transfusions > 0	276 (35.4%)	62 (54.9%)	79 (62.2%)	<0.001
Number of transfusions	2 (2–4)	2 (2–4)	3 (2–5)	0.304
Pregnancies > 0	150 (19.2%)	54 (47.8%)	57 (44.9%)	<0.001
Number of pregnancies	2 (1–3)	3 (2–3)	2 (1–3)	0.158
Any sensitizing event	372 (47.7%)	103 (91.2%)	123 (96.9%)	<0.001
Blood type AB0				<0.001
A	325 (41.7%)	53 (46.9%)	71 (55.9%)	
B	30 (3.8%)	3 (2.7%)	10 (7.9%)	
AB	16 (2.1%)	1 (0.9%)	5 (3.9%)	
0	409 (52.4%)	56 (49.6%)	41 (32.3%)	
Super-urgent status	10 (1.3%)	1 (0.9%)	3 (2.4%)	0.446
Outcomes				
In active list	92 (11.8%)	31 (27.4%)	53 (41.7%)	<0.001
TR with DD	533 (68.3%)	54 (47.8%)	42 (33.1%)	<0.001
TR with LD	44 (5.6%)	2 (1.8%)	2 (1.6%)	0.016
In contraindication list	93 (11.9%)	20 (17.7%)	25 (19.7%)	0.007
Death	19 (2.4%)	6 (5.3%)	5 (3.9%)	0.164
Time in list (months)[b]	38.8 ± 23.9	48.2 ± 26.9	68.8 ± 42.3	<0.001
Time in cohort (months)[b]	31.0 ± 19.5	38.6 ± 21.0	43.0 ± 21.6	<0.001
Time in dialysis (months)[c]	19.3 (7.6–39.1)	27.1 (12.9–69.7)	61.8 (29.8–112.5)	<0.001
PRA max%[c]	0 (0–2)	2 (2–12)	35 (5–69)	<0.001
PRA > 50%	5 (0.9%)	6 (5.5%)	45 (36.0%)	<0.001
PRA > 80%	1 (0.2%)	1 (0.9%)	18 (14.4%)	<0.001

cPRA, calculated panel reactive antibody; DD, deceased donor; LD, living donor; TR, transplant.

[a]Categorical data are presented as numbers (frequencies, %).

[b]Continuous data are presented as mean (± SD, standard deviation).

[c]Continuous data are presented as median (IQR, interquartile range).

PRA determination

Kidney transplantation candidates are tested for HLA antibodies in the initial study with classic cell-based PRA-CDC and solid-phase assays (SPA). PRA-CDC is determined by a CDC cross-match assay with 50 mononuclear cells isolated by ficoll-hypaque method, from HLA-A and -B typed donors. Microscopic visual assessment of cell death percentage indicates if DSA are present. On the other hand, the SPA used are based on synthetic microspheres (beads) coated with HLA antigens. In the initial study, the SPA performed is a screening test with beads coated with purified HLA class I and class II antigens, LabScreen mixed assay (LSM12; One Lambda Inc., Canoga Park, CA). If HLA antibodies are detected in the screening assay, their specificities are determined by a SAB assay, Labscreen Single Antigen (One Lambda Inc.). This assay includes beads with a single recombinant HLA antigen allowing specificities identification, considering a bead positive when MFI value is greater than 1000. Data analysis and cPRA calculation were performed using HLAfusion™ software, considering HLA-A, -B, -DRB1 phenotypic frequencies of 227 Portuguese deceased organ donors. The cPRA value considered in the analysis was the last determination for prevalent patients and the initial study value for incident patients.

Thereafter, nonsensitized patients are monitored every 3 months with screening SPA only, while HLA-sensitized candidates are tested using both PRA-CDC and screening SPA. Annually, all patients are monitored with PRA-CDC and SAB assay.

Statistical analysis

Continuous data were described using mean (standard deviation, SD) or median (interquartile range, IQR) and categorical data were expressed as numbers (frequencies). Categorical data including demographic, clinical, and immunological features were compared using Pearson χ^2 test or χ^2 for trend, as appropriate. Continuous variables were compared with one-way analysis of variance (ANOVA) or Kruskal–Wallis, as appropriate.

Deceased organ offer rate was obtained by Poisson log linear regression considering cPRA groups, stratified by AB0 groups.

Median waiting time for a kidney transplant from a DD by blood type and cPRA was calculated using Kaplan–Meier

Table 2 Deceased organ offer rate by blood group and cPRA[a]

		Mean months/ 1 offer/candidate	95% CI	*P*
Overall ($n=987$)		6.7	6.3–7.1	—
Blood type A ($n=434$)	Global	6.0	5.7–6.2	A *versus* B = 0.642
				A *versus* AB = 0.404
				A *versus* O = <0.001
	1. cPRA 0% ($n=312$)	4.8	4.6–5.1	1 *versus* 2 = 0.001
	2. cPRA 1%–79% ($n=52$)	5.9	5.3–6.6	1 *versus* 3 = <0.001
	3. cPRA ≥ 80% ($n=70$)	15.8	13.7–18.1	2 *versus* 3 = <0.001
Blood type B ($n=40$)	Global	5.8	5.0–6.6	B *versus* AB = 0.675
				B *versus* O = <0.001
	1. cPRA 0% ($n=27$)	4.0	3.5–4.7	1 *versus* 2 = 0.024
	2. cPRA 1%–79% ($n=3$)	7.0	3.9–12.7	1 *versus* 3 = <0.001
	3. cPRA ≥80% ($n=10$)	28.7	16.3–50.5	2 *versus* 3 = 0.015
Blood type AB ($n=20$)	Global	5.5	4.5–6.6	AB *versus* O = <0.001
	1. cPRA 0% ($n=14$)	4.2	3.3–5.3	1 *versus* 2 = 0.704
	2. cPRA 1%–79% ($n=1$)	3.5	1.6–7.8	1 *versus* 3 = <0.001
	3. cPRA ≥80% ($n=5$)	9.3	6.4–13.6	2 *versus* 3 = 0.133
Blood type 0 ($n=493$)	Global	10.6	10.1–11.1	—
	1. cPRA 0% ($n=398$)	9.9	9.4–10.4	1 *versus* 2 = 0.320
	2. cPRA 1%–79% ($n=56$)	10.6	9.3–12.1	1 *versus* 3 = <0.001
	3. cPRA ≥ 80% ($n=39$)	29.8	20.9–38.9	2 *versus* 3 = <0.001

cPRA, calculated panel reactive antibody.

[a]Data are presented as mean and 95% confidence interval (CI).

method, with comparison between patients' groups being done by log-rank test.

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).

Results

In our cohort of 1020 patients waiting for a kidney transplant, 780 (76.5%) are nonsensitized, 113 (11.0%) sensitized, and 127 (12.5%) were highly sensitized, considering cPRA ranges of 0%, 1%–79%, and greater than 80%, respectively. Comparison of demographical, clinical, and immunological characteristics across the three groups defined by cPRA is shown in Table 1. Analyzing patients with higher cPRA values, it was noticeable that they were predominantly female ($P<0.001$), had more sensitizing events ($P<0.001$), longer dialysis waiting time ($P<0.001$), and were less transplanted with DD ($P<0.001$). Also noteworthy is that only 14.2% (18 of 127) of the HS patients had a scoring PRA, accessed by CDC, greater than 80% (Table 1).

The DD organ offer rate according to blood type and cPRA groups is displayed in Table 2. Overall, there were 4257 organ offers that represented 1 offer every 6.7 months per candidate. The DD organ offer rate by blood type tended to decrease with the increase of cPRA for the candidates of A and B blood groups. For AB blood type patients, this difference was only seen in the extreme cPRA values perhaps due to the lower number of patients in these groups. Curiously, for 0 blood group patients, no difference on DD organ offer was observed between nonsensitized and sensitized patients, emphasizing the long waiting time that even nonsensitized 0 blood group patients have to face (Table 2).

One hundred and twenty-five patients were evaluated with at least one kidney LD and 48 (37.5%) were transplanted. LD kidney transplantation was more common in nonsensitized (5.6%) than in sensitized (1.8%) and HS (1.6%) patients ($P=0.016$). Moreover, the ratio between the median number of LD evaluated per LD kidney graft recipients was 1, 1, and 3 in nonsensitized, sensitized, and HS patients, respectively ($P=0.009$).

The longitudinal analysis ($n=776$) revealed that the median waiting time for transplant was greater for 0 blood group patients (65.3 months), when compared to the remaining blood types: A (35.1 months), B (22.8 months), and AB (14.5 months) (Table 3). The waiting time for A blood type patients increased with cPRA value ($P<0.001$) as the percentage of patients transplanted over time was significantly lower. For 0 blood group patients, this difference was only significant between nonsensitized patients and the remaining groups ($P<0.001$), as showed by the cumulative incidence of kidney transplants. For blood type B and AB patients, the difference was only statically significant between nonsensitized and HS ($P<0.001$ and $P=0.013$, respectively).

A multivariable Cox regression model showed that older patients [hazard ratio (HR) = 1.020, $P<0.001$], HS (*versus* nonsensitized, HR = 0.090, $P<0.001$), and sensitized (*versus* nonsensitized, HR = 0.380, $P<0.001$) had a lower chance of been transplanted (Table 4). Additionally, when compared to A blood type candidates, blood type B and AB patients had a higher chance of been transplanted (HR = 1.574, $P=0.019$;

Table 3 Median waiting time for a kidney transplant by blood type and cPRA[a]

	cPRA	# patient transplanted	Median waiting time	Log-rank *P*
Overall ($n=776$)		601 (77.4%)	47.5	—
Blood type A ($n=344$)	Global	290 (84.3%)	35.1	A *versus* B = 0.366
				A *versus* AB = 0.117
				A *versus* O = <0.001
	1. cPRA 0% ($n=254$)	239 (94.1%)	30.7	1 *versus* 2 = <0.001
	2. cPRA 1%–79% ($n=38$)	29 (76.3%)	47.4	1 *versus* 3 = <0.001
	3. cPRA ≥80% ($n=52$)	22 (42.3%)	129.3	2 *versus* 3 = <0.001
Blood type B ($n=35$)	Global	31 (88.6%)	22.8	B *versus* AB = 0.638
				B *versus* O = 0.001
	1. cPRA 0% ($n=25$)	25 (100%)	16.9	1 *versus* 2 = 0.215
	2. cPRA 1%–79% ($n=3$)	2 (66.7%)	27.4	1 *versus* 3 = <0.001
	3. cPRA ≥80% ($n=7$)	4 (57.1%)	144.0	2 *versus* 3 = 0.156
Blood type AB ($n=17$)	Global	14 (82.4%)	14.5	AB *versus* O = <0.001
	1. cPRA 0% ($n=13$)	12 (92.3%)	13.5	1 *versus* 2 = 0.867
	2. cPRA 1%–79% ($n=1$)	1 (100%)	16.6	1 *versus* 3 = 0.013
	3. cPRA ≥80% (n = 3)	1 (33.3%)	70.5	2 *versus* 3 = 0.083
Blood type 0 ($n=380$)	Global	266 (70.0%)	65.3	—
	1. cPRA 0% ($n=308$)	232 (75.3%)	62.0	1 *versus* 2 = 0.001
	2. cPRA 1%–79% ($n=42$)	21 (50.0%)	75.1	1 *versus* 3 < 0.001
	3. cPRA ≥80% ($n=30$)	13 (43.3%)	91.1	2 *versus* 3 = 0.176

cPRA, calculated panel reactive antibody; DD, deceased donor; LD, living donor; TR, transplant.
[a]Data are presented as total number (#), percentage, and median (months).

Table 4 Multivariable Cox regression analysis of the chance of receiving kidney graft from a DD ($n=776$)[a]

	HR	95% CI	*P*
Age	1.020	1.012–1.027	<0.001
AB0 group			<0.001
A	Reference		
B	1.574	1.079–2.296	0.019
AB	2.582	1.495–4.460	0.001
0	0.255	0.211–0.308	<0.001
cPRA			<0.001
0%	Reference		
1–79	0.380	0.284–0.510	<0.001
≥80%	0.090	0.062–0.131	<0.001

CI, confidence interval; cPRA, calculated panel reactive antibody; DD, deceased donors; HR, hazard ratio.
[a]Data are presented as HR and 95% CI.

HR = 2.582, $P = 0.001$, respectively), while the opposite occurred in 0 blood type patients (HR = 0.255, $P < 0.001$) (Table 4).

Discussion

Our retrospective cohort study showed that 0 blood group patients are in great disadvantage when compared to the remaining candidates, waiting longer to have a kidney graft offer from a DD (10.6 months) and, consequently, having an extended waiting time on dialysis (65.3 months). Additionally, and regardless blood type, cPRA value greater than 80% was a tremendous unfavorable factor, with HS patients having fewer kidney offers, prolonged waiting time and being less transplanted.

Portuguese allocation system is aware of these disadvantages and, in order to seek fairness regarding blood group differences, introduced the AB0-identical rule in a legislation update in 2007. With this change, blood group 0 kidneys are allocated to non-0 recipients only for clinical super-urgent patients. This change had a striking effect in decreasing waiting times as reported by Lima et al., where the overall waiting time on dialysis decreased from a median of 65–51 months (112–77 for 0 blood type patients) in the following years (2008–2011) (23). A disadvantage of this legislative change was the loss of points for HLA matching to dialysis time, which will have future impact in allosensitization degree for patients needing a second transplant.

Regarding patients allosensitization status, we consider that the present allocation program is not reasonable when it assigns score extra points based only on cytotoxic PRA (24). By doing so, the Portuguese allocation system works as a double-edged sword because it considers the less sensitive PRA-CDC assay in order to provide additional points to HS patients, but imposes a rigid cutoff in SAB assay to determine unacceptable HLA mismatches. Previously, we published data showing that no antibody-mediated rejection occurred in nondesensitized patients with preformed DSA with a MFI below 3000 (25). This approach is an important explanation for the HS patients low transplant rate as they are excluded based on vXM, and we are convinced that the introduction of cPRA in the allocation matching would provide a more pertinent measure of sensitization and transplantability, a fairer assignment of additional

points for HS patients, and it could reverse the observed trend of organ offer and transplantation rates, as already published (26). In fact, this stringent cutoff in the vXM may be just when considering low or nonsensitized patients, but we believe it is not reasonable when considered high sensitized patients. Although we understand that transplantation across an antidonor HLA antibody is not the best option, this may be the best chance a HS patient will ever have, allowing them to be transplanted with low-moderate level DSA and a negative cytotoxic cross-match, with a suitable immunosuppression therapy. Additionally, this inflexible cutoff leads to false unacceptable HLA mismatches listing, not clinically relevant, and denying some patients the opportunity of having a cross-match assay performed (27), as SAB assay has been associated with a number of technical issues (28) such as, difficulty to set a cutoff (29), antigen denaturation in recombinant beads (30), complement interference (31, 32), and the antibody identification with SAB assays of HLA antibodies in male nonimmunized candidates (33).

Another important drawback of the Portuguese legislation is the lack of a national program for HS patients, diminishing greatly their donor pool, in particular of HLA full matched kidneys, as graft survival is superior with zero mismatches (34). Portugal is a small country where the logistic of graft exchanges between transplant centers is easily achieved without increasing cold ischemia. It has been reported that unexpected positive cross-matches, and consequently the reshipment of incompatible kidney, would increase cold ischemia time (35), but we believe that HS patient's sera exchange between histocompatibility laboratories, allowing the execution of cell-based cross-match before organ shipment, would prevent unexpected positive cross-matches that could occur due to incomplete or error in donors HLA typing. In fact, even organ exchange between other countries can be a reality in the near future as Portugal became a South Alliance for Transplants (SAT) member, a transnational alliance in the field of organ, tissue, and cell donation and transplantation.

Regarding LD transplantation, again as expected, the transplant rate of HS patients was significantly lower. Also, the national KPD program implemented 5 years ago did not have a very significant role in LD transplantation, with only nine kidney transplants performed, due to insufficient number of pairs enrolled, as it success depends greatly of the number of incompatible pairs included (36, 37). Besides, our program does not allow altruistic donations (38), ABOi (39, 40), or introduction of compatible pairs (41), that would certainly increase match rates.

To conclude, this study emphasizes the importance of adopting new strategies for 0 blood type and HS patients, otherwise they will continue to accumulate in the waiting list, having a remote chance of receiving a transplant. For 0 blood type, recipients with believe that the continued engagement in LD programs (42) enhanced by the introduction of ABOi, compatible pairs, altruistic donations, and desensitization (43) in KPD would allow raising transplantation rate. Concerning HS candidates, we believe that a national AM program, along with the following essential changes, would have a huge impact on HS patient's transplantability (44, 45). First, reassessment of HLA unacceptable antigens, as not all HLA specificities determined by SAB assays are necessarily real unacceptable antigens. HLA matchmaker algorithm should be used in reanalysis considering that HLA antibodies recognize HLA epitopes rather than HLA antigens (46). Second, update level of sensitization to cPRA since classic CDC alone underestimates patient's allosensitization, with important variations between laboratories. Finally, extending the current local allocation nationally would triple donor pool, increasing the chances of finding a donor match.

With this study, we hope to open a new door for a constructive discussion towards a fairer kidney allocation system.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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Appendix 2

Eplet based virtual PRA increases transplant probability in highly-sensitized patients

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Research Article

Eplet-based virtual PRA increases transplant probability in highly-sensitized patients

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ABSTRACT

Background: The reduced access of highly-sensitized (HS) patients to kidney transplantation (KTx) is one of the major challenges for transplant community. Therefore, the aim of our study was to estimate the impact of three different vPRA calculations, assessed traditionally and using eplet-based analysis, in donor offers. Methods: At 01-01-2020, 157 HS patients are waitlisted for deceased donor KTx and were included in this study. Total vPRA (vPRAt) was calculated considering all patient allosensitization history, using 1 k MFI cut-off. Current vPRA (vPRAc) refers only to the last year SAB assays, using 1 k MFI cut-off. For eplet vPRA (vPRAe) every SAB assay was analyzed by HLAMatchmaker and HLAfusion software. Matching runs have been performed taking vPRA calculation as unacceptable antigens (UAs).

Results: All patients had at least one previous sensitizing event and patients with 100% vPRA were predominantly candidates for retransplantation ($P < 0.001$), had higher PRA-CDC ($P < 0.001$), and longer dialysis vintage waiting time ($P < 0.001$). Inter-group movement analysis between vPRA measures showed that 70 (45%), 124 (79%) and 80 (51%) patients were reclassified to a lower group when considering vPRAt to vPRAc, vPRAt to vPRAe and vPRAc to vPRAe, respectively. The median percentage of change in estimated number of match runs needed for 95% probability of finding an acceptable donor was significantly more pronounced by increasing vPRAt intervals, when considering the reclassification from vPRAt to vPRAe ($P < 0.001$) or vPRAc to vPRAe ($P = 0.045$), while from vPRAt to vPRAc it was not ($P = 0.899$).

Conclusions: Our study demonstrated that the use of total or current vPRA calculations are impairing HS patients, by decreasing transplant probability, leading to dramatically longer waiting times, when compared to eplet based vPRA.

1. Introduction

Reduced transplantability rates within highly-sensitized (HS) patients is a global health issue identified more than thirty years ago [1]. This disadvantaged group was known to have prolonged waiting times due to positive crossmatches against almost all donors tested [2] and, since then, scientific community struggle to increase chance of finding compatible donors for these patients [3,4]. Additionally, patient's HLA allosensitization status, historically determined with panel reactive antibodies (PRA) by complement-dependent cytotoxicity (CDC), was deeply underestimated. This method, described by Terasaki and McClelland in 1964 [5], was time-consuming, lacked sensitivity and did not allow the discrimination of non-cytotoxic or non-HLA antibodies. This low-throughput method was particularly ineffective for HS patients, being almost impossible to identify HLA specificities.

With the introduction of solid-phase immunoassays (SPI), particularly single antigen bead (SAB) assays, HLA antibodies identification has undergone a substantial progress, improving crossmatch prediction and allowing the implementation of virtual PRA (vPRA) [6]. The introduction of vPRA allowed a more consistent and reliable measure of

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allosensitization enabling the introduction of virtual crossmatch (vXM), increasing transplantation rates among sensitized patients [7,8]. Unacceptable antigens (UA) accurate assignment prevents offers to patients that will result in positive crossmatches avoiding organs to travel unnecessarily, promoting efficiency and ultimately reducing cold ischemia time [9,10].

Although the unquestionable SAB assays value, it is now recognized that these assays also need to be interpreted with caution due to several technical limitations [11–13] and difficulties to establish clinical relevant cut-offs for antibodies specificities identified by this more sensitive technique [14,15]. The improvement accuracy for UA assignment is of most importance for HS patients that, due to reduced donor offers, have prolonged waiting times [16]. In fact, the probability of finding an acceptable match, calculated by the eq. 1-(vPRA)^n where n value is the number of potential donors, decreases exponentially for patients approaching vPRA of 100% [17]. To overcome this, alternative allocation programs that improved transplantation efficiency, have been implemented worldwide [18,19].

In Portugal, highly-sensitized patient's allocation is regional since our legislation for deceased donor allocation was updated in 2008, aggravating this problem by lowering donor pool. Since then, waiting time for these patients has increased and Portugal now bears with 683 (34.6%) patients, for whom it is very difficult to find a compatible donor. Furthermore, we consider all sera tested by SAB assay, with mean fluorescence intensity (MFI) value higher than 1 k, as an absolute contraindication for transplant. This conservative strategy results in overestimated allosensitization, immediately excluding patients without physical crossmatch, preventing them organ offers and transplantation. We believe that eplet-based analysis can improve greatly UA assignment, increasing the probability of donor offer and transplantation within HS patients.

Therefore, the aim of our study was to compare three different vPRA, determined traditionally and using eplet-based analysis, estimating their impact in donor offers within HS population.

2. Materials & methods

2.1. Study population

At 01-01-2020, 1973 patients were waitlisted for deceased donor kidney transplantation in Portugal, with 606 (30.71%) in the Northern region and studied by our center. 157 (25.9%) of these patients are highly-sensitized with vPRA higher than 98%, that represents 22.9% of the overall Portuguese HS population ($n = 683$), and were included in this study.

2.2. HLA antibodies assessment

Patient's allosensitization status was determined by cellular and SPI. The cellular assay consists in standard 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 allowed the determination of cytotoxic PRA using Lambda Scan® Plus II Analysis software, version 5.9.

SPI were carried out using coded-colour microbeads coated with purified class I or class II HLA antigens based on Luminex Xmap® Technology (LABScreen® Single Antigen Bead (SAB) kit, OneLambda, Canoga Park, CA, USA), according to manufacturer instructions using EDTA-treated sera. SAB analysis was carried out with HLAfusion™ software, version 4.4 (One Lambda, Inc., CA, USA).

2.3. In silico calculation of virtual panel reactive antibodies

vPRA was determined using a Portuguese national population of 1100 deceased organ donors typed for HLA-A, -B, -C, -DRβ1 and -DQβ1 loci using polymerase chain reaction (PCR) amplification with specific sequence primers (Olerup® SSP HLA typing kits, Stockholm, Sweden) and confirmed with reverse sequence-specific oligonucleotide (LABType® SSO typing kits, One Lambda, Canoga Park, CA, USA). HLA antigen frequencies were performed on each of the five *loci* and no deviation from the Hardy- Weinberg equilibrium (HWE) was observed.

For total virtual PRA (vPRAt) the mean fluorescence intensity (MFI) higher than 1 k, considering all patient allosensitization history, was considered positive for assignment UA. For the analysis three intervals groups for vPRA were considered, [97.50%–99.50%]($n = 34$, 22%), [99.50%–99.99%], ($n = 46$, 29%) and 100% ($n = 77$, 49%). Current virtual PRA (vPRAc) refers only to the last year SAB determinations, using the same 1 k MFI cut-off. For eplet vPRA (vPRAe) every SAB assay was analyzed by HLAfusion™ software, version 4.4 (One Lambda, Inc., CA, USA) and HLAMatchmaker algorithm, ABC antibody analysis with Acceptable Mismatch Determinations v3.1 and DRDQDP Analysis and Mismatch Acceptability v3.0, respectively for HLA class I and class II.

Table 1
Baseline characteristics of the overall cohort and comparison of vPRAt intervals.

	Total $N = 157$	A.[97.50%–99.50%] $N = 34$ (22%)	B.[99.50%–99.91%] $N = 46$ (29%)	C.100% $N = 77$ (49%)	P
Age, median (IQR)	52.4 (45.0–58.6)	55.2 (45.1–63.1)	48.9 (42.7–56.0)	51.9 (45.1–58.5)	0.055*
Female, n (%)	87 (55)	25 (74)	24 (52)	38 (49)	0.053**
Previous sensitizing events, n (%)	157 (100)	–	–	–	–
- Previous transfusions, n (%)	125 (80)	23 (68)	36 (78)	66 (86)	0.090**
- Previous pregnancy, n (%)[a]	64 (74)	18 (72)	20 (83)	26 (68)	0.422
- Previous transplant, n (%)	120 (76)	17 (50)	37 (80)	66 (86)	**<0.001***,**
Blood group AB0, n (%)					0.749
- A	73 (47)	15 (44)	20 (43)	38 (49)	
- B	15 (10)	2 (6)	4 (9)	9 (12)	
- AB	9 (6)	1 (3)	4 (9)	4 (5)	
- 0	60 (38)	16 (47)	18 (39)	26 (34)	
Dv months, median (IQR)	106.9 (66.9–161.8)	65.0 (41.4–103.1)	94.8 (65.6–150.1)	134.9 (90.3–180.4)	**<0.001***,**,***
- Dv ≥ 5 years, n (%)	126 (80)	19 (56)	37 (80)	70 (91)	**<0.001***,**
- Dv ≥ 8 years, n (%)	87 (55)	9 (26)	22 (48)	56 (73)	**<0.001****,***
- Dv ≥ 10 years, n (%)	67 (43)	7 (21)	17 (37)	43 (56)	**0.002****,***
PRA-CDC (%),median (IQR)	18 (4–55)	2 (0–13)	8 (0–32)	39 (16–65)	**<0.001***,**,***
- PRA-CDC ≥50%, n (%)	47 (30)	4 (12)	9 (20)	34 (44)	**0.001****,***
- PRA-CDC ≥80%, n (%)	19 (12)	0	5 (11)	14 (18)	0.024**

IQR, interquartil range; Dv, Dialysis vintage time; PRA, panel reactive antibodies; CDC, complement-dependent cytotoxicity.
* A. *vs* B. $P < 0.05$.
** A. *vs* C. $P < 0.05$.
*** B. *vs* C. $P < 0.05$.
[a] Considering only women ($n = 87$ in all cohort).

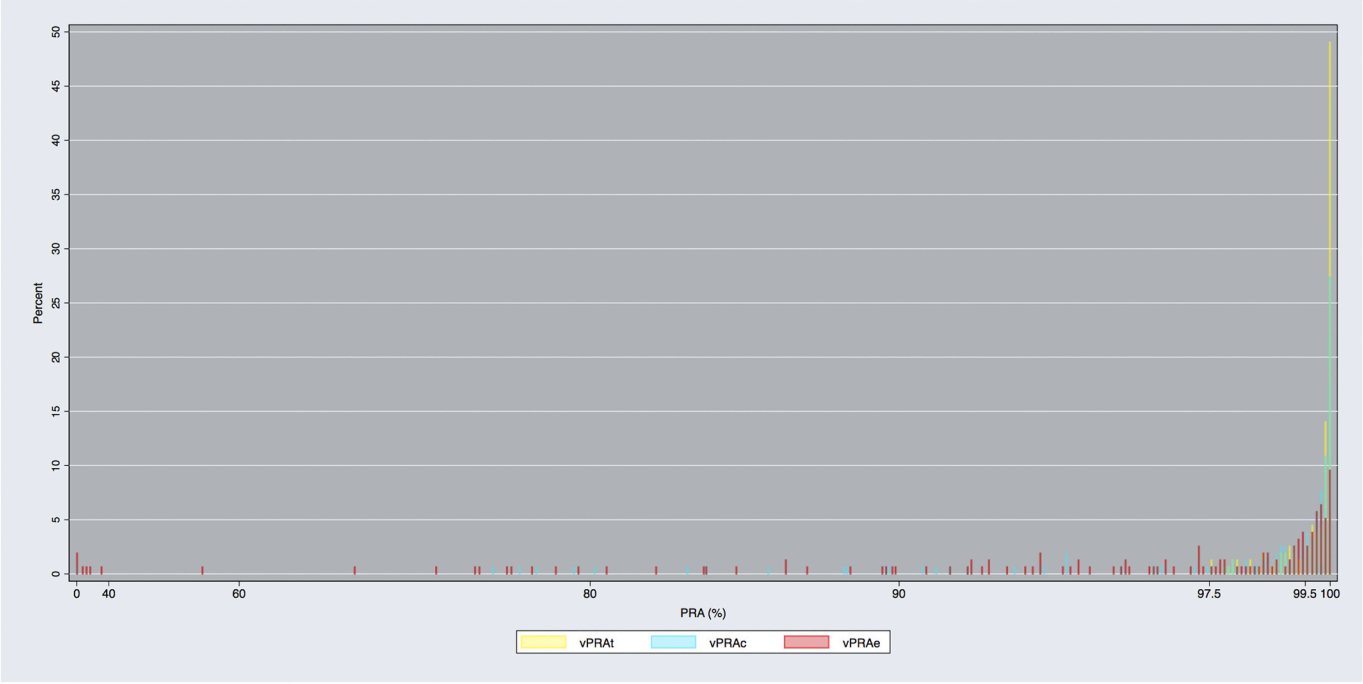

Fig. 1. Virtual PRA inter-group reclassification overlay histogram.

Discrepancies found in eplet analysis, between HLAmatchmaker and HLAfusion software, were resolved by HLA epitope registry [20]. Allelic specific specificities found in the SAB that could not be explained with an antibody-verified eplet (AbVer), or high ElliPro score (HiElliPro), were not considered.

2.4. Statistical analysis

Continuous data were described using 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 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. The Wilcoxon signed rank test was used to compare paired changes between vPRA different calculations.

We used the Spearman's rho correlation to examine the relationship between scores from the three virtual PRA calculations: vPRAt, vPRAc and vPRAe. Finally, we grouped patients by vPRA intervals: 1) 97.5%–99.5%, 2) 99.5%–99.9% and 3) 100% and analyzed inter-group movement of patients between the three vPRA groups to assess transplantability impact of using based eplet virtual PRA. The strength of association between vPRA values was assessed by Goodman and Kruskal's Gamma rank correlation and Cohen's kappa for agreement.

Estimation of the number (n) of match runs needed for 95% probability of finding an acceptable donor was calculated as previously

Table 2
Intergroup movement between total and current virtual panel reactive antibodies calculations.

			vPRAt		
			[97.50%–99.50%] N = 34 (22%)	[99.50%–99.91%] N = 46 (29%)	100% N = 77 (49%)
		Estimated number of match runs needed for 95% probability of finding an acceptable donor	[150–600]	[600–3330]	~30,000
vPRAc	[0%–97.50%] N = 28 (18%)	[1–150]	17 *61%* 50%	6 *21%* 13%	5 *18%* 6%
	[97.50%–99.50%] N = 36 (23%)	[150–600]	17 *47%* 50%	13 *36%* 28%	6 *17%* 8%
	[99.50%–99.91%] N = 50 (32%)	[600–3330]	0	27 *54%* 59%	23 *46%* 30%
	100% N = 43 (27%)	~30,000	0	0	43 *100%* 56%
		Agreement test	Kappa: 0.383 P < 0.001		
		Rank correlation test	Gamma: 0.831 P < 0.001		

Italic: row (current virtual PRA, vPRAc) percentages; Underline: column (total virtual PRA, vPRAtt) percentages.
Same group: 87 (55%).
Reclassified from vPRA to 1-degree lower group by vPRAa: 53 (34%).
Reclassified from vPRA to >1-degree lower group by vPRAe: 17 (11%).
Reclassified as HS from vPRA to non-HS by vPRAa (<97.50%): 28 (18%).

described [17]. The percentage of change of eMR was calculated usind the formula %eMR = (eMR_{final} – $eMR_{initial}$) / eMR_{final} *100.

A two-sided P-value <0.01 was considered as statistically significant. Statistical calculations were performed using Stata/MP, version 15.1 (Stata Corp, College Station, TX).

3. Results

3.1. Baseline characteristics

Patient's cohort characteristics are presented in Table 1. All patients had at least one previous sensitizing event, such as transplant (n = 120, 76%), blood component transfusions (n = 125, 80%), and pregnancies (n = 64 of 87 female patients, 74%). As expected patients with 100% of vPRA were predominantly candidates for retransplantation (P < 0.001), had higher PRA-CDC (P < 0.001), and longer dialysis vintage waiting time (P < 0.001).

It is noteworthy that only 19 (12%) patients are classified as HS by the Portuguese legislation with PRA-CDC higher than 80%. In fact, only 47 (30%) patients had PRA-CDC higher than 50% and would be granted extra-points in Portuguese allocation process. It is also important to note that the median dialysis vintage time in our cohort is 106.9 (66.9–161.8). This is significantly increased in the vPRA = 100% group with a medium dialysis vintage time of 134.9 (90.3–180.4).

3.2. vPRA calculations correlations and inter-group reclassification

As expected, spearman rank-order coefficient showed strong correlation between vPRA calculations, vPRAt *vs.* vPRAc (ρ = 0.715, P < 0.001), vPRAt *vs.* vPRAe (ρ = 0.531, P < 0.001) and vPRAc *vs.* vPRAe (ρ

Table 3
Intergroup movement between total and eplet virtual panel reactive antibodies calculations.

			vPRAt		
			[97.50%–99.50%] N = 34 (22%)	[99.50%–99.91%] N = 46 (29%)	100% N = 77 (49%)
		Estimated number of match runs needed for 95% probability of finding an acceptable donor	[150–600]	[600–3330]	~30,000
vPRAe	[0%–97.50%] N = 66 (42%)	[1–150]	30 *45%* 88%	16 *24%* 35%	20 *30%* 26%
	[97.50%–99.50%] N = 39 (25%)	[150–600]	4 *10%* 12%	16 *41%* 35%	19 *49%* 25%
	[99.50%–99.91%] N = 37 (24%)	[600–3330]	0	14 *38%* 30%	23 *62%* 30%
	100% N = 15 (10%)	~30,000	0	0	15 *100%* 20%
		Agreement test Rank correlation test	Kappa: 0.049 P = 0.116 Gamma: 0.637 P < 0.001		

Italic: row (eplet virtual PRA, vPRAe) percentages; Underline: column (total virtual PRA, vPRAt) percentages.
Same group: 33 (21%).
Reclassified from vPRAt to 1-degree lower group by vPRAe: 69 (44%).
Reclassified from vPRAt to >1-degree lower group by vPRAe: 55 (35%).
Reclassified as HS from vPRAt to non-HS by vPRAe (<97.50%): 66 (42%).

Table 4
Intergroup movement between current and eplet virtual panel reactive antibodies calculations.

			vPRAc			
			[0%–97.50%] N = 28 (18%)	[97.50%–99.50%] N = 36 (23%)	[99.50%–99.91%] N = 50 (32%)	100% N = 43 (27%)
		Estimated number of match runs needed for 95% probability of finding an acceptable donor	[1–150]	[150–600]	[600–3330]	~30,000
vPRAe	[0%–97.50%] N = 66 (42%)	[1–150]	27 *41%* 96%	25 *38%* 69%	9 *14%* 18%	5 *8%* 12%
	[97.50%–99.50%] N = 39 (25%)	[150–600]	1 *3%* 4%	11 *28%* 31%	17 *44%* 34%	10 *26%* 23%
	[99.50%–99.91%] N = 37 (24%)	[600–3330]	0	0	23 *62%* 46%	14 *38%* 33%
	100% N = 15 (10%)	~30,000	0	0	1 *7%* 2%	14 *93%* 33%
		Agreement test Rank correlation test	Kappa: 0.319 P < 0.001 Gamma: 0.809 P < 0.001			

Italic: row (current virtual PRA, vPRAc) percentages; Underline: column (total virtual PRA, vPRAt) percentages.
Same group: 75 (48%).
Reclassified from vPRAa to 1-degree lower group by vPRAe: 56 (36%).
Reclassified from vPRAa to >1-degree lower group by vPRAe: 24 (15%).
Reclassified from vPRAa to 1-degree higher group by vPRAe: 2 (1%).
Reclassified as HS from vPRAa to non-HS by vPRAe (<97.50%): 39 (25%).

Table 5
Comparisons of vPRA calculations median and median change, estimated number and percentage of change in estimated number of match runs needed for 95% probability of finding an acceptable donor (eMR).

	Total $N = 157$	1.[97.50%–99.50%] $N = 34$ (22%)	2.[99.50%–99.91%] $N = 46$ (29%)	3.100% $N = 77$ (49%)	P
vPRA (%), median (IQR)					
vPRAt	99.91 (99.64–100)	98.95 (98.36–99.18)	99.82 (99.73–99.91)	100 (100–100)	–
vPRAc	99.73 (98.64–100)	97.45 (90.64–98.73)	99.64 (99.00–99.82)	100 (99.73–100)	**<0.001***,**,***
vPRAe	98.64 (93.27–99.73)	90.59 (76.73–95.64)	98.73 (94.55–99.64)	99.46 (97.36–99.91)	**<0.001***,**,***
vPRA change (%), median (IQR)					
vPRAt to vPRAc	−0.09 (−0.82–0) Signed-rank P < 0.001	−1.32 (−8.55–0)	−0.09 (−0.82–0)	0 (−0.27–0)	**<0.001***,**,***
vPRAt to vPRAe	−1.27 [−6.36-(−0.18)] Signed-rank P < 0.001	−8.41 [−22.18-(−3.00)]	−1.09 [−5.36-(−0.27)]	−0.55 [−2.64-(−0.09)]	**<0.001***,**
vPRAc to vPRAe	−0.82 [−4.64-(−0.09)] Signed-rank P < 0.001	−5.41 [−10.73-(−1.46)]	−0.50 [−4.45-(−0.09)]	−0.46 (−1.91–0)	**<0.001***,**
eMR by vPRA, median (IQR)					
vPRAt	3290 (822–29,949)	286 (182–365)	1645 (1096–3290)	29,949 (29949–29,949)	–
vPRAc	1096 (218–29,949)	118 (30–234)	822 (298–1645)	29,949 (1096–29,949)	**<0.001***,**,***
vPRAe	218 (43–1096)	31 (11–67)	234 (53–822)	548 (112–3290)	**<0.001***,**,***
% of change in eMR, median (IQR)					
vPRAt to vPRAc	−40.1 (−91.7–0) Signed-rank P < 0.001	−60.0 (−87.3–0)	−50.0 (−81.9–0)	0 (−96.3–0)	0.899
vPRAt to vPRAe	−94.5 [−98.8-(−66.7)] Signed-rank P < 0.001	−89.2 [−96.2-(−75.7)]	−82.9 [−97.7-(−50.0)]	−98.2 [−99.6-(−89.0)]	**<0.001****,***
vPRAc to vPRAe	−66.8 [−92.9-(−16.2)] Signed-rank P < 0.001	−55.6 [−84.2-(−14.0)]	−59.8 [−80.1-(−21.6)]	−79.5 (−97.8–0)	0.045

% of change: (final-initial)/final*100.
* 1. *vs* 2. P < 0.01.
** 1. *vs* 3. P < 0.01.
*** 2. *vs* 3. P < 0.01.

= 0.738, P < 0.001).

Inter-group reclassification overall analysis is displayed in an overlaid histogram in Fig. 1.

Table 2 shows inter-group reclassification between vPRAt and vPRAc and 87 (55%) patients remained in the same interval group. Reclassification from vPRAt to one-degree lower group by vPRAc occurred in 53 (34%) patients and 17 (11%) were reclassified from vPRAt to more than one-degree lower group by vPRAc. Also, 28 (18%) patients were reclassified to non-HS when considering only the current sera in vPRA calculation. Kappa and gamma correlation values between vPRAt *vs.* vPRAc were 0.383 (P < 0.001) and 0.831 (P < 0.001), respectively.

When comparing vPRAt and vPRAe, as expected, the inter-group movement is higher remaining only 33 (21%) patients in the same interval group (Table 3). Reclassification occurred for 124 patients, 69 (44%) from vPRAt to one-degree lower group by vPRAe and 55 (35%) from vPRAt to more than one-degree lower group by vPRAe. In fact, with eplet based vPRAe, 66 (42%) would be reclassified as non-HS. Kappa and gamma correlation values between vPRAt *vs.* vPRAe were 0.049 (P = 0.116) and 0.637 (P < 0.001), respectively.

Inter-group movement between current allosensitization calculated with vPRAc and vPRAe is lower with 75 (48%) patients remaining in the same interval group (Table 4). Reclassification occurs for 56 (36%) from vPRAc to one-degree lower group by vPRAe and 24 (15%) from vPRAc to more than one-degree lower group by vPRAe. Only 25% of the patients ($n = 39$) were reclassified to non-HS. It is noteworthy that 2 (1%) patients were reclassified from vPRAc to one degree higher by vPRAe. Kappa and gamma correlation values between vPRAc *vs.* vPRAe were 0.319 (P < 0.001) and 0.809 (P < 0.001), respectively.

3.3. Estimated number of match runs needed to find an acceptable donor

Median and median change between current and eplet vPRA calculations, considering vPRAt interval groups, were significantly different (P < 0.001) (Table 5).

The median estimated number of match runs (eMR) needed for 95% probability of finding an acceptable donor by vPRAt intervals are significantly different for vPRAc and vPRAe (P < 0.001). Furthermore, also the percentage of change in eMR by vPRAt to vPRAe was significantly more pronounced by increasing vPRAt intervals (P < 0.001). This percentage of change of eMR was not so pronounced between vPRAc to vPRAe (P = 0.045) and was not observed for the percentage change of eMR from vPRAt to vPRAc (P = 0.899) (Fig. 2).

3.4. Comparison between vPRA values, according to sensitizing events and dialysis vintage time

Total vPRAt median change to current or eplet vPRA, vPRAc and vPRAe, according to previous sensitizing events such as transfusions, pregnancies and transplants, vintage dialysis and PRA-CDC is showed in Table 6. In the full cohort, median reduction from vPRAt to vPRAe was significantly less pronounced in candidates to retransplant (P < 0.001)

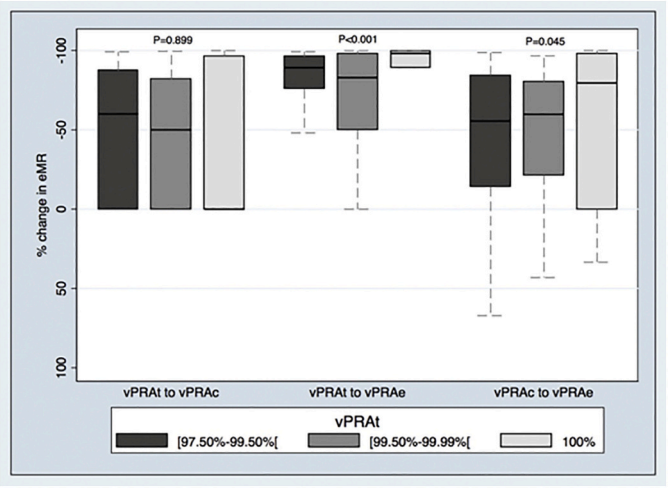

Fig. 2. Comparisons of percentage of change in estimated number of match runs (eMR) needed for 95% probability of finding an acceptable donor.

Table 6
Comparison between vPRAt change to vPRAc and vPRAe, according to sensitizing events and dialysis vintage over 10 years.

Full cohort ($n = 157$)				
	vPRAt to vPRAc change (%), median (IQR)	P	vPRAt to vPRAe change (%), median (IQR)	P
Previous transfusions		0.705		0.251
No	−0.09 (−1.32–0)		−3.27 [−7.82-(−0.27)]	
Yes	−0.09 (−0.73–0)		−1.09 [−6.09-(−0.18)]	
Previous pregnancy ($n = 87$)		0.725		0.491
No	−0.09 (−1.64–0)		−0.73 [−7.82-(−0.09)]	
Yes	−0.09 (−1.14–0)		−1.77 [−6.36-(−0.23)]	
Previous transplant		0.044		**<0.001**
No	−0.09 (−2.55–0)		−4.91 [−16.82-(−1.18)]	
Yes	−0.05 (−0.73–0)		−0.68 [−4.64-(−0.18)]	
Dialysis vintage ≥10 years		0.121		**<0.001**
No	−0.09 (−1.64–0)		−2.95 [−9.18-(−0.45)]	
Yes	−0.01 (−0.46–0)		−0.27 [−1.55-(−0.09)]	
PRA-CDC ≥50%		**0.005**		**0.002**
No	−0.18 (−1.09–0)		−2.18 [−8.73-(−0.27)]	
Yes	0 (−0.18–0)		−0.55 [−1.36-(−0.09)]	
vPRAt [97.50%–99.99%]				
	vPRAt to vPRAc change (%), median (IQR)	P	vPRAt to vPRAe change (%), median (IQR)	P
Previous transfusions		0.634		0.186
No	−0.27 [−2.55-(−0.01)]		−4.91 [−11.09-(−1.82)]	
Yes	−0.18 (−2.91–0)		−2.00 [−12.55-(−0.27)]	
Previous pregnancy ($n = 38$)		0.398		0.657
No	−0.82 (−9.55–0)		−7.18 [−16.82-(−0.36)]	
Yes	−0.09 (−2.91–0)		−2.91 [−9.27-(−0.36)]	
Previous transplant		0.444		0.100
No	−0.27 [−4.09-(−0.01)]		−5.14 [−16.82-(−1.73)]	
Yes	−0.23 (−1.91–0)		−2.27 [−11.09-(−0.27)]	
Dialysis vintage ≥10 years		0.911		**0.008**
No	−0.23 (−3.91–0)		−4.86 [−14.18-(−1.14)]	
Yes	−0.27 (−1.59–0)		−0.50 [−7.59-(−0.09)]	
PRA-CDC ≥50%		0.097		0.019
No	−0.27 (−4.09–0)		−4.45 [−15.45-(−0.36)]	
Yes	−0.01 (−0.82–0)		−0.64 [−1.46-(−0.18)]	
vPRAt = 100%				
	vPRAt to vPRAc change (%), median (IQR)	P	vPRAt to vPRAe change (%), median (IQR)	P
Previous transfusions		0.283		0.310
No	0 (−0.09–0)		−0.36 (−2.91–0)	
Yes	0 (−0.27–0)		−0.55 [−2.64-(−0.18)]	
Previous pregnancy ($n = 49$)		0.986		0.283
No	0 (−0.41–0)		−0.18 [−1.68-(−0.05)]	
Yes	0 (−0.27–0)		−0.64 [−3.46-(−0.09)]	
Previous transplant		0.255		0.010
No	−0.09 (−0.27–0)		−3.46 [−24.73-(−0.36)]	
Yes	0 (−0.18–0)		−0.46 [−1.73-(−0.09)]	
Dialysis vintage ≥10 years		0.348		**0.005**
No	−0.05 (−0.27–0)		−1.64 [−5.46-(−0.27)]	
Yes	0 (−0.18–0)		−0.27 [−0.91-(−0.09)]	
PRA-CDC ≥50%		0.283		0.523
No	0 (−0.36–0)		−0.55 [−2.91-(−0.18)]	
Yes	0 (−0.09–0)		−0.50 [−1.36-(−0.09)]	

and those with dialysis vintage ≥10 years (P < 0.001) or PRA-CDC ≥50% (P = 0.002).

The same effect was observed, when considering only vPRAt = 100% patients, for retransplantation (P = 0.010) and dialysis vintage ≥10 years (P = 0.005), or, in the remaining cohort (vPRAt [97.50%–99.99%]), for dialysis vintage ≥10 years (P = 0.008) and PRA-CDC ≥50% (P = 0.019).

3.5. Comparison of percentage of change in estimated number of match runs (eMR)

The comparison of percentage of change in eMR, needed for 95% probability of finding an acceptable donor, from vPRAt to vPRAc or vPRAe is showed in Table 7. Considering all cohort, the percentage of change in eMR was significantly less pronounced in candidates with dialysis vintage ≥10 years (P = 0.049). This was also observed for patients with 100% of vPRA (P = 0.005) and for the remaining cohort, with vPRA between 97.50% and 97.99% (P = 0.015). For patients with vPRA of 100, also the percentage of change in eMR was less significant for patients with a previous transplant patients (P = 0.010) (Fig. 3).

4. Discussion

The extremely sensitive SAB assays are used worldwide as the golden standard method for UA assignment and vPRA calculation. The introduction of vPRA, replacing traditional cytotoxic PRA, resulted in overwhelming progress bringing more equity to HS patients [21]. However, vPRA can reflect variability depending on considered *loci,* serum dates and/or cut-off values. Additionally, several SAB assays technical limitations assays have been described, such as false positive reactions, with an important impact in vPRA values and transplantability [22].

Total virtual PRA is used in Portuguese allocation algorithm to assign UA for all candidates in the waiting list fot a kidney transplant. This conservative strategy was already described to be inefficient for transplanting highly-sensitized patients that are accumulating in the waitlist with extremely prolonged waiting times [16]. In fact, in our study 120 (76%) of HS patients are re-transplant candidates and 126 (80%)

Table 7
Comparison % of change in estimated number of match runs (eMR) needed for 95% probability of finding an acceptable donor from vPRAt to vPRAc or vPRAe.

Full cohort				
	vPRAt to vPRAc % of change in eMR (%), median (IQR)	P	vPRAt to vPRAe % of change in eMR (%), median (IQR)	P
Previous transplant		0.282		0.522
No	−50.2(−89.0–0)		−95.0 [−98.9-(−81.7)]	
Yes	−10.8 (−92.1–0)		−94.5 [−98.6-(−64.2)]	
Dialysis vintage ≥10 years		0.385		0.049
No	−53.9 (−92.6–0)		−96.3 [−99.2-(−76.6)]	
Yes	−9.0 (−91.6–0)		−94.5 [−98.4-(−50.0)]	
PRA-CDC ≥50%		0.049		0.734
No	−54.2(−93.8–0)		−94.9 [−98.7-(−75.0)]	
Yes	0 (−89.0–0)		−94.5 [−98.8-(−50.0)]	
vPRAt [97.50%–99.99%]				
	vPRAt to vPRAc % of change in eMR (%), median (IQR)	P	vPRAt to vPRAe % of change in eMR (%), median (IQR)	P
Previous transplant		0.950		0.597
No	−50.0 [−72.0-(−0.2)]		−87.5 [−96.2-(−75.2)]	
Yes	−54.1 (−87.4–0)		−85.4 [−97.3-(−57.2)]	
Dialysis vintage ≥10 years		0.941		0.015
No	−53.9 (−86.4–0)		−89.4 [−97.9-(−75.0)]	
Yes	−50.0 [−84.2-(−4.5)]		−62.6 [−94.2-(−46.4)]	
PRA-CDC ≥50%		0.051		**0.008**
No	−58.1 (−90.9–0)		−90.2 [−97.7-(−66.7)]	
Yes	−9.0 (−57.7–0)		−58.4 [−85.8-(−50.0)]	
vPRAt = 100%				
	vPRAt to vPRAc % of change in eMR (%), median (IQR)	P	vPRAt to vPRAe % of change in eMR (%), median (IQR)	P
Previous transplant		0.255		0.010
No	−89.0 (−96.3–0)		−99.7[−99.96-(−97.3)]	
Yes	0 (−94.5–0)		−97.8 [−99.4-(−89.0)]	
Dialysis vintage ≥10 years		0.348		**0.005**
No	−44.5 (−96.3–0)		−99.4 [−99.8-(−96.3)]	
Yes	0 (−94.5–0)		−96.3 [−98.9-(−89.0)]	
PRA-CDC ≥50%		0.283		0.523
No	0 (−97.3–0)		−98.2 [−99.7-(−94.5)]	
Yes	0 (−89.0–0)		−98.0 [−99.3-(−89.0)]	

patients have dialysis vintage time over five years.

Using all history of HLA specificities identified by IgG-SAB assays with 1 k cut-off to determine UA as an absolute contraindication to transplant, without performing a physical crossmatch, it's not reasonable [23]. In fact, desensitization protocols are often used to overcome humoral incompatibility for HS patients where the final goal is to achieve a window of opportunity where crossmatches reach negativity [24]. Using vPRAt for UA assignment we may be missing opportunities and preventing transplantation based on historic specificities, as it includes sera without prozone treatment [12,25,26] and tested under therapies with known interaction with SAB assays [27,28].

Our study showed that almost half of the HS patients (45%, $n = 70$) would be reclassified to a lower vPRA interval, when considering current vPRA, decreasing the number of eMR needed for 95% probability of finding a compatible donor, and hence increasing transplant probability.

However, using only the current vPRA with a fixed 1 k MFI cutoff may also be misleading and should be interpreted with caution. In fact, IgG-SAB assays are semi-quantitative with a limited number of HLA antigens *per* bead and MFI values do not reflect the antibodies titers [13]. Besides bead saturation, also antigen density is different between beads being erroneous to employ a universal cut-off for all *loci*. In fact, flow crossmatch positivity due to HLA-C and HLA-DPB1 donor specific antibodies (DSA) are associated with higher MFI values, when compared to other *loci* [29]. Furthermore, non-specific background reactivity has been described to particularly beads in specific lots, particularly in patients with autoimmune pathologies, leading to false positivity and vPRAt increment. In addition, denatured proteins on bead surface and the HLA antigen purification process can expose antibody binding epitopes that are not expressed *in vivo* [30–32]. This phenomenon has been reported as «natural antibodies» as they were first identified in unsensitized male sera [33].

The SAB assay limitations interferes with both current and total vPRA values in a flawed technique and, although vPRAc can improve results over vPRAt, we must not underestimate that absence of measurable DSAs in the current sera does not exclude the presence of memory B cells and a potential anamnestic response [34].

Notwithstanding, increasing cut-off value for UA assignment would also result in lower percentage of vPRA and increased transplant probability, but eplet analysis allowed us to introduce clinical relevance in the interpretation. Eplet-based analysis considers as acceptable HLA antigens without an explainable antibody-verified eplet or low ElliPro score. ElliPro is a prediction program based on three-dimensional structures of antigen-antibody complexes developed by Ponomarenko [35] that enables the characterization of clinically relevant eplet repertoires in HLA matching [36]. Also, this molecular approach allows the understanding of allelic specific reactivities, usually considered as antigen unacceptability, increasing acceptable antigens list. On the other hand, also false negativity could occur due to shared public eplets in multiple antigens leading to less antibody binding per bead. In this case, HLA antigens with MFI value below 1 k aren't reported as positive in vPRAc but are considered when eplet analysis in done resulting in higher vPRA.

Another important limitation of HLA class I and class II IgG-SAB assay is panel representation as it contains only 98 different HLA alleles each. Using eplet based antibody assignments allow us to identify the reactive(s) eplet(s) and subsequently identifying also non-luminex DSAs.

Eplet based vPRA granted the reclassification of 124 (79%) and 80 (51%) patients to a lower group when considering vPRAt and vPRAc to vPRAe, respectively. In fact, 66 (42%) and 39 (25%) patients would actually be reclassified as non-HS patients with vPRA lower than 98%. This was particularly significant when considering the third group of patients with 100% ($P < 0.001$).

This represented a huge impact on the percentage of change of eMR needed for 95% probability of finding an acceptable donor for the whole cohort, when considering vPRAt to vPRAe ($P < 0.001$), being less

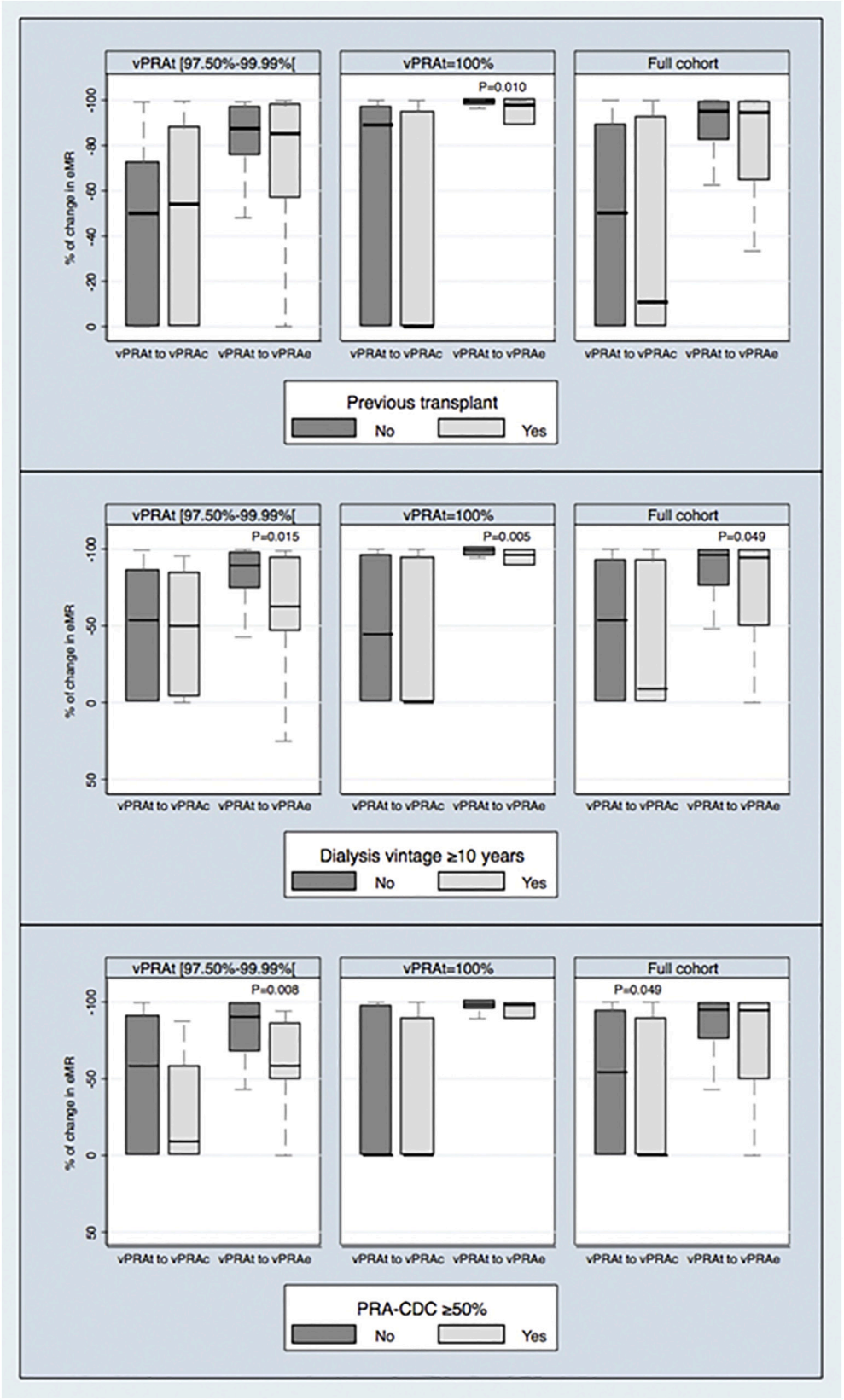

Fig. 3. Comparison % of change in estimated number of match runs (eMR) needed for 95% probability of finding an acceptable.

pronounced for vPRAc to vPRAe (P = 0.045). This was not observed in the percentage of change in eMR from vPRAt to vPRAc (P = 0.899). Our study showed also that median percentage of change in eMR from vPRAt to vPRAe was less pronounced in candidates to retransplant with 100% of vPRA (P = 0.010) and for patients with dialysis vintage ≥10 years (P = 0.049 for all cohort, P = 0.015 for vPRA = 100% and P = 0.005 for patients with vPRA between 97.50% and 97.99%). This observation reinforces the strength of vPRAe measure, which has an important decrease within first transplant candidates with lower cytotoxic PRA, and this impact is less pronounced in patients considered to be at highly immunological risk [37–39].

A limitation of our study is that, although presenting a strategy to increase donor offer and transplant probability, we did not perform cellular assays. As such, we must be aware that a proportion of described eplets lack antibody-verified status and a percentage of cellular based crossmatches could be positive and all DSAs should be included in the immunologic risk analysis.

To conclude, our study demonstrated that the use of total or current vPRA calculations are impairing HS patients, decreasing transplantability leading to dramatically longer waiting times, when compared to eplet based vPRAe. We believe that HLA antibodies identified in SAB that failed to be explained by eplet analysis, or have a low ElliPro score, should be listed as risk factors, instead of an absolute contraindication, increasing greatly donor offers, wet-crossmatch testing and ultimately transplant probability.

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Authorship

Sandra Tafulo - Conceptualization; Data curation; Methodology; Writing - original draft; Writing - review & editing.

Jorge Malheiro - Conceptualization; Formal analysis; Writing - review & editing.

Leonídio Dias - Conceptualization; Supervision.

Manuela Almeida - Conceptualization; Investigation.

La Salete Martins – Conceptualization; Investigation.

Sofia Pedroso – Conceptualization; Investigation.

Ermelinda Osório - Methodology.

Luísa Lobato – Conceptualization; Funding acquisition; Supervision.

António Castro-Henriques - Conceptualization; Supervision.

Data availability statement

The data that support the findings of this study are available from the corresponding author, Sandra Tafulo, upon reasonable request.

Disclosure

The authors declare no conflicts of interest.

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Appendix 3

Improving HLA matching in living donor kidney transplantation using kidney paired exchange program.

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Improving HLA matching in living donor kidney transplantation using kidney paired exchange program

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A B S T R A C T

Background: The inclusion of compatible pairs within kidney paired exchange programs has been described as a way to enhance these programs. Improved immunological matching for the recipient in compatible pair has been described to be a possible benefit.
Methods: The main purpose of our study was to determine if the introduction of compatible pairs in the Portuguese kidney paired exchange program would result in a better match for these patients, but also to assess if this strategy would increase the number of incompatible pairs with a possible match.

We included 17 compatible pairs in kidney paired exchange pool of 35 pairs and performed an *in-silico* simulation determining HLA eplet mismatch load between the co-registered and matched pairs using HLA MatchMaker, version 3.0.
Results: Our study showed that the inclusion of fully HLA-A, -B, −DR mismatched compatible pairs within the national Portuguese KEP increased matched rate within ICP (0.71%) and improved HLA eplet matching within compatible pairs. 16 of 17 (94.12%) of the CP obtained one or more transplants possibilities and 13 (81.25%) would have been transplanted with significantly lower HLA class I and class II total and antibody-verified eplet mismatch load (83.9 ± 16.9 *vs.* 59.8 ± 12.2, $P = .002$ and 30.1 ± 5.5 *vs.* 21.2 ± 3.0, $P = .003$, respectively).
Conclusions: This strategy is a viable alternative for compatible pairs seeking a better matched kidney and Portuguese KEP program should allow them this possibility.

1. Introduction

The incidence of end-stage renal disease (ESRD) is increasing worldwide and kidney transplantation (KT) is the best treatment for renal replacement therapy. This culminated in a profound imbalance with an increasing gap between organ supply and demand. Several strategies have been implemented in order to overcome organ shortage, such as expanded criteria of deceased donors [1,2], donation after circulatory-death [3] and living donation [4,5].

The first successful living donor transplant was performed in 1954 between monozygotic twins [6] and, since then, multiple studies aimed to assess beneficial *versus* medical risks [7,8]. Furthermore, international guidelines recommendations to assist medical professionals in the living donation process, were published [9]. However, it is undoubtable that living donation is a superior alternative when compared to deceased donation, because it has been associated with better long-term of patient and graft survival rates, especially due to decease donor characteristics [10,11]. Also, longer times on pre-transplantation dialysis therapy are associated with poorer outcomes following transplantation [12–14].

Until recently, direct living donors were rejected due to blood type (ABOi) or HLA incompatibility (HLAi) but this immunological barrier can now, in some extent, be surpassed with desensitization protocols [15–18]. A noteworthy alternative to transplant these incompatible

Abbreviation: ABOi, ABO incompatibility; AbVerEp, antibody-verified eplets; CP, compatible pairs; DSA, donor specific antibodies; EptMM, total eplet mismatch load; EptMM-I, HLA class I total eplet mismatch; EptMM-II, HLA class II total eplet mismatch; EpvMM, antibody-verified eplet mismatch load; ESRD, end-stage renal disease; HLAi, HLA incompatibility; ICP, incompatible pairs; KEP, kidney exchange programs; KT, kidney transplantation; MFI, Mean fluorescence intensity; SAB, single antigen bead; vPRA, virtual panel reactive antibodies

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pairs (ICP), with lower risk and without the need of expensive desensitization, prolonged hospitalizations and early readmissions [19–21], are kidney exchange programs (KEP). The concept was introduced in 1986 by Felix Rapaport [22] and currently the success of KEP worldwide is unquestionable [23–28]. However, these programs are not able to present solutions for every ICP, and also struggle to find new solutions, such as participation in wider international programs [29], the use of desensitization programs within KEP [30] and the introduction of compatible pairs (CP) [31,32]. Also, the close communication between histocompatibility laboratory and clinic is of extreme importance in order to correctly define unacceptable mismatches [33].

Portugal implemented a national KEP almost ten years ago (Portuguese Ordinance n° 802/2010, of August 23) and, as a relatively small country, the median number of ICP is limited and the program lacks efficiency.

The main purpose of our study was to determine if the introduction of CP in the Portuguese KEP program would result in a better match for these patients, but also to assess if this strategy would increase the number of ICP with a possible match.

2. Materials & methods

2.1. Donor and recipient pairs

Each donor-recipient pair is considered compatible if there is no insurmountable HLA or ABO allosensitization against the donor. HLAi is assessed considering all patient history, cellular and solid-phase assays and, for ABOi, we consider anti-A/B isoagglutinin IgG titles below 512. As such, incompatible pairs are considered untransplantable and are proposed to integrate the Portuguese national KEP. The program includes five transplantation units and performs quarterly match cycles with ICP, ABOi or HLAi. The compatibility is determined by scoring four characteristics: a) blood group, identical blood group pairs (30 points) *versus* compatible blood groups (0 points); b) compatibility probability, calculated of each pair as described by Keizer *et al* [34] adding 30 points if lower than 25%, 20 points if between 25 and 50%, 10 points if between 50 and 75% and 0 points if higher than 75%; c) age difference, with 10 points if the difference is less than 20 years; d) time in renal replacement therapy, scoring 0.05 points for each month.

To assess the effect of inclusion of CP we used the Portuguese national KEP pool from October 2019 that consisted of thirty five ICP. The CP included were transplanted directly in Hospital Santo António – Centro Hospitalar do Porto from 2009 and 2017 and were fully mismatched for HLA-A, -B, −DR *loci*. One pair (CP7) was ABOi and two pairs (CP8 and CP15) were HLAi, requiring desensitization protocols to be overcome the incompatibility and be transplanted.

2.2. HLA eplet mismatch

HLA intermediate resolution typing for HLA-A,-B, −C, −DRβ1 and DQα1/β1 *loci* was performed for all pairs using reverse sequence-specific oligonucleotide (LABType® SSO typing kits, One Lambda, Canoga Park, CA, USA). HLA allelic typing was determined as previously described [35].

HLA eplet mismatch was calculated for each CP pair using HLAMatchmaker, version v3.0, which included all antibody-verified eplets (AbVerEp) in the registry until November 1, 2019 (http://www.epregistry.com.br). A better match was defined when CP would have at least one matching possibility with lower HLA class I and II eplet mismatch load.

2.3. Allocation matching

The allocation was performed with the 35 ICP only and, afterwards, match cycles simulations were performed including each of the selected CP. Unacceptable antigens were determined by single antigen bead (SAB) IgG assays (LABScreen® Single Antigen kit, OneLambda, Canoga Park, CA, USA). Mean fluorescence intensity (MFI) of each bead was measured using LABScan™100 flow analyzer (Luminex®, Austin, TX, USA). Positive reaction threshold was considered a MFI value of ≥1000 and virtual panel reactive antibodies (vPRA) was calculated using Eurotransplant calculated PRA (ETRL HLA database version 2.0, https://www.etrl.org/Virtual%20PRA/). Only ABO compatible matches were considered.

2.4. Comparison of estimated probability of graft failure between CPD and KPD

Comparison of estimated graft failure (GF), at 5 and 10 years, between the original compatible pair donor (CPD) and kidney paired donor (KPD) was performed using the calculator described by Ashby et al. [36], available online (https://kecc.shinyapps.io/SurvivalCalculator/). The recipient characteristics include age, gender, race, height (cm) and weight (kg), time on dialysis, blood type, history of diabetes and hepatitis C, previous transplants and vPRA. Insurance status is public primary payer for all patients that don't have history of diabetes. Donor's characteristics include age, gender, relation to recipient (unrelated, first or second degree), race, blood type, height (cm) and weight (kg), HLA mismatches (0–6) and HLA-DR (0–2) antigen mismatches and history of smoking history.

3. Results

3.1. Kidney paired exchange incompatible pairs

The Portuguese KEP pool of ICP included 17 ABOi, 17 HLAi and one pair with both types of incompatibility ($n = 35$). This group is characterized by blood group unbalance with 25.7% O, 60.0% A, 8.6% B and 5.7% of AB blood type. Besides disproportion of blood group, the extremely elevated degree of allosensitization worsens transplant probability among this group. The median vPRA was 59.29%, with 21 (60%) patients with vPRA higher than 50% and 16 (45.7%) with vPRA higher than 95%. Regarding gender the differences are smoother with 21 (58.3%) female potential donors and 17 (47.2%) female recipients.

3.2. Compatible pairs

The characteristics of CP included in the match simulations are described in Table 1. Donor's blood type was mainly O (64.7%) while recipient's were predominant A blood type (58.8%).

Although all CP were equally mismatched, with six HLA antigen mismatches, the total number of HLA class I eplet mismatch (EptMM-I) from recipients of CP ranged from 26 to 74, and 19 to 85 for HLA class II eplet mismatch load (EptMM-II).

3.3. Match simulation without compatible pairs

The match performed without the inclusion of CP identified nine possible transplants between ICP in 4-way, 3-way and a 2-way loop. Transplanted patients characteristics are described in Table 1. This match allowed the transplantation of seven (77.8%) patients with vPRA higher than 50%, three of them being higher than 95%.

3.4. Match simulation with compatible pairs

In the simulation match cycle with 35 ICP, 16 out of 17 (94.1%) CP had at least one cross-over kidney transplant possibility and 13 out of 16 (81.25%) patients succeed to find a better HLA eplet matched donor (81.4 ± 17.6 *vs.* 64.4 ± 19.5; $P = .007$) – Fig. 1. Considering the results, the comparison of eplet mismatch load, total (EptMM) and antibody-verified (EpvMM), between cross-over kidney donors that would be considered ($n = 13$) and CP original donor, are presented in

Table 1
Characteristics of the pairs included in match simulation of the Portuguese kidney exchange program.

Pair-ID	Blood group		Gender		Virtual	HLA class I EpMM		HLA class II EpMM	
	D	R	D	R	vPRA	AbVer	Other	AbVer	Other
Compatible pairs transplanted directly (n = 17)									
CP1	O	O	F	M	0,00	17	29	23	27
CP2	O	A	F	M	0,00	12	24	15	3
CP3	O	O	F	M	0,00	20	35	12	15
CP4	O	A	F	M	0,00	15	40	21	51
CP5	A	A	F	M	0,00	16	27	10	24
CP6	O	O	F	F	15,79	22	52	6	13
CP7[a]	A	O	F	M	0,00	10	31	22	32
CP8[b]	O	O	M	F	21,12	16	41	15	26
CP9	O	A	M	F	0,00	13	15	11	14
CP10	O	O	M	F	0,00	21	31	18	25
CP11	A	A	F	M	0,00	17	32	15	19
CP12	A	A	F	M	78,15	13	29	6	12
CP13	A	A	F	M	6,870	14	22	12	20
CP14	O	A	F	M	0,00	14	25	11	19
CP15[b]	A	A	F	M	55,66	16	33	12	21
CP16	O	O	F	M	0,00	10	23	11	23
CP17	O	A	M	F	11,63	9	17	12	20
Incompatible pairs with at least one match possibility without CP inclusion (n = 9)									
2-way match run									
ICP 1	A	O	F	M	56,16				
ICP 2	O	A	F	F	99,26				
3-way match run									
ICP 3	A	A	M	F	99,75				
ICP 4	A	O	M	F	99,48				
ICP 5	O	A	F	M	9,11				
4-way match run									
ICP 6	O	A	F	F	49,16				
ICP 7	O	O	M	F	89,77				
ICP 8	A	A	F	F	52,79				
ICP 9	A	O	F	M	4,92				
Additional Incompatible pairs with at least one match possibility with CP simulation (n = 7)									
HLA incompatibility									
ICP 10	O	B	F	M	99,88				
ICP 11	A	AB	F	M	97,44				
ICP 12	A	A	F	M	100,00				
AB0 incompatibility									
ICP 13	A	O	M	M	22,99				
ICP 14	A	O	F	M	0,00				
ICP 15	A	O	F	M	0,00				
ICP 16	A	O	F	M	0,00				
Incompatible pairs with no match possibility with CP simulation (n = 19)									
HLA incompatibility									
ICP 17	AB	AB	M	F	99,56				
ICP 18	O	A	M	F	100,00				
ICP 19	A	A	F	M	100,00				
ICP 20	O	O	F	M	84,82				
ICP 21	A	A	M	F	99,97				
ICP 22	O	O	F	F	99,99				
ICP 23	O	O	M	M	99,55				
ICP 24	B	B	F	F	98,39				
AB0 incompatibility									
ICP 25	A	O	M	F	98,65				
ICP 26	A	O	M	F	99,99				
ICP 27	AB	B	F	M	1,79				
ICP 28	A	B	M	F	99,35				
ICP 29	A	O	M	F	10,74				
ICP 30	B	A	F	M	0,00				
ICP 31	A	O	F	M	0,00				
ICP 32	B	O	M	F	30,99				
ICP 33	A	O	F	M	7,39				
ICP 34	A	O	F	M	6,61				
AB0 and HLA incompatibility									
ICP 35	A	O	F	F	56,49				

[a] ABOi

[b] HLAi; CP, compatible pair; D, donor; R, recipient; F, feminine; M, masculine; vPRA, virtual panel reactive antibodies; HLA, Human Leucocyte antigens; EpMM, eplet mismatches; AbVer, antibody-verified; Other, no antibody-verified eplets.

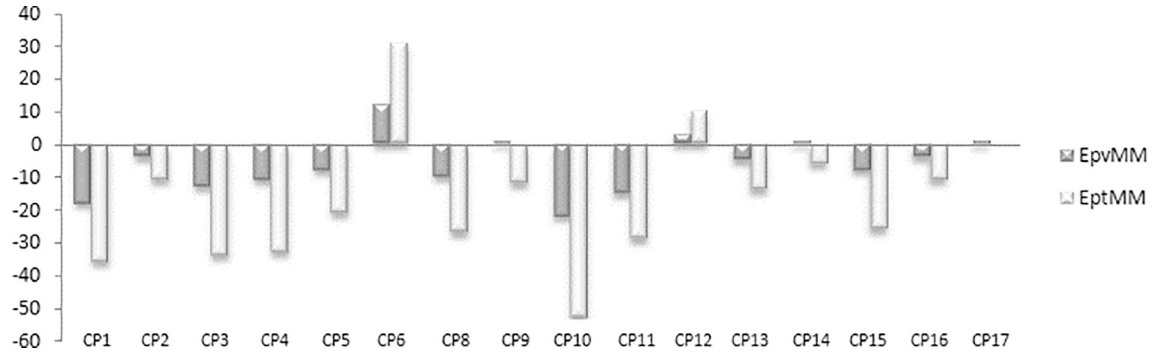

Fig. 1. Change in HLA eplet mismatch load, antibody-verified (grey) and total (white) in CP with a matched donor within Portuguese KEP, compared the original donor.

Table 2.

HLA class I and class II EptMM and EpvMM was significantly different between CP and KEP donors (83.9 ± 16.9 *vs.* 83.9 ± 16.9, $P = .002$ and 30.1 ± 5.5 *vs.* 21.2 ± 3.0, $P = .003$, respectively). This difference remained statistically significant if HLA class I (45.8 ± 8.1 *vs.* 32.2 ± 9.7, $P = .002$ and 15.5 ± 3.0 *vs.* 10.9 ± 3.5, $P = .003$, respectively) and class II (38.1 ± 12.2 *vs.* 27.7 ± 12.8, $P = .016$ and 15.3 ± 4.0 *vs.* 9.7 ± 4.7, $P = .004$, respectively) were analyzed independently.

We found no difference between age (49.8 ± 9.9 *vs.* 48.5 ± 7.8, $P = .674$) and gender of the donor (10 *vs.* 8 female donors, $P = .727$) in both groups.

3.5. Matching rate with compatible pairs

Considering the inclusion of the 13 CP that would benefit by entering this program we observed an increased transplantation rate with 7 additional patients with a matching possibility in the same ICP pool (Table 1).

3.6. Comparison of estimated probability of graft failure between CPD and KPD

Table 3 summarizes the characteristics and estimated probability of GF, at 5 and 10 years, for the thirteen patients considered for KEP by HLA eplet matching analysis, with CPD and KPD. This estimated kidney graft failure calculator showed that nine patients (69,2%) would benefit from KEP donor, when compared to the original donor.

4. Discussion

Our study shows that the inclusion of fully mismatches CP with national Portuguese KEP increased matched rate within ICP. This was expected as we observed an improved balance for ABO blood groups

Table 2
Comparison of eplet mismatch load between compatible and simulation pairs, obtained after match in Portuguese kidney exchange program.

	CPD ($N = 13$) Mean ± SD	KPD (N = 13) Mean ± SD	Mean of difference (95% CI)	*P*	Pos ↑ Zero = Neg ↓
Ept_I + II	83.9 ± 16.9	59.8 ± 12.2	−24.1 (−32.1; −16.0)	0.002	0 0 13
Epv_I + II	30.1 ± 5.5	21.2 ± 3.0	−8.9 (−13.1; −4.7)	0.003	2 0 11
Ept_I	45.8 ± 8.1	32.2 ± 9.7	−13.7 (−19.1; −8.3)	0.002	0 1 12
Epv_I	15.5 ± 3.0	10.9 ± 3.5	−4.5 (−6.9; −2.2)	0.003	1 1 11
Ept_II	38.1 ± 12.2	27.7 ± 12.8	−10.4 (−17.9; −2.9)	0.016	2 0 11
Epv_II	15.3 ± 4.0	9.7 ± 4.7	−5.5 (−9.0; −2.1)	0.004	0 1 10
Donor age	49.8 ± 9.9	48.5 ± 7.8	−1.2 (−10.2; 7.7)	0.674	6 1 6
Female donor, n (%)	10 (77)	8 (62)	–	0.727	–

CPD, compatible pair donor; KPD, kidney exchange program donor; Ept, total eplet mismatch load; Epv, antibody-verified eplet mismatch load; I, HLA class I; II, HLA class II; I + II, HLA class I and class II; ↑, number of patients with increased value of Ep or age, considering KPD versus CPD; =, number of patients with equal value of Ep or age, considering KPD *versus* CPD; ↓, number of patients with decreased value of Ep or age, considering KPD *versus* CPD.

Table 3
Comparison of estimated graft failure between compatible pair donors and kidney paired donors, obtained after match in Portuguese kidney exchange program, using the online calculator described by Ashby et al. [36].

Recipient								Compatible paired donor *vs.* Kidney Paired Donor									
#	Age (Y)	Gender	Height (cm)	Weight (Kg)	ABO	TD (Y)	vPRA (%)	Age (Y)	Gender	Height (cm)	Weight (Kg)	ABO	HLA_MM (ABDR/DR)	Smoking history	GF_5Y (%)	GF_10Y (%)	Best Donor*
R1	62	M	175	84	O	< 1Y	0,00	49	F	157	70	O	6 / 2	No	18,7	51,7	
								46	F	154	66	O	3 / 1	No	16,7	46,4	*
R2	56	M	180	74	A	< 1Y	0,00	59	F	162	79	O	6 / 2	No	17.0	40.7	
								52	M	172	62	A	5 / 1	Yes	11.9	37.1	*
R3	49	M	166	74	O	1-2Y	0,00	58	F	150	64	O	6 / 2	No	18.3	43.3	
								42	M	163	66	O	3 / 1	Yes	11.4	32.1	*
R4	38	M	160	68	A	< 1Y	0,00	35	F	150	53	O	6 / 2	No	11.7	28.1	*
								52	M	172	62	A	5 / 2	Yes	12.7	34.7	
R5	37	M	169	69	A	1-2Y	0,00	60	F	150	57	A	6 / 2	No	18.0	42.8	
								39	F	165	76	A	5 / 1	No	12.9	31.0	*
R8	42	F	163	58	O	< 1Y	21,12	46	M	169	68	O	6 / 2	No	11.7	30.0	*
								47	F	152	69	O	5 / 2	No	12.6	30.9	
R9	39	F	165	79	A	1-2Y	0,00	40	M	167	64	O	6 / 2	No	13,6	31,9	*
								41	F	157	80	A	4 / 2	No	13,8	31,3	
R10	23	F	169	50	O	2-3Y	0,00	37	M	173	100	O	6 / 2	Yes	24.6	58.9	
								48	F	152	69	O	3 / 0	No	20.4	42.9	*
R11	59	M	165	67	A	2-3Y	0,00	57	F	158	55	A	6 / 2	No	16.2	42.9	
								57	M	172	62	A	4 / 0	Yes	13.7	38.1	*
R13	63	M	166	68	A	1-2Y	6,87	62	F	157	60	A	6 / 2	No	20,5	53,2	
								43	F	157	80	A	3 / 1	No	18,2	50,8	*
R14	64	M	168	76	A	< 1Y	0,00	55	F	158	90	O	6 / 2	No	18,5	51,0	
								43	F	157	80	A	5 / 1	No	16,5	46,9	*
R15	36	M	174	79	A	< 1Y	55,66	36	F	167	63	A	6 / 2	No	11.9	30.1	*
								42	F	161	51	A	5 / 1	No	14.8	33.0	
R16	48	M	191	82	O	< 1Y	0,00	53	F	165	63	O	6 / 2	Yes	13,3	37,6	
								54	F	154	66	O	6 / 2	No	12,9	33,6	*

Y, years; TD, time on dialysis; vPRA, virtual panel reactive antibodies; HLA_MM, HLA antigen mismatches; GF_5Y, percentage of graft failure within 5 years with the donor; GF_10Y, percentage of graft failure within 10 years with the donor.

and vPRA. In fact, allosensitization degree of Portuguese patients is very high, aggravated by the lack of a national program for highly sensitized patients and, together with O blood type imbalance, a well-known problem [37], results in lower transplantability among these patients [38,39]. This problem is reflected within Portuguese KEP making extremely difficult to obtain a compatible match, revealed by the low transplantability of this program (median of 2.67 transplants per year). We believe it is very promising to understand that this strategy would help antagonize this inequity.

Furthermore, both HLAi pairs that were desensitized to be transplanted directly and included in the CP sub-cohort, would find a compatible match without donor specific antibodies (DSA), sparing desensitization. On the other hand, the matching simulation did not find a matched pair for ABOi pair, CP7 desensitized and transplanted directly. This is not surprising as this patient was blood type O and these donors were in minority (25.7%) within ICP in KEP. In fact, ABOi barrier can be crossed with outcomes equivalent to ABO-compatible transplantation. Several groups have described that acceptance of ABOi matching significantly enhances transplant rates in KEP [40].

However, to include CP in KEP we must assure benefit and that was the main reason why HLA mismatching was assessed using the HLA eplets, rather than traditional HLA antigens, as this has been demonstrated to be a more accurate strategy [35,41]. Our study showed that, in the pool of 35 ICP, 16 CP (94.1%) obtained one or more transplants possibility within the program, of which 13 (81.25%) were able to be transplanted with a better matched donor considering HLA class I and class II eplet mismatch load. The introduction of CP within KEP to seek immunological benefit has been described by other groups [42,43]. Beyond that, also age, gender and size improved matching could be a reciprocal benefit offer, as these factors also affects transplant outcome [44,45]. Massie AB et al. developed the living kidney donor profile index (LKDPI) score that allows comparison with kidney donor profile index (KDPI) for deceased donors [46] and Ashby et al. developed an online calculator of estimated graft failure for living donors [36]. This calculator allows the comparison of HLA antigen, age, gender and body size mismatches and 9 patients, considering the 13 patients with a better HLA eplet match within KEP, would benefit to be transplanted with KPD with a lower probability of GF at 5 and 10 years.

To the best of our knowledge, this is the first study based on antibody-verified eplets using the latest HLAmatchmaker version, a strategy proved to be particularly useful to prevent both anamnestic responses and allosensitization in kidney transplantation [47].

The major limitation of our study simulation is the absence of high-resolution typing for HLA eplet mismatch. However, Fidler *et al* have shown that, in a predominantly Caucasian cohort, two-digit alleles converted to four-digit alleles reliably calculate the number of eplet mismatches at both class I and II loci compared to four-digit molecular HLA typing method [48]. Besides, the negative virtual crossmatch obtained for these pairs does not consider HLA-DPB *loci* that could result in a positive cell crossmatch.

Although some studies raise some ethical issues and not all CP would approve their participation in KEP [49], we believe that the altruistic nature of living donors [50], allied to prove increasing benefit to the intended recipient, would also be a favorable factor in the decision of CP to participate in KEP [51,52].

We believe it is our duty to offer the best option for each patient and the inclusion of HLA mismatched CP within KEP allocation matching, in order to seek for alternative better-matched donors, would allow the clinical team to evaluate all possibilities and provide a better medical advice.

Disclosure

The authors declare no conflicts of interest.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, [Sandra Tafulo], upon reasonable request.

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Appendix 4

HLA class II antibody-verified eplet mismatch load improves prediction of *dn*DSA development after living donor kidney transplantation.

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ORIGINAL ARTICLE

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HLA class II eplet mismatch load improves prediction of *dn*DSA development after living donor kidney transplantation

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Abstract

HLA donor-specific antibodies developed de novo after transplant remain a major cause of chronic allograft dysfunction. Our study main purpose was to determine whether HLA MM, assessed traditionally and by HLA total and AbVer eplet mismatch load (EptMM and EpvMM) assessed with HLAMatchMaker, had impact on *dn*DSA development after living donor kidney transplantation (LDKT). We retrospectively analysed a cohort of 96 LDKT between 2008 and 2017 performed in Hospital Santo António. Seven patients developed *dn*DSA-II and EpvMM and EptMM were greater in *dn*DSA-II group compared to the no *dn*DSA-II (18.0 ± 8.7 versus 9.9 ± 7.9, $p = .041$ and 41.3 ± 18.9 versus 23.1 ± 16.7, $p = .018$), which is not observed for AgMM (2.29 versus 1.56; $p = .09$). In a multivariate analysis, we found that preformed DSA (HR = 7.983; $p = .023$), living unrelated donors (HR = 8.052; $p = .024$) and retransplantation (HR = 14.393; $p = .009$) were predictors for *dn*DSA-II (AUC = 0.801; 0.622–0.981). HLA-II EpvMM (HR = 1.105; $p = .028$; AUC = 0.856) showed to be a superior predictor of *dn*DSA-II, when compared to AgMM (HR = 1.740; $p = .113$; AUC = 0.783), when adjusted for these clinical variables. Graft survival was significantly lower within *dn*DSA-II patient group (36% versus 88%, $p < .001$). HLA molecular mismatch analysis is extremely important to minimize risk for HLA-II *dn*DSA development improving outcome and increasing chance of retransplant lowering allosensitization.

KEYWORDS

antibodies, histocompatibility, HLA, immune response, Immunology, matching, medicine, transplantation

1 | INTRODUCTION

Human Leukocyte antigens (HLA) donor-specific antibodies (DSA) developed de novo after transplant, in particular against HLA class II, remain a major cause of chronic allograft dysfunction (Terasaki & Cai, 2008; Wiebe et al., 2012). HLA mismatching is a well-known risk factor for *de novo* DSA (*dn*DSA) (Opelz & Döhler, 2013), alongside with early T cell-mediated rejection, young age and inadequate immunosuppression due to minimization or nonadherence.

HLA mismatching traditionally is determined considering the donors HLA molecules not shared by the recipient. However, after the development of HLAMatchmaker by Rene Duquesnoy (Duquesnoy, 2002), several authors have been demonstrating the importance of matching at the epitope level (Dankers et al., 2004;

Duquesnoy et al., 2003; Silva et al., 2010; Wiebe et al., 2015), particularly HLA class II eplet mismatch load (Kishikawa et al., 2018; Wiebe et al., 2013).

HLA Matchmaker is a theoretical algorithm that compares donor and recipient HLA molecules at the structural level to infer the number of mismatched functional epitopes, named eplets (Duquesnoy, 2006). Eplets are small configurations of polymorphic amino acid residues on the HLA molecular surface and are classified as antibody-verified (AbVer) when verified as targets of DSA. The total eplet mismatch load (EptMM) includes AbVer eplet mismatches (EpvMM) and eplets which have not yet been antibody-verified experimentally, named nonverified eplets.

Hence, our study main purpose was to determine whether HLA mismatches, assessed traditionally considering the HLA molecule and by HLA AbVer and total eplet mismatch (EpvMM and EptMM) load assessed with HLAMatchMaker, had impact on *dn*DSA development after living donor kidney transplantation (LDKT).

2 | MATERIALS & METHODS

2.1 | Patient population

In this study, we retrospectively analysed a cohort of 210 LDKT between 1 January 2008 and 31 December 2017 performed in Hospital de Santo António—Centro Hospitalar Universitário do Porto. Patients presenting post-transplant anti-HLA antibodies assessment performed with single-antigen bead (SAB) assays were considered, defining the 96 LDKT recipients as the studied cohort. At transplant, all patients had a negative T- and B-lymphocyte cytotoxic crossmatch (standard NIH technique, not enhanced with anti-human globulin) in current sera.

Median follow-up after transplant was 52.4 (33.7–77.7) months.

The Institutional Review Board at Centro Hospitalar do Porto approved this study.

2.2 | HLA typing and mismatch determination

HLA intermediate resolution typing for HLA-A, HLA-B, HLA-C, HLA-DRB1 and DQA1/DQB1 *loci* was performed for all pairs using reverse sequence-specific oligonucleotide (LABType® SSO typing kits, One Lambda). HLA allelic typing for HLA-A, HLA-B, HLA-C, HLA-DRB1/3/4/5 and HLA-DQA1/B1 loci was assigned based on the observed National Marrow Donor Program (NMDP) code, linkage disequilibrium and the most probable phase genotype for Caucasian population frequencies using HaploStats (available via http://www.haplostats.org/) and HLA eplet mismatch load, total and antibody-verified eplets, was calculated using HLAMatchmaker software, version 2.0. HLA antigen mismatch analysis was performed traditionally by counting HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 broad and split HLA antigens.

2.3 | HLA antibody and DSA assignment

HLA antibodies were assessed on ethylenediaminetetraacetic acid (EDTA) treated sera collected pre- and post-transplant using IgG-SAB assay based on Luminex Xmap® Technology (LABScreen® Single Antigen kit, OneLambda). Mean fluorescence intensity (MFI) of each bead was measured using LABScan™100 flow analyzer (Luminex®). Positive reaction threshold for was considered a MFI value of ≥1,000.

2.4 | Rejection diagnosis and treatment

Kidney graft rejection was defined as biopsy-proven, classified according to Banff '17 classification (Haas et al., 2018). Antibody-mediated rejection (AMR) was treated with pulse steroids, intravenous immunoglobulin 2 g/Kg (maximum 140 g) divided into 2–4 doses associated with plasmapheresis (at least 3–5 sessions) and rituximab (single-dose of 375 mg/m^2). Patients with *dn*DSA emergence but without signs of graft dysfunction received no specific treatment, besides optimization of tacrolimus (trough level 8–10 ng/ml) and MMF dose.

2.5 | Induction protocol and maintenance immunosuppression

Induction therapy was used in a majority of patients 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 patients with high HLA mismatch, previous transplant and/or those with high (>20%) cytotoxic panel reactive antibodies (PRA). All patients had similar triple maintenance immunosuppression, consisting of tacrolimus or cyclosporine, mycophenolate mofetil, and prednisolone.

2.6 | Statistical analysis

Continuous data were described using mean ± standard deviation (*SD*) or median (IQR), and categorical data were expressed as number (and percentages). Categorical data were compared using Pearson chi-square test or Fisher exact test, and continuous variables were compared with Student's *t*-test or Mann–Whitney *U* test, as appropriate.

De novo DSA incidence and graft survival curves were visualized using Kaplan–Meier method, with comparison between patients' groups being done by log-rank test. In the case of death with a functioning graft, time was censored at the time of death.

Potential predictors of *dn*DSA incidence and graft failure were explored by univariate and multivariable Cox proportional hazards models. Independent predictors were identified using a backward

elimination method, with a *p*-value <.05 necessary for retention in the model, as previously proposed (Heinze & Dunkler, 2017).

A 2-sided *p*-value <.05 was considered as statistically significant. Statistical calculations were performed using STATA/MP, version 15.1 (Stata Corp).

3 | RESULTS

The clinical and immunological characteristics of the studied cohort are described in Table 1. Thirty-two patients (33%) had preformed anti-HLA antibodies of which eight (8%) were donor-specific. HLA class I split and broad antigen mismatch (AgMM), EptMM and EpvMM mean values were 3.28 ± 1.91, 3.21 ± 1.86, 13.0 ± 8.1 and 7.6 ± 4.8, respectively. The Pearson's correlation values were 0.821 ($p < .001$) and 0.771 ($p < .001$) between HLA split AgMM and EptMM and EpvMM, 0.822 ($p < .001$) and 0.783 ($p < .001$) between HLA broad AgMM and EpMM, total and AbVer, respectively. The mean values for HLA class II split and broad AgMM were 1.89 ± 1.27 and 1.61 ± 1.19. HLA class II EptMM and EpvMM mean values were 24.4 ± 17.4 and 10.5 ± 8.2, respectively. Pearson's correlation values between HLA split AgMM and EpMM, total and AbVer, were 0.829 ($p < .001$) and 0.810 ($p < .001$), while HLA broad AgMM and EpMM were 0.823 ($p < .001$) and 0.826 ($p < .001$).

Thirteen patients (14%) experienced one rejection episode, eight cellular-mediated and five antibody-mediated rejections.

Six patients developed HLA class I *dn*DSA (Table 2). The incidence of *dn*DSA for HLA class I at 6-years was 7%. No significant difference was found for HLA broad and split antigen and total and AbVer eplet mismatches in *dn*DSA-I group compared to the no *dn*DSA-I group (2.50 ± 2.07 versus 3.26 ± 1.85, $p = .321$; 2.83 ± 1.94 versus 3.31 ± 1.91, $p = .509$; 9.2 ± 6.5 versus 13.3 ± 8.2, $p = .256$ and 4.7 ± 2.6 versus 7.8 ± 4.9, $p = .101$, respectively). In a multivariate analysis, no predictors for *dn*DSA-I were identified (Table 3).

Regarding HLA class II, seven patients developed *dn*DSA during follow-up time (Table 4). HLA class II total and AbVer eplet mismatches were greater in *dn*DSA-II group compared to the no *dn*DSA-II (41.3 ± 18.9 versus 23.1 ± 16.7, $p = .018$ and 18.0 ± 8.7 versus 9.9 ± 7.9, $p = .041$), which is not observed for HLA class II antigen broad and split mismatches (2.29 ± 0.49 versus 1.56 ± 1.22, $p = .090$ and 2.43 ± 0.79 versus 1.84 ± 1.30, $p = .248$). As expected, AMR was greater within *dn*DSA-II group (3.0 versus 2.0; $p = .002$). The incidence of *dn*DSA-II at 6 years was 17% (Figure 1). For HLA broad antigen mismatch considering 0–1, 2 and 3–4 mismatches were 0%, 24% and 33%, respectively ($p = .060$)—Figure 1a. Regarding total eplet mismatches, tercile intervals of less than 17, between 17 and 32, and higher than 32 were 0%, 13% and 41%, respectively ($p = .028$)—Figure 1b. Finally, AbVer eplet mismatch load terciles of less than 6, between 6 and 14, and higher than 14 were 0%, 4% and 42% ($p = .006$)—Figure 1c.

In a multivariate analysis, we found that preformed DSA (HR = 7.983; 95%IC: 1.329–47.968; $p = .023$), living unrelated donors (HR = 8.052; 95%IC: 1.313–49.394; $p = .024$) and retransplantation (HR = 14.393; 95%IC: 1.946–106.441; $p = .009$) were predictors for *dn*DSA-II (AUC = 0.801; 95% CI: 0.622–0.981)

TABLE 1 Clinical and immunological characteristics of the studied cohort ($n = 96$)

	Total
Follow-up (months), median (IQR)	52.4 (33.7–77.7)
Recipient age (years), mean ± *SD*	40.8 ± 12.8
Donor age (years), mean ± *SD*	49.4 ± 9.7
Female recipient, *n* (%)	32 (33)
Female donor, *n* (%)	64 (67)
Living unrelated donor, *n* (%)	38 (40)
Dialysis vintage (months), median (IQR)	12.6 (0–24.1)
Preemptive KT, *n* (%)	32 (33)
Retransplant, *n* (%)	10 (10)
Previous blood transfusion, *n* (%)	23 (24)
Previous pregnancy, *n* (%)[a]	14 (44)
Cytotoxic PRA (%), median (IQR) [Min-Max]	0 (0–0) [0–77]
Cytotoxic PRA ≥ 5%, *n* (%)	4 (4)
Virtual PRA (%), median (IQR)	0 (0–20)
Induction, *n* (%)	
No	1 (1)
Basiliximab	88 (92)
Antithymocyte globulin	7 (7)
Calcineurin inhibitor, *n* (%)	
Cyclosporine	0 (0)
Tacrolimus	96 (100)
Anti-HLA antibodies, *n* (%)	32 (33)
Preformed DSA, *n* (%)	8 (8)
AB0-incompatible, *n* (%)	5 (5)
Desensitized, *n* (%)	9 (9)
AB0i, *n*	5
HLAi, *n*	4
HLA-I antigen broad MM, mean ± *SD*	3.21 ± 1.86
HLA-II antigen broad MM, mean ± *SD*	1.61 ± 1.19
HLA-I antigen split MM, mean ± *SD*	3.28 ± 1.91
HLA-II antigen split MM, mean ± *SD*	1.89 ± 1.27
HLA-I eplet total MM, mean ± *SD*	13.0 ± 8.1
HLA-II eplet total MM, mean ± *SD*	24.4 ± 17.4
HLA-I eplet AbVer MM, mean ± *SD*	7.6 ± 4.8
HLA-II eplet AbVer MM, mean ± *SD*	10.5 ± 8.2
Biopsy-proven rejection, *n* (%)	13 (14)
Cellular, *n*	8
Antibody-mediated, *n*	5

Abbreviations: AbVer, antibody-verified; DSA, donor-specific antibodies; HLA, human leucocyte antigen; HLA-I, HLA class I; HLA-II, HLA class II; IQR, interquartile range; PRA, panel reactive antibodies; *SD*, standard deviation.

[a]Analysis considering only women ($n = 32$).

TABLE 2 Clinical and immunological characteristics considering *dn*DSA for HLA class I

	No *dn*DSA-I *N* = 90	*dn*DSA-I *N* = 6	*p*
HLA-I antigen broad MM, mean ± *SD*	3.26 ± 1.85	2.50 ± 2.07	.321
HLA-I antigen split MM, mean ± *SD*	3.31 ± 1.91	2.83 ± 1.94	.529
HLA-I eplet total MM, mean ± *SD*	13.3 ± 8.2	9.2 ± 6.5	.256
HLA-I eplet AbVer MM, mean ± *SD*	7.8 ± 4.9	4.7 ± 2.26	.101
Acute cellular rejection, *n* (%)	7 (8)	1 (17)	.415
Antibody-mediated rejection, *n* (%)	5 (6)	0	1

Abbreviations: AbVer, antibody-verified; HLA-I, HLA class I; MM, mismatches; *SD*, standard deviation.

TABLE 3 Multivariate models of *dn*DSA for HLA class I

	HR	95% CI	*p*
Model 1			
No predictor detected			
Model 2			
HLA-I eplet total MM	0.942	0.841–1.054	.295
Model 3			
HLA-I eplet AbVer MM	0.874	0.709–1.077	.207
Model 4			
HLA-I antigen broad MM	0.903	0.559–1.458	.676
Model 5			
HLA-I antigen split MM	0.975	0.617–1.543	.915

Note: Model 1 included the following variables: recipient age and gender, dialysis type and vintage, donor age and gender, unrelated living donor, induction IS, retransplant, virtual PRA and preformed DSA, but excluded HLA class II eplet and antigen mismatches. Final model with independent predictors was defined by stepwise backward selection ($p < .05$ used for retention in the model). Models 2 to 5: univariate (unadjusted) analysis for HLA-I total eplet MM, HLA-I AbVer eplet MM, HLA-I antigen broad MM and HLA-I antigen split mismatch, respectively.

TABLE 4 Clinical and immunological characteristics considering *dn*DSA for HLA class II

	No *dn*DSA-II *N* = 89	*dn*DSA-II *N* = 7	*p*
HLA-II antigen broad MM, mean ± *SD*	1.56 ± 1.22	2.29 ± 0.49	.090
HLA-II antigen split MM, mean ± *SD*	1.84 ± 1.30	2.43 ± 0.79	.248
HLA-II eplet total MM, mean ± *SD*	23.1 ± 16.7	41.3 ± 18.9	.018
HLA-II eplet AbVer MM, mean ± *SD*	9.9 ± 7.9	18.0 ± 8.7	.041
Acute cellular rejection, *n* (%)	7 (8)	1 (14)	.467
Antibody-mediated rejection, *n* (%)	2 (2)	3 (43)	.002

Abbreviations: HLA-II, HLA class II; MM, mismatches; SD, standard deviation.

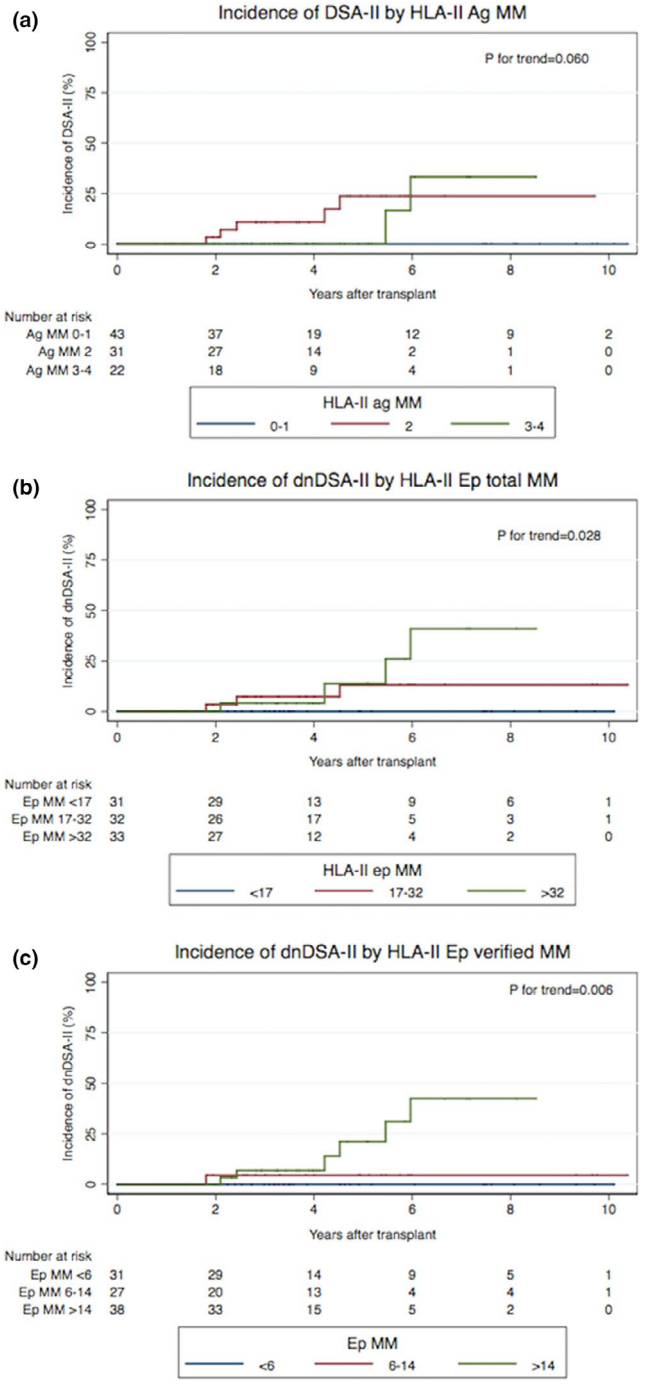

FIGURE 1 Incidence of *dn*DSA for HLA class II considering (a) antigen mismatches; (b) total eplet mismatches; and (c) antibody-verified eplet mismatch load

(Table 5, Model 1). HLA class II total and AbVer eplet mismatches (HR = 1.042; 95%IC: 1.004–1.082; $p = .031$; AUC = 0.852 and HR = 1.105; 95%IC: 1.011–1.208; $p = .028$; AUC = 0.856) showed to be superior predictors of *dn*DSA-II, when compared to broad or split antigen mismatches (HR = 1.740; 95%IC: 0.877–3.452; $p = .113$; AUC = 0.783 and HR = 1.677; 95%IC: 0.847–3.318; $p = .138$; AUC = 0.818), when adjusted for Model 1(Table 5, Model 3, 5, 7 and 9 respectively).

TABLE 5 Multivariate models for *dn*DSA for HLA class II.

	HR	95% CI	*p*	Model C-statistics (95% CI)
Model 1				
Preformed DSA	7.983	1.329–47.968	.023	0.801 (0.622–0.981)
Living unrelated donor	8.052	1.313–49.394	.024	
Retransplant	14.393	1.946–106.441	.009	
Model 2				
HLA-II eplet total MM, unadjusted	1.056	1.015–1.098	.007	0.755 (0.630–0.919)
Model 3				
HLA-II eplet total MM, adjusted to Model 1	1.042	1.004–1.082	.031	0.852 (0.718–0.986)
Model 4				
HLA-II eplet AbVer MM, unadjusted	1.141	1.042–1.249	.004	.738 (0.565–0.911)
Model 5				
HLA-II eplet AbVer MM, adjusted to Model 1	1.105	1.011–1.208	.028	0.856 (0.726–0.748)
Model 6				
HLA-II antigen broad MM, unadjusted	1.645	0.883–3.063	.117	.659 (0.570–0.748)
Model 7				
HLA-II antigen broad MM, adjusted to Model 1	1.740	0.877–3.452	.113	.783 (0.605–0.961)
Model 8				
HLA-II antigen split MM, unadjusted	1.458	0.804–2.643	.214	.590 (0.498–0.683)
Model 9				
HLA-II antigen split MM, adjusted to Model 1	1.677	0.847–3.318	.138	.818 (0.628–1)

Note: Model 1 included the following variables: recipient age and gender, dialysis type and vintage, donor age and gender, unrelated living donor, induction IS, retransplant, virtual PRA, preformed DSA, excluding HLA class II eplet and antigen mismatches, were included. Final model with independent predictors was defined by stepwise backward selection ($p < .05$ used for retention in the model); models 2, 4, 6 and 8: univariate (unadjusted) analysis for each predictor; models 3, 5, 7 and 9: multivariate (adjusted for independent predictors detected in Model 1) analysis for each predictor.

FIGURE 2 Kaplan–Meier graft survival curve comparisons by *dn*DSA status

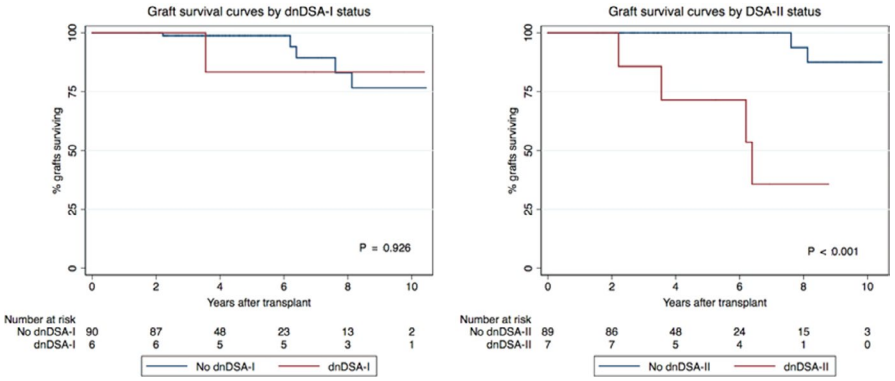

TABLE 6 Multivariate model for allograft failure

	HR	95% CI	*p*
Rejection	16.026	1.420–180.887	.025
*dn*DSA-II	20.447	1.994–209.687	.011

Note: Multivariable model adjusted for recipient age and gender, donor age and gender, living related versus unrelated donor, retransplant, preformed DSA, virtual PRA, *dn*DSA-I.

Six patients (6.25%) experienced graft failure deemed as alloimmune-related. Overall graft survival was 79% at 9 years of follow-up (Figure 2). Graft survival was significantly lower within *dn*DSA-II patients group (36% versus 88%, $p < .001$). No significant difference was observed for *dn*DSA-I (83% versus 77%, $p = .926$).

Finally, in a multivariable model adjusted for recipient age and gender, donor age and gender, living related versus unrelated donor, retransplant, preformed DSA, virtual PRA (vPRA), *dn*DSA-I, we

found that rejection episodes (HR = 16.026; 95%IC: 1.420–180.87; $p = .025$) and HLA class II *dn*DSA development (HR = 20.447; 95%IC: 1.994–209.687; $p = .011$) were independent predictors of allograft failure (Table 6).

4 | DISCUSSION

Our study demonstrates that the number of HLA class II, total and particularly AbVer, eplet mismatch load were greater in *dn*DSA-II group compared to no *dn*DSA-II, which is not verified when mismatches are determined traditionally considering the HLA class II broad or split molecule as a whole. Also, antibody-mediated rejection was significantly higher within HLA class II *dn*DSA positive subcohort (3.0 versus 2.0; $p = .002$).

We also demonstrated in a multivariate analysis that HLA class II total and AbVer eplet mismatch load were independent predictors for HLA class II *dn*DSA development (HR = 1.042; 95%IC: 1.004–1.082; $p = .031$; AUC = 0.852 and HR = 1.105; 95%IC: 1.011–1.208; $p = .028$; AUC = 0.856). This was not observed for HLA class II broad or split antigen mismatch that failed to achieve statistical significance in the multivariate model (HR = 1.740; 95%IC: 0.877–3.452; $p = .113$; AUC = 0.783 and HR = 1.677; 95%IC: 0.847–3.318; $p = .138$; AUC = 0.818).

On the other hand, neither HLA class I broad and split antigen or HLA class I total or AbVer eplet mismatch load had any predictive value for HLA class I dnDSA, in our cohort. This was already demonstrated by several studies using total eplet mismatch analysis, (Duquesnoy, 2017a; Duquesnoy & Askar, 2007; Kishikawa et al., 2018; Snanoudj et al., 2019; Tafulo et al., 2019; Wiebe et al., 2013). Several authors have reported that *dn*DSAs are mainly HLA class II and donor-specific *dn*DSA against HLA class II are associated with worse prognosis, when compared to HLA class I (Hidalgo et al., 2009; Wiebe et al., 2012). Although the reason for this is unclear, a number of mechanisms have been postulated such us different regulation of antibody response and different expression of the target antigen (Wiebe et al., 2012). Despite this, as HLA class I DSAs are also associated with graft rejection and failure, the clinical relevance of HLA class I eplet mismatch load should be further investigated.

The major limitation of our study is due to the sample size but, on the other hand, is a very uniform cohort of living donors, younger patients under uniform immunosuppression therapy. Another limitation is the absence of high-resolution typing for HLA eplet mismatch analysis. However, Fidler *et* al have showed in a Caucasian cohort that two-field typing prediction based on two-digit typing is reliable calculation (Fidler et al., 2018).

We believe it urges the application of this molecular mismatch approach on routine practice to minimize the risk for developing HLA class II *de novo* DSA, improving outcome and especially not shutting down forever the possibility of a retransplant due to broad HLA allosensitization. For these reasons, HLA eplet mismatch analysis should be implemented in the near future, not only for direct living donation, but also in kidney paired exchange programmes (Ferrari et al., 2017), to assess risk following minimization of immunosuppression (Snanoudj et al., 2019), and to improve allocation systems within deceased donation (Duquesnoy, 2017b).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTIONS

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.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, [Sandra Tafulo], upon reasonable request.

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Appendix 5

Degree of HLA Class II Eplet Mismatch Load Improves Prediction of Antibody-Mediated Rejection in Living Donor Kidney Transplantation.

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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).

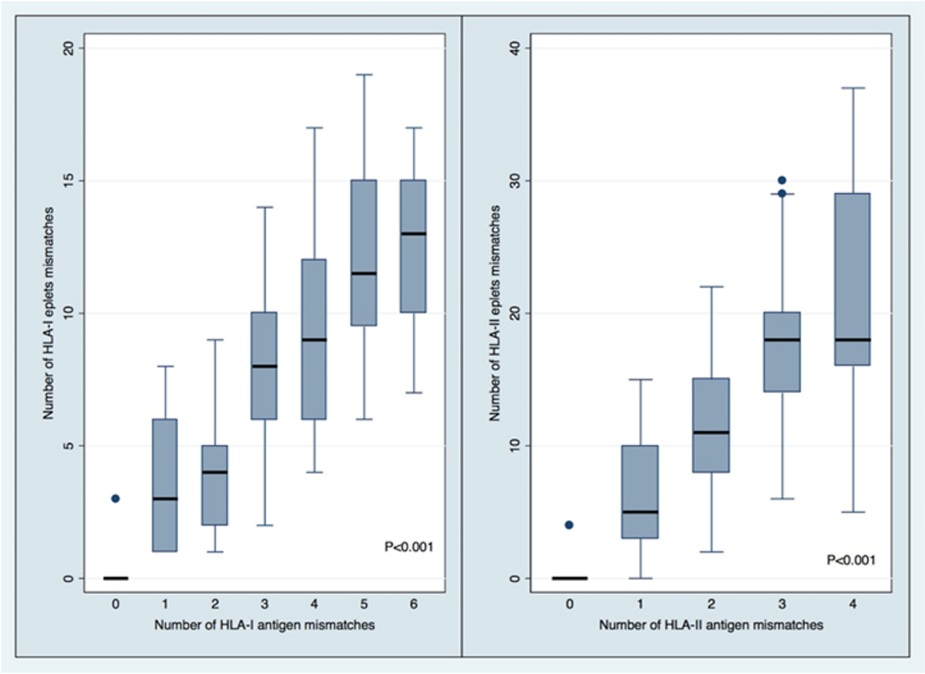

Fig. 1. Association between the number of HLA class I and class II antigen mismatches with the eplet mismatch load.

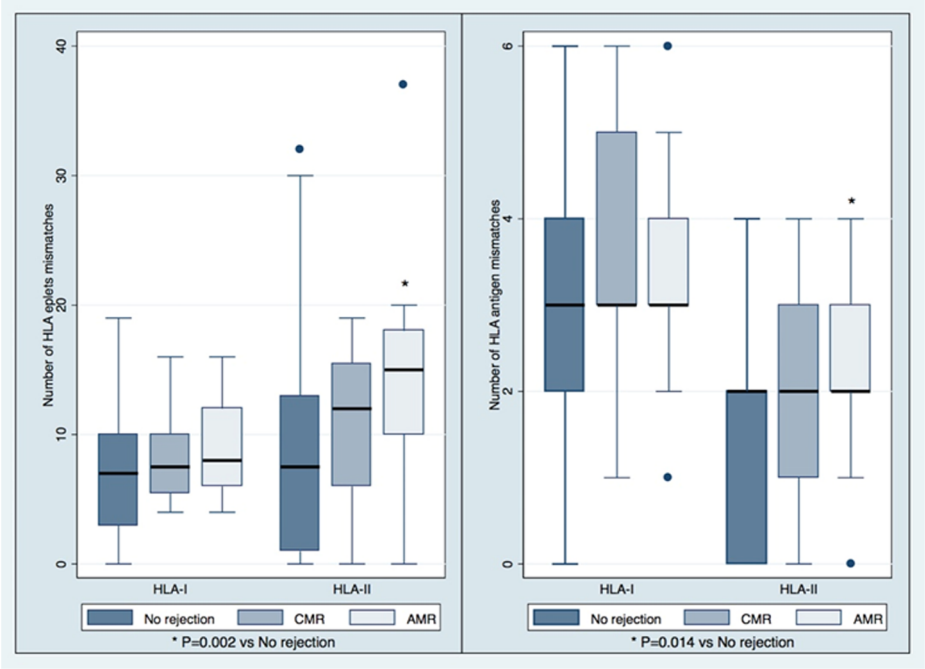

Fig. 2. Association between the number of HLA class I and class II eplet and antigen mismatches with rejection episodes considering: no rejection, CMR and AMR.

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.

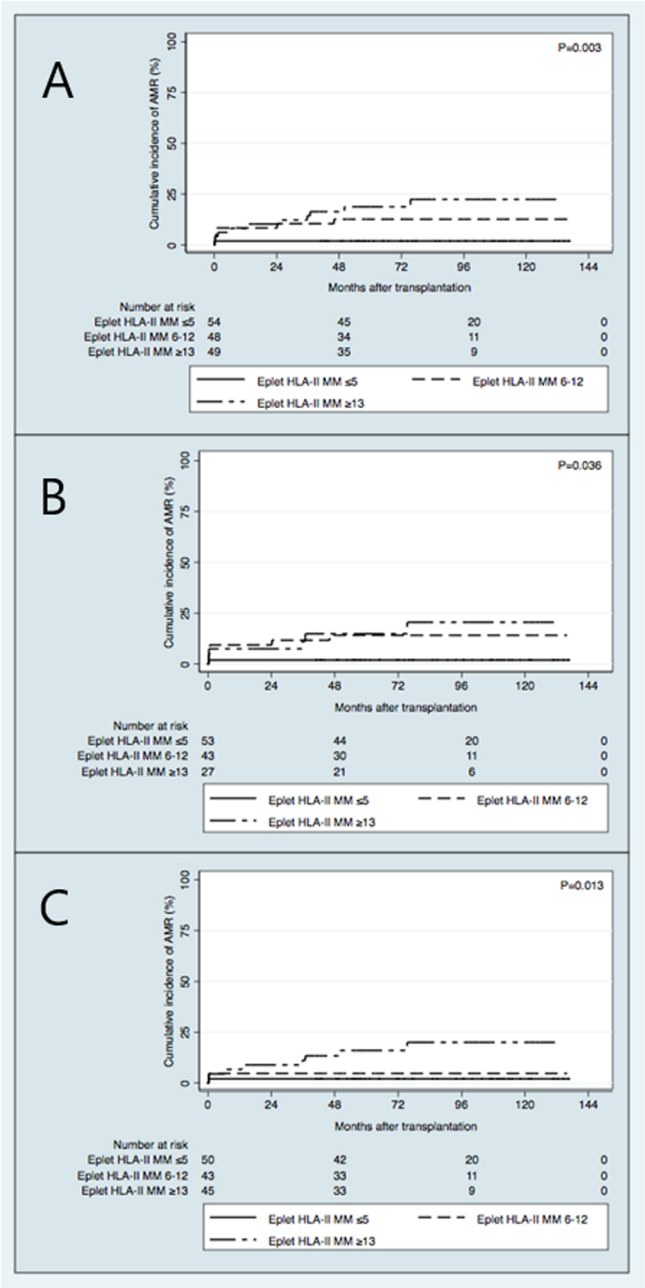

Fig. 3. Cumulative incidence curves at 96 month for antibody-mediated rejection considering HLA class II EpMM load in terciles: a) overall cohort (T1 = 2%, T2 = 13%, T3 = 22%, $P = 0.003$); b) patients with 0–2 HLA class II antigen MM (T1 = 2%, T2 = 14%, T3 = 20%, $P = 0.036$); c) Patients without preformed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

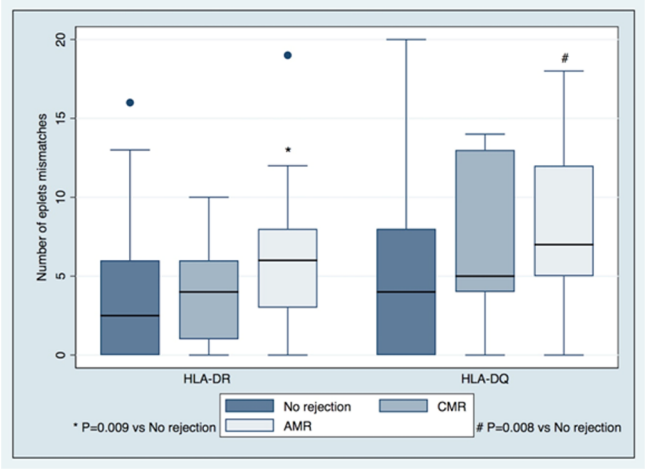

Fig. 4. Comparison of HLA-DR and HLA-DQ eplet mismatches according to rejection status (significant differences are shown).

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

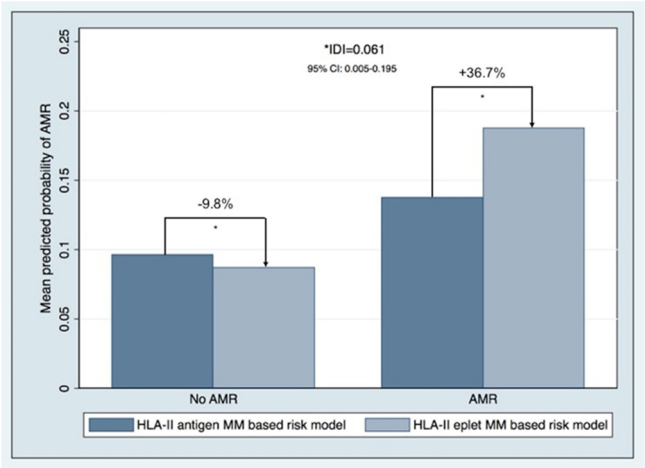

Fig. 5. Improvement in calculated risk of AMR considering HLA-II eplet mismatch in addition to classic HLA-II broad antigen mismatch.

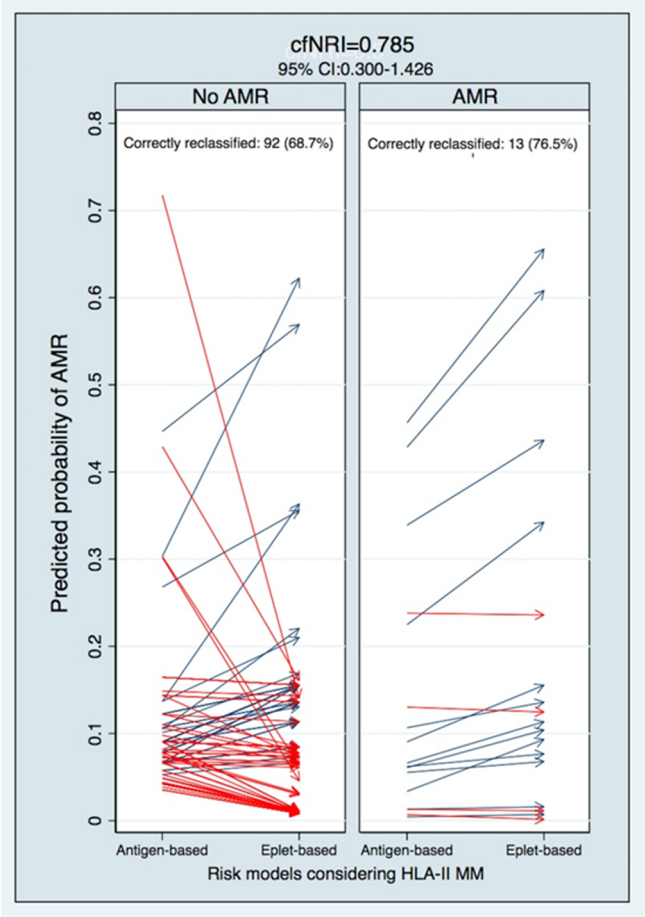

Fig. 6. Improvement in calculated risk of AMR considering HLA-II eplet mismatch in addition to classic.

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).

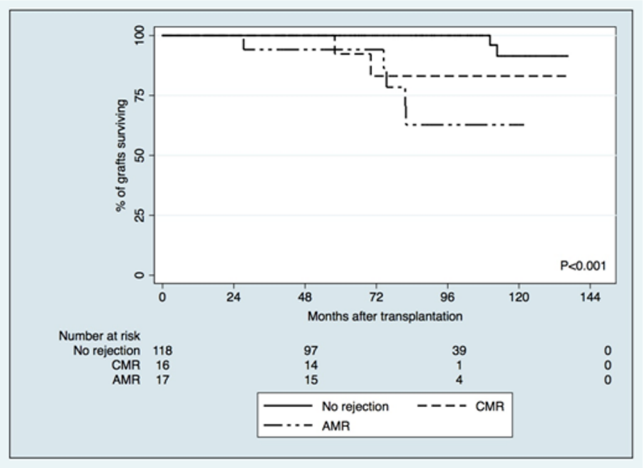

Fig. 7. Graft survival Graft at 120 months for patients with no rejection episodes, patients with CMR and patients with AMR.

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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