Universitat Autònoma de Barcelona

ADVANCES IN IMMUNE MONITORING IN KIDNEY TRANSPLANTATION

Laura Llinàs Mallol

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Doctoral Thesis submitted by

Laura Llinàs Mallol

For the degree of Doctor

Directors

Dr. Julio Pascual Santos

Dr. Marta Crespo Barrio

Dr. Dàlia Raïch Regué

Tutor

Dr. Juan Pedro-Botet Montoya

Doctoral Thesis Program in Medicine

Department of Medicine, Universitat Autònoma de Barcelona

Barcelona, 2021

Design and layout: Oriol Nebot

Als meus pares.

Acknowledgements

The development of this doctoral thesis could not have been done without the support, encouragement and help from a lot of people during these four years.

A mis directores de tesis doctoral. A Julio, por abrirme las puertas del Servicio de Nefrología para empezar un proyecto de fin de máster y por todas las oportunidades durante estos años. A Marta, porque sin ti esta tesis no sería posible. Gracias por creer en mí desde el primer minuto y por enseñarme tantas cosas, siempre con disponibilidad absoluta. Por sacar lo mejor de mí con esfuerzo, exigencia y valorando el trabajo bien hecho. I a la Dàlia, per tot el suport durant aquests anys, per ensenyar-me tantes coses del món de la investigació i de la vida, i pel gran equip que hem format durant aquests anys.

A todos mis compañeros de la Unidad de Trasplante Renal del Hospital del Mar, por ayudarme a ser un poco nefróloga de trasplante. Especialmente a Dolo, por ayudarme y apoyarme siempre y porque trabajar juntas es tan fácil. A María José, por confiar en mí; y a Anna y a Carla, por todos los buenos momentos compartidos estos años.

A tots els companys del laboratori del GREN, per l'ajuda i suport que m'heu donat durant aquests anys. A la Marta, per encoratjar-me i solucionar-me dubtes sempre que ho he necessitat. A la Vanesa, per recolzar-nos mútuament en aquest camí ple de reptes i posar-hi alegria, entusiasme i kit-kats quan ha sigut necessari. Al David, perquè sempre tenies una bona solució als meus dubtes al laboratori. I a la Sara i l'Alberto, per donar-me suport en la recta final d'aquest camí.

A les meves companyes de la USR: Anna, Montse, Maria i Sara, per ajudar-me sempre que ho he necessitat i perquè sense vosaltres, cap dels projectes que fem seria possible.

A Miguel, por confiar en mí para el proyecto Marató y por compartir conocimientos y el punto de vista crítico tan necesario para seguir avanzando; y a Jose y a Dulce, por vuestro apoyo con la citometría de flujo y las muestras de los pacientes.

I am also grateful to all the Transplant Immunology Research Group for their warm welcome during my stay in Leiden. To Dr. Heidt, for giving me the opportunity to join your team. To Gonca, for being a great supervisor, and for all the wonderful opportunities you gave me during my stay. To Yvonne, for showing me all the skills in the lab and also how to better plan

and organize my experiments. And finally, thanks to Kim, Cynthia, Ellen, Els, Jackie and Manon for being so nice with me during my stay abroad. Dank je wel!

A la Laura, per tot el suport i l'amistat compartida en quatre anys de carrera en comú i cinc de veïnes de despatx, i els que ens queden. I a la Sílvia, perquè entre les converses de bany i passadís arreglem el món (no), i fem pinya (això sí!).

A tots els meus amics i amigues, per la vostra amistat i suport durant tants anys. Molt especialment a la Laura, a la Marta, al Sergi, a la Núria, a la Júlia, al Josep, al Fran, a l'Edwards, a la Rita i a la Laura.

I finalment, a la meva família, que sou el meu pilar fonamental. Als meus pares, que sempre heu confiat en mi i m'heu inculcat el valor de l'esforç i el treball constant. Als meus avis, que sempre vau creure que arribaria on em proposés, gràcies per haver-me cuidat tant. I al Joan, per ser el meu company de vida i perquè no vols sortir als agraïments d'aquesta tesi perquè saps que t'ho mereixes més que ningú.

Abbreviations

ABMR: antibody-mediated rejection

$ABMR_h$: antibody-mediated rejection histology

ADCC: antibody-dependent cellular cytotoxicity

AECA: anti-endothelial cell antibodies

AAMS: amino acid mismatch score

APCs: antigen-presenting cells

ATG: antithymocyte globulin

AT_1R: angiotensin-II type 1 receptor

AT_1R-Ab: antibodies against angiotensin-II type 1 receptor

BCR: B cell receptor

CAN: chronic allograft nephropathy

CDC: complement-dependent cytotoxicity

CI: confidence interval

CNI: calcineurin inhibitors

CsA: cyclosporine A

dnDSA: *de novo* donor-specific antibodies

dnHLA-DSA: *de novo* donor-specific antibodies against human leukocyte antigens

DSA: donor-specific antibodies

EC: endothelial cells

EC-XM: crossmatch with primary aortic endothelial cells

EDTA: ethylenediamine tetraacetic acid

eGFR: estimated glomerular filtration rate

EMS: electrostatic mismatch score

ESDR: end-stage renal disease

ETAR: endothelin-I type A receptor

ETAR-Ab: antibodies against endothelin 1 type A receptor

FcγR: antibody constant region heavy chain γ receptor

GEE: generalized estimating equations

HLA: human leukocyte antigens

HLA-DSA: donor-specific antibodies against human leukocyte antigens

HS: healthy subjects

IFTA: interstitial fibrosis and tubular atrophy

KDPI: kidney donor profile index

KIR: inhibitory killer immunoglobulin-like receptors

KT: kidney transplantation

KTR: kidney transplant recipients

MDRD-4: modification of diet in renal disease study equation

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

MICA: major histocompatibility complex class I related chain A

MICA-Ab: antibodies against major histocompatibility complex class I related chain A

MPA: mycophenolic acid

mTOR: serine/threonine protein kinase mechanistic target of rapamycin

mTORC1: serine/threonine protein kinase mechanistic target of rapamycin complex 1

mTORC2: serine/threonine protein kinase mechanistic target of rapamycin complex 2

mTORi: serine/threonine protein kinase mechanistic target of rapamycin inhibitors

NA: not applicable

NK: Natural Killer cell

NKG2A: CD94/Natural Killer group 2 member A receptor

NKG2C: CD94/Natural Killer group 2 member C receptor

ONT: National Organization of Transplants (Spain)

PBL: peripheral blood lymphocytes

pCOR: protein to creatinine ratio in urine

PRA: panel reactive antibodies

PTDM: post-transplant diabetes mellitus

SAB: single antigen bead assays

SD: standard deviation

SEM: standard error of the mean

SM: steroid maintenance

SOT: solid-organ transplantation

SW: steroid withdrawal

TCMR: T-cell mediated rejection

TFH: T follicular helper cells

TNF- α: tumour necrosis factor-α

Treg: T regulatory cell

UNOS: United Network for Organ Sharing (U.S.)

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Summary

Kidney transplantation (KT) is the best therapeutic option for patients with end-stage renal disease and has the goal to provide long-term stable graft function to those patients. However, graft survival is still limited in time, and antibody-mediated rejection (ABMR) is nowadays recognised as a leading cause of allograft rejection and graft loss. Antibodies directed against HLA antigens present on the donor (HLA-DSA) have been classically associated with ABMR damage. Nevertheless, cases of ABMR without detectable HLA-DSA do exist, and this strengthens the hypothesis that other mechanisms of damage may be playing a role in those cases. The use of immunosuppressive drugs is intended to prevent the immunological rejection of the graft, but its use may be related to the development of serious drawbacks. Changes in the immunosuppression treatment after KT are frequent, but their impact on peripheral blood immune cells and HLA-DSA development has not been elucidated yet. The main aim of this doctoral thesis is to assess whether the immune monitoring of KT recipients in a combined effort comprising HLA and non-HLA antibodies, HLA epitope mismatch analysis and immune cells subpopulations may help to better tailor their immunological risk.

In the first two studies, two changes in immunosuppressive treatment were evaluated: steroid withdrawal and conversion from tacrolimus to mTOR inhibitors. Both immunosuppressive strategies proved to be safe in terms of renal function and HLA-DSA development and triggered a significant redistribution of peripheral blood lymphocyte subsets. Steroid withdrawal mainly impacted the B-cell compartment, whereas conversion to mTOR inhibitors exerted its principal effects in the NK-cell niche. In the third study, we aimed to assess the role of HLA and non-HLA antibodies, together with the HLA epitope mismatch analysis, in the development of histological ABMR. Nearly a third of histological ABMR cases did not show circulating HLA-DSA at biopsy time, but no non-HLA antibody associated with these cases. Those patients presented lower class II and DRB HLA epitope mismatches compared with ABMR histology cases that also presented HLA-DSA, dismissing the possibility of low, undetected HLA-DSA. The detection of pre-transplant antibodies against angiotensin-II type 1 receptor (AT_1R) associated with the development of ABMR histology in the presence of HLA-DSA and suggested that HLA-DSA and AT_1R antibodies may function in synergy. Finally, the analysis of HLA epitope mismatches proved to be superior to the classical HLA antigen mismatches analysis for the prediction of *de novo* HLA-DSA development.

To conclude, the results presented in this doctoral thesis support that immune monitoring is a useful tool for better immunological risk assessment in KT recipients.

Resumen

El trasplante renal (TR) es la mejor estrategia terapéutica para los pacientes con enfermedad renal crónica, y tiene el objetivo de proporcionar una función renal estable a largo plazo. Sin embargo, la supervivencia del injerto es todavía limitada en el tiempo, y el desarrollo de rechazo mediado por anticuerpos (RMA) es una causa prevalente de pérdida del injerto renal. La detección de anticuerpos dirigidos a antígenos HLA presentes en el donante (HLA-ADS) ha sido clásicamente asociada al RMA. Sin embargo, existen casos de RMA en los cuáles no hay HLA-ADS detectables, lo que sugiere que otros mecanismos de daño también pueden ser relevantes. El uso de fármacos inmunosupresores busca prevenir el rechazo inmunológico del injerto, pero su uso puede asociarse con el desarrollo de efectos secundarios. Por ello, los cambios en la pauta inmunosupresora son frecuentes, pero su impacto en la distribución de poblaciones linfocitarias en sangre periférica y el desarrollo de HLA-ADS no ha sido dilucidado todavía. El objetivo principal de esta tesis doctoral es evaluar si la monitorización inmunológica de receptores de TR, en un esfuerzo combinado que incluya anticuerpos HLA y no-HLA, análisis de incompatibilidades por epítopos HLA y de poblaciones linfocitarias en sangre periférica, permite definir mejor su riesgo inmunológico.

En los dos primeros estudios, se evaluaron dos cambios de pauta inmunosupresora: la retirada de esteroides (RE) y la conversión a inhibidores del mTOR (C-imTOR). Ambas estrategias se mostraron eficaces a nivel de función renal y prevención de HLA-ADS, y provocaron una redistribución significativa de poblaciones linfocitarias en sangre periférica. La RE impactó principalmente en las subpoblaciones de células B, y la C-imTOR en las subpoblaciones de células NK. En el tercer estudio, nos propusimos analizar el papel en el desarrollo de RMA de los anticuerpos HLA y no-HLA y el análisis de incompatibilidades por epítopos HLA. Casi un tercio de los TR con RMA no mostraron HLA-ADS, pero ningún anticuerpo no-HLA se asoció con estos casos. Estos TR mostraron menor número de incompatibilidades por epítopos HLA de clase II comparados con los TR con RMA y HLA-ADS, contradiciendo la posibilidad de que en estos casos haya HLA-ADS no detectados. La detección pre-trasplante de anticuerpos contra el receptor-1 de la angiotensina-II (AT_1R) se asoció con el desarrollo de RMA en presencia de HLA-ADS, sugiriendo que los HLA-ADS y los anticuerpos anti-AT_1R pueden funcionar sinérgicamente. Finalmente, el análisis de incompatibilidades por epítopos HLA se mostró más eficaz que el análisis clásico de incompatibilidades por antígenos HLA para predecir el desarrollo de HLA-ADS *de novo*.

En conclusión, los resultados de esta tesis doctoral corroboran que la monitorización inmunológica en receptores de TR es eficaz para estratificar mejor su riesgo inmunológico.

1. INTRODUCTION

1. Introduction

1.1. Kidney transplantation and alloimmune responses

Kidney transplantation (KT) is the treatment of choice for patients with end-stage renal disease (ESRD), conferring better survival rates compared to remaining on dialysis (1). The survival benefit of KT has been confirmed even when kidneys from expanded criteria, elderly and extremely aged donors are used, or in the case of recipients with comorbidities (2-6). In 1954, a team led by the surgeon Joseph Murray performed the first successful KT, being donor and recipient identical twins (7). Eleven years later, in 1965, this achievement was replicated in Spain by the team headed by Dr. Josep María Gil-Vernet and Dr. Antoni Caralps at the Hospital Clinic of Barcelona (8). Since 1989, with the set-up of the National Organization of Transplants (ONT) in Spain, the annual KT activity has presented a continuous increase over the years. In 2019, 3423 KT were performed in Spain, followed by 2700 KT in 2020, a reduction attributable to the COVID-19 pandemic (9) (**Figure 1**).

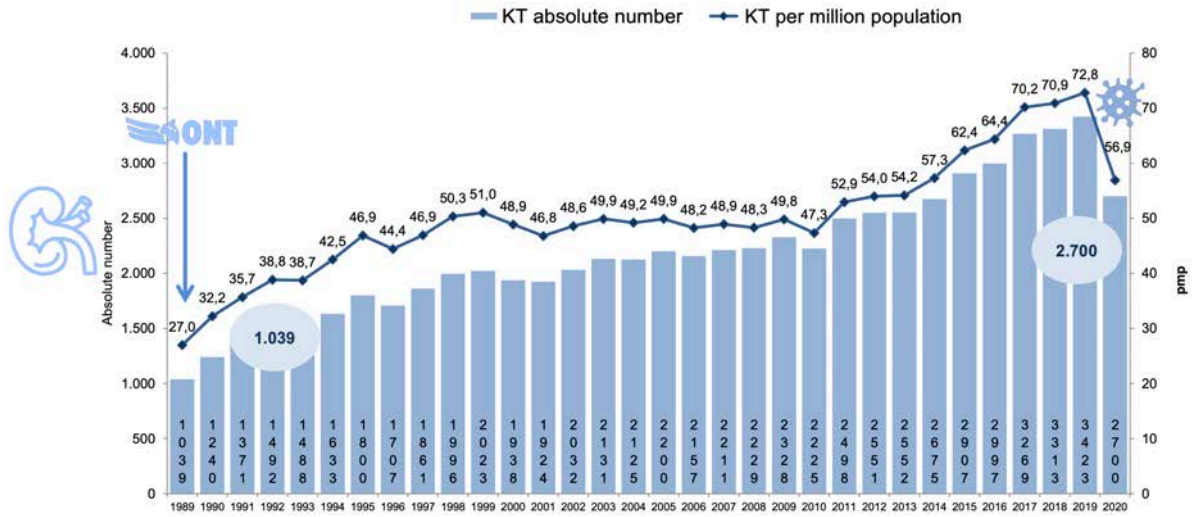

Figure 1. *Evolution of the number of KT performed in Spain as reported by the ONT by year (1989-2020 period). Adapted from ONT 2020 Annual Report.*

Nowadays, graft survival is still limited in time, and there is a noteworthy prevalence of patients included in the KT waiting list after losing a renal allograft. During 2020, 17.6% of patients who entered the KT waiting list in Catalonia had lost a previous KT, and this percentage has remained constant since 2014 (data obtained from the Catalan Transplant Organization Registry). Current 1- and 5-year death-censored graft survival rates in Europe are reported to be 92% and 84.4% in patients who received a KT between 2006 and 2015, with little improvement over the last few years (10). Thus, one of the main challenges in KT is to improve the useful half-life of the renal allografts. Several studies have addressed the

multiple causes of renal allograft loss, which were classically summarized in a large entity termed chronic allograft nephropathy (CAN) (11). CAN included heterogeneous post-transplant clinical courses attributable to both immunologic and nonimmunologic causes, leading to allograft loss (12). Several research in the field has endeavoured to better characterize the pathogenesis and mechanisms involved in CAN (13). In 2012, a study highlighted the dominant role of antibody-mediated rejection (ABMR) in KT that progress to kidney allograft failure (14). Allograft rejection was the attributable cause of graft failure in 36/56 (64%) cases. In 2016, our group analysed the risk of graft loss in KT recipients with allograft biopsies categorized in one of the six Banff 2013 diagnostic categories, and also found that ABMR conferred the higher risk of graft loss, considering the normal biopsy as a reference (15). Other studies drafted similar conclusions (16, 17). Nowadays, allograft rejection - mainly the ABMR phenotype - has been recognized as the leading cause of graft dysfunction and graft loss after KT (18-20).

Allograft rejection is the consequence of the response of the recipient's immune system after recognizing foreign molecules in the graft. It is therefore due to the failure in achieving a successful matching between donor and recipient. Currently, there are two well-known compatibility barriers in solid-organ transplantation (SOT): the first one is the blood ABO group, and the second is the existence of the histocompatibility leukocyte antigen (HLA) system, the human equivalent to the major histocompatibility complex (MHC) of animals.

The ABO antigen system represents the first major alloantigens recognized in humans, and were originally described in 1901 on erythrocytes (21). Four ABO blood groups have been described (group A, B, AB and O) considering the presence of one, both or neither of A and B antigens on erythrocytes and other cells of the human organism. Humans develop natural antibodies against the AB antigens that they do not have. On the other hand, MHC molecules are responsible of presenting peptides to T cells, which can recognize them through their T cell receptor (TCR). TCR can only recognize foreign antigens if they are presented by MHC molecules (22-24), preventing T cells from responding in an uncontrolled manner.

The HLA system is highly polymorphic and was characterized in the 1950s by using agglutinating antibodies in the sera of multiparous women and patients who had undergone multiple blood transfusions (25-27). Two classes of HLA molecules are relevant in the transplantation compatibility setting: class I (including HLA-A, HLA-B and HLA-C) and class II (including HLA-DRB1/DRA1, HLA-DQB1/DQA1 and HLA-DPB1/DPA1). HLA genes are located on the short arm of chromosome 6, and to date, more than 24.000 HLA allelic variants

have been identified (28). This remarkable rate of polymorphism is the ultimate responsible of HLA recognition as foreign antigens after SOT. KT in the presence of donor-specific ABO or HLA antibodies may lead to a quick and fatal hyper-acute rejection, which was described in the early 1960s (29, 30). Since 1969, the pre-transplant complement-dependent cytotoxicity (CDC) crossmatch reaction between the donor cells and the recipient sera - together with pairing for compatible ABO blood groups - became mandatory in KT to prevent these cases of hyper-acute rejection (31). Nowadays, desensitization protocols allow successful KT across the ABO blood group barrier (32).

The immune responses (alloresponses) against non-self-antigens that are expressed on the graft lead to several mechanisms of organ transplant injury. Recognition of foreign antigens by T cells via their TCR can take place in three different ways:

- Direct allorecognition: after transplantation, donor antigen-presenting cells (APCs, mainly dendritic cells) bearing mismatched molecules can be recognized by alloreactive T cells of the recipient, initiating an immune response (33, 34).
- Indirect allorecognition: recipient APCs present donor antigens to alloreactive T cells on the secondary lymphoid organs (35), activating T cells which undergo clonal expansion and differentiation.
- Semidirect allorecognition: recipient APCs acquire intact allogeneic MHC-peptide complexes from donor cells and present them to directly reactive recipient T cells (36, 37).

Three consecutive signals are needed for T lymphocyte activation. The first signal is the recognition of an antigen via the TCR of T cells, which is presented by MHC of dendritic cells. The second signal consists of the costimulatory signals provided by APCs mainly through CD80 and CD86 to CD28, accompanied with other costimulatory pairs such as CD40-CD40L and ICOS/ICOSL (38). Afterwards, signals 1 and 2 will activate signal transduction pathways in T cells through the CD3 complex, triggering the expression of cytokines such as interleukin-2 or interleukin-15. Those cytokines, together with other cytokines secreted by APCs to the milieu, will activate the serine/threonine protein kinase mechanistic target of rapamycin (mTOR) pathway providing the signal 3, that stimulates T cell proliferation and differentiation (39). After that, activated T lymphocytes in the secondary lymphoid organs return to the graft, where they may produce interstitial infiltration and tubulitis with intimal endarteritis or fibrinoid necrosis (40). Besides, there is increasing evidence that B cell alloresponses, which are predominantly T cell dependent, are also crucial in graft rejection (41, 42). The humoral response starts when naïve B cells enter into secondary lymphoid organs following chemokine signals secreted by stromal and follicular dendritic cells present in the B cell

follicle (43-45). There, they may encounter an alloantigen, usually presented by APCs such as follicular dendritic cells or specialized macrophages (46). B cells need two signals for their activation. Firstly, the antigen recognition through their B cell receptor (BCR), which triggers the downregulation of the chemokine receptor CXCR5 and the upregulation of CCR7 and EBI2 (47). As a consequence, B cells will exit the follicle and migrate to the T-B interface in order to receive T cell help from T follicular helper cells (Tfh). Secondly, after migration to the T-B interface, the interaction between B cells and Tfh, mediated by co-stimulatory molecules such as CD28, CD154 and CD40L on T cells and CD80/86 and CD40 on B cells, together with secretion of IL-4 and IL-21 by Tfh (42, 45, 47-49) (**Figure 2**).

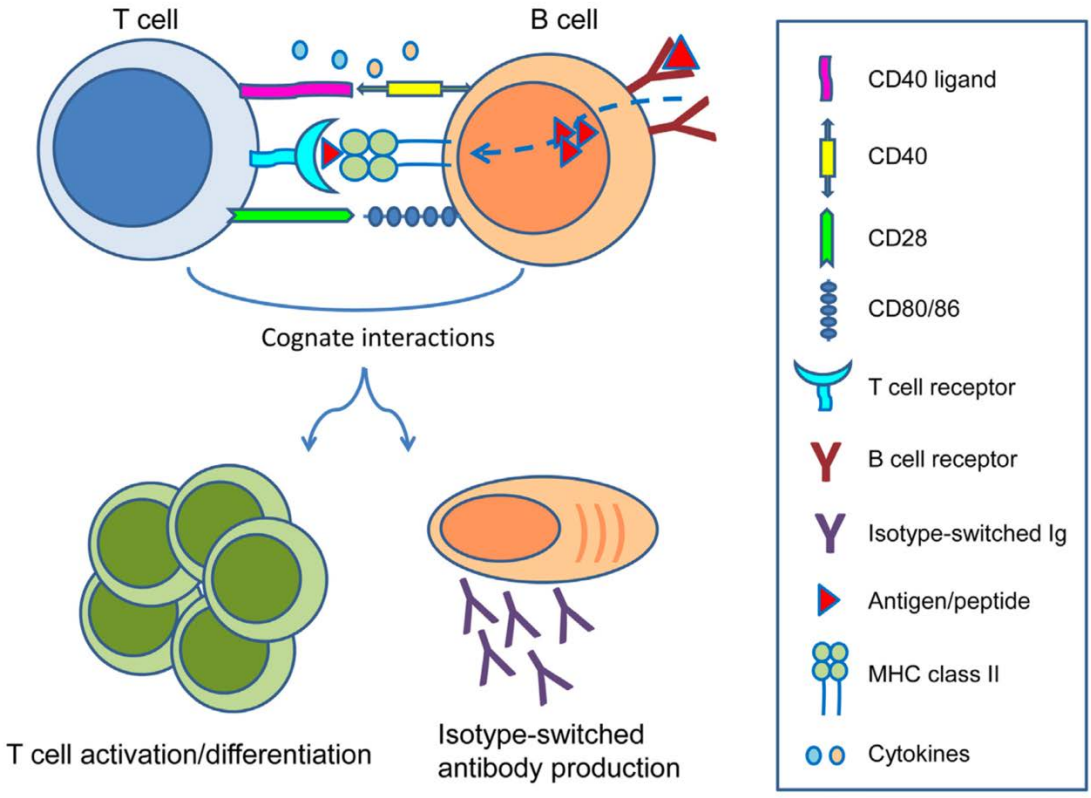

Figure 2. *Representative figure of the interactions between B and T cells following B cell processing and presentation of antigenic peptides. Extracted from (45).*

Afterwards, B cells can differentiate into short-lived plasma cells secreting low-affinity antibodies or into long-lived plasma cells or circulating memory B cells after class switching and somatic hypermutation in a germinal center (42, 50). Both bone marrow based long-lived plasma cells, and blood circulating memory B cells will be able to secrete high-affinity antibodies. After KT, histological evidence of antibody-mediated damage is characterized by antibody interactions with the vascular endothelium, initially identified by complement activation in the form of C4d deposition, and now also by acute microvascular inflammation and injury, such as capillaritis and glomerulitis or thrombotic microangiopathy (42).

1.2. Rationale for immune monitoring before and after kidney transplantation

KT has the ultimate goal to provide life-time stable graft function to patients with ESRD, however several threats may put into risk this final objective. At the beginning, KTs - if not performed between identical twins - were unsuccessful due to hyper-acute allograft rejection (29, 30). This highlighted the importance of inducing an immunosuppressive state in the KT recipient in order to prevent the immunological rejection of the graft. The use of total body irradiation and prednisolone were the first efforts towards this achievement (51). Later on, other immunosuppressive drug combinations were tested (52), resulting in fewer acute rejection episodes and improved 1-year graft survival (53). However, these dramatic advances come together with the evidence that immunosuppression treatments are related to several unwanted side-effects, such as a high incidence of malignancies (54), post-transplant diabetes mellitus (PTDM) (55), cardiovascular complications (56) and infections (57-59). On the other side, the development of allograft rejection is still a major cause of graft loss (14, 15, 18) regardless of the use of immunosuppression strategies. All these adverse outcomes constitute serious setbacks in improving long-term graft survival after KT. Consequently, many efforts have been made in order to better identify which KT recipients are at greater risk of developing these complications. Nowadays, there is evidence that immune monitoring may be useful for stratifying KT recipients at risk of developing cancer (60, 61), infections (62, 63), acute allograft rejection (64) or ABMR (65-68). Moreover, immune monitoring can also help in the identification of KT recipients which may have long-term functioning allografts (69-71). All in all, immune monitoring before and after KT pursues the goal to characterize useful biomarkers that may help tailoring treatments to achieve the best outcome for an individual KT patient (72). Ultimately, these tools may help to better understand the “operational tolerance”, which is defined as a well-functioning transplant in the absence of immunosuppression treatment (73).

1.3. Available tools for immune monitoring in kidney transplant recipients: state of the art

1.3.1 The modern-classics I: HLA matching and HLA donor-specific antibodies

As previously mentioned, the first successful KT occurred with donor and recipient being identical twins (7). In 1987 the United Network for Organ Sharing (UNOS, U.S.) started a kidney-sharing program to increase the number of HLA-matched KT (considering HLA-A, HLA-B and HLA-DR). Results from the first period of this program (1987-1999) showed greater graft outcomes and lower incidence of graft rejection in HLA-matched compared to HLA-mismatched KT (74, 75) (**Figure 3**).

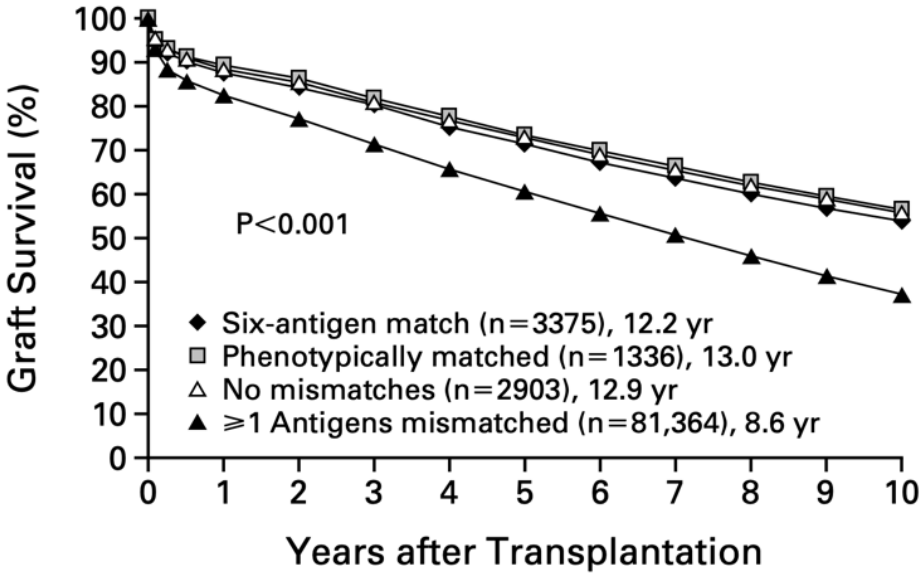

Figure 3. *HLA-matched transplantation prolongs graft survival. Graft survival in HLA-mismatched KT (black triangles) was significantly lower compared to graft survival of groups with six-antigen matched KT, phenotypically matched KT, and KT without HLA mismatches. Extracted from (75).*

Meanwhile, other multicentre analyses depicted similar results (76), which justified the careful consideration of HLA matching in the decision process of organ allocation. However, in 2004 a UNOS data analysis including 33.443 deceased donor KT showed that the impact of HLA compatibility on graft outcomes diminished in 1998 comparing with 1995-1997 data (77). Authors suggested that the evolving clinical practice, including the use of safer and more powerful immunosuppressive drugs, was the most plausible reason and proposed to revise the organ allocation algorithms. However, subsequent data from the Collaborate Transplant Study database including 135.970 KT from deceased donors between 1985-2004 arrived to the contrary conclusion, highlighting the relevance of HLA matching before KT (78).

Nowadays it is well established that the number of HLA mismatches adversely affects KT graft outcomes (79-82). The emergence of new contributing factors, such as the continuous increase in the use of expanded criteria donors due to the widespread organ shortage, has modified the current importance of HLA matching in allocation algorithms. In fact, the Kidney Donor Profile Index (KDPI), a U.S. kidney quality index based on 10 clinical variables (age, race, history of high blood pressure or diabetes, among others, but excluding HLA matching) (83) is currently in use in the U.S for organ allocation. It should be noted that several concerns have been raised regarding KDPI use in Spain and Europe, precluding further application of this index in our environment (84).

In the meantime, from the late 1980s onwards, the detection of HLA donor-specific antibodies (HLA-DSA) has been increasingly associated with higher KT failure (66, 85, 86). The first technique developed for HLA antibody detection was the CDC reaction, which is performed between the donor cells and the recipient sera. Since 1969, the CDC crossmatch reaction entered the clinical routine before KT, due to the fact that a positive CDC reaction is a strong predictor of hyper-acute rejection (31). Currently, more sensitive tools, such as solid-phase immunoassays - mainly on Luminex platforms-, have improved the ability to detect, identify and semi-quantify HLA antibodies. This has been of utmost importance for better understanding the role of HLA-DSA in KT. In the early 2010s it was reported that recipients who receive a KT after a negative CDC crossmatch assay may present detectable preformed HLA-DSA by Luminex, and that these pre-KT HLA-DSA were related to poorer outcomes after KT (87, 88). A meta-analysis from 7 retrospective cohort studies (1119 KT recipients) confirmed that the presence of pre-KT HLA-DSA almost doubled the risk for ABMR development and increased the risk of graft failure compared to the absence of preformed HLA-DSA (89). In this study it was highlighted the use of flow cytometry crossmatch for HLA-DSA detection, a technique with higher sensitivity than CDC, but lower than current techniques based on solid-phase assays such as Luminex.

On the other side, detection of *de novo* HLA-DSA (dnHLA-DSA) post-KT has also been linked to lower graft survival (68), and associates with later occurrence of ABMR and lower graft survival compared with preformed HLA-DSA (90, 91). However, in apparent contrast, there is evidence that not all HLA-DSA may cause graft injury (92-94). In 2018, a study from our group reported that both dnHLA-DSA and persistent preformed HLA-DSA were more crucial risk factors for ABMR development than cleared preformed HLA-DSA (91). In a larger study in 2019, Senev *et al.* reported that the persistence of preformed HLA-DSA post-KT had a negative impact on graft survival (95). Several groups have aimed to identify those HLA-DSA characteristics that may portend worse prognosis, such as HLA-DSA strength (96), complement binding ability (C1q or C3d) (97, 98), HLA sub-class (class I or II) (19) or IgG subclasses (99). Nowadays, HLA-DSA are an established biomarker for ABMR development and greater risk of allograft loss.

1.3.2 The modern-classics II: Immune cells and renal biopsies

Immune responses are generally classified into two main categories: the rapid mechanisms of innate immunity, and the more potent and specific adaptive immune responses (100). Although some immune cells participate in these two types of response, it is recognized that the innate immune system comprises phagocytic cells (granulocytes and macrophages), which internalize and kill pathogens (101) and natural killer (NK) cells, which are involved in early defence against certain intracellular pathogens and tumours (102-105). On the other side, the acquired or adaptive immune system include T cells (*t*hymus-derived cells), key actors providing cellular immunity (106, 107), and B cells (*b*one marrow-derived cells), that provide humoral immunity (106, 108). Monitoring of immune cells before and after KT has gained interest in the last few years as a surrogate biomarker of immune responses. Importantly, immune cell phenotyping has proved to be useful in predicting cancer development (60, 61) or CMV infection risk stratification (62, 109, 110). In this sense, NK cells have been linked to HLA-DSA and ABMR detection in KT recipients (111-113). Our group reported that KT recipients with HLA non-DSA and HLA-DSA antibodies displayed lower proportions of NK cells, and increased proportions of $CD56^{bright}$ and $NKG2A^{+}$ NK subsets in comparison with cases without detectable anti-HLA antibodies (112). Of note, immune cell monitoring has also been of importance in studies pursuing to understand mechanisms of KT tolerance. Indeed, increased proportions of peripheral blood B cells, particularly activated, memory and early memory B cells (114) and with regulatory properties (115) were reported in KT recipients with stable graft function without immunosuppression treatment. However, in 2016, Rebollo-Mesa *et al.* demonstrated that it was biased by immunosuppression treatment (116), highlighting the importance of further studies regarding the impact of immunosuppression treatment on immune cells.

In 1991, a group of renal pathologists and nephrologists standardized the nomenclature and criteria for the histologic diagnosis of renal allograft rejection in Banff (Canada) (117). They proposed a classification into six categories: normal (1), hyperacute rejection (2), borderline changes (3), acute rejection (4), CAN (5) and other changes (6), widely known as the Banff classification. Every two years the *Banff Foundation for Allograft Pathology* holds a new meeting to discuss and update the classification criteria. The first Banff meetings were key to better characterize the T-cell mediated rejection (TCMR) (117). In the 1990s, acute ABMR, a form of allograft rejection associated with the detection of HLA-DSA, emerged as a separate clinicopathological entity (65, 66, 118). The key features of acute ABMR were the detection of C4d deposition and the presence of microvascular inflammation in peritubular capillaries, together with endothelial damage (119). In 2003, the Banff group presented an addendum to

the Banff'97 classification (120), in which for the first time, two types of allograft rejection were considered: ABMR and TCMR. The Banff'2019 classification is the last consensus classification available (121) (**Table 1**).

Table 1. *Summary of the current Banff classification categories for KT according to the 2019 update (121). Next Banff meeting is expected to be held in October 2021.*

2019 Banff classification for renal allograft rejection diagnosis	
Category 1	Normal biopsy or nonspecific changes
Category 2	Antibody-mediated changes - Active ABMR - Chronic active ABMR - Chronic (inactive) ABMR - C4d staining without evidence of rejection
Category 3	Borderline (suspicious) for acute TCMR
Category 4	TCMR - Acute TCMR (grades IA-IB, IIA-IIB, III) - Chronic active TCMR (grade IA-IB, II)
Category 5	Polyomavirus nephropathy
Category 6	Other changes

As a result of the widely use of the Banff classification, protocol kidney allograft biopsies may be used as another monitoring tool. Several groups have reported the utility of protocol biopsies at different follow-up points after KT, which may uncover pathologies in their subclinical stages (94, 122-124). Moreover, protocol biopsies may be helpful in delineating the impact of another clinical event. In 2017 Schinstock *et al.* evaluated the relationship between dnHLA-DSA detection and ABMR histology, reporting that when dnHLA-DSA was detected, ABMR was present only in 25% of patients, but increased up to 52.9% one year after (94). Although some evidence supports the use of surveillance biopsies for clinical decision-making (125), their use remains under controversy, especially regarding their optimum timing, and are currently restricted to research investigation in KT units.

1.3.3 The modern-modern I: Non-HLA antibodies

In KT, first evidence of the role of non-HLA antibodies was found in reports of accelerated ABMR in KT recipients from HLA-identical siblings (126-128). In 2005 it was reported for the first time a relationship between non-HLA sensitization and KT outcomes (129, 130). KT biopsies presenting histological findings suggestive of ABMR but without evidence of circulating HLA-DSA do exist (131, 132). In fact, biopsies which fulfilled all criteria for category 2 diagnosis except for HLA-DSA detection, were included in the Banff 2013 classification as suspicious for ABMR (133). Based on the hypothesis that other antibodies may play a lead role, some groups evaluated the role of non-HLA antibodies in KT recipients (134, 135).

Nowadays, two main types of non-HLA antibodies have been described (136). First, alloantibodies directed against polymorphic antigens that differ between donor and recipient, such as the HLA-related MICA or MICB (137). Second, antibodies that recognized self-antigens - autoantibodies- such as the angiotensin-II type 1 receptor (AT_1R) (130, 138), endothelin-I type A receptor (ETAR) (139), vimentin (140, 141), perlecan (142) or autoantigens expressed by endothelial cells (AECA) (143-145), among others. It is known that the prevalence of non-HLA antibodies is high in KT recipients. Gareau *et al.* reported a prevalence of 89% (90/101) positive recipients for at least one non-HLA antibody at the time of KT, while 82% (9/11) patients without a non-HLA antibody before KT developed at least one non-HLA antibody *de* novo (146). The correct identification of deleterious non-HLA antibodies has been challenging due to the high prevalence of them, together with the heterogeneous post-KT clinical course of patients included in some of the studies to evaluate them (147).

1.3.4 The modern-modern II: HLA eplet mismatch

Each known HLA antigen consists of a unique set of different polymorphic amino acid configurations, which are named HLA epitopes and are the binding place for antibodies (148). HLA molecules share individual epitopes, therefore, epitopes that are not present on self-HLA molecules are considered foreign by the immune system (149). Each structural epitope contains a cluster of 2-5 amino acids involved in antigen/antibody binding: the functional epitope, termed eplet (150, 151) (**Fig. 4**).

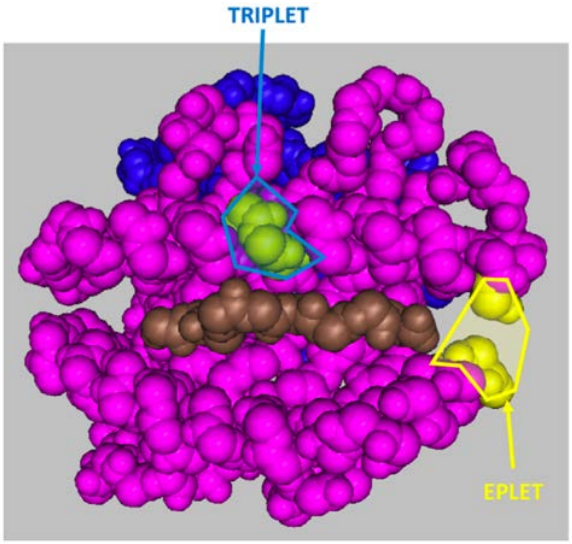

Figure 4. *Eplet depiction in an HLA molecule. An eplet can be a linear sequence of amino acid triplets (triplet) or contain linearly discontinuous amino acids that cluster together in the three-dimensional structure of the HLA molecule. Extracted from (148) (Tambur'15).*

Based on this principle, Duquesnoy *et al.* (152) developed the HLAMatchmaker tool to analyse the potential mismatched HLA epitopes between KT donor and recipient. Over the past few years, this immune monitoring tool has gained interest in the field of KT (149, 153). Higher number of HLA eplet mismatches have been associated with the development of *de novo* HLA-DSA, transplant glomerulopathy and immune allograft injury (154-159). Each whole-antigen HLA mismatch can be associated with a broad range of HLA eplet mismatches (157). Consequently, evaluation of HLA matching at epitope level has been proposed as a better strategy to prevent HLA-DSA development compared with classical HLA antigen matching (154, 160), and as a predictive biomarker for facilitating personalized immunosuppression adjustment (157).

Epitope mismatch load analysis or molecular mismatch is proposed as the best option for risk stratification (161). New tools such as HLA-EMMA are able to analyse compatibility on amino acid level and help to characterize the most immunogenic amino acid mismatches (162). PIRCHE, amino acid mismatch score (AAMS) or electrostatic mismatch score (EMS) are another molecular mismatch tools that show excellent performance (163). However, clinical relevance of epitope mismatch analysis remains under discussion and its application -apart from retrospective studies- is not generalized by now.

1.4. Key techniques in immune monitoring: Flow cytometry and Luminex in the front row

The flow cytometry technique was born in 1964 due to the close collaboration between an electrical engineer, Mack Fulwyler, and the inventor of the technology behind ink jet printers, Richard Sweet (164, 165). The aim of this new technique was 1) to measure simultaneously two or more characteristics of a cell and 2) to separate them according to the ratio of such characteristics (165). Only a few years later, in 1969, the association between the biochemist César Milstein and the biologist Georges Köhler -which had developed the Nobel prize-winning monoclonal antibody technology- together with the geneticist Leonard Herzenberg, developed fluorescent dye-tagged specific monoclonal antibodies (165, 166). This was of utmost importance for flow cytometry development, being the first step for distinguishing multiple subsets of immune cells according to the emitted fluorescence, and the technique rapidly grew. The fluorescent dyes reagent market is now already valued at more than $1 billion per year, and the number of simultaneous measurements on each cell type has raised from 1-2 to more than 30 (167). Currently, flow cytometry has become one of the preferred tools for the analysis of the immune system cellular phenotype and different functional characteristics (168). Flow cytometry can be used to characterize cell-surface proteins, intracellular phosphoproteins (169), cytokine production (170) and also proliferation (171), migration and degranulation (172) of several immune cells. Nowadays, as a consequence of the increased analytical complexity of flow cytometry, efforts are being made in the development of the spectral cytometry, which enables us to analyse the acquired full spectrum data (173), facilitating the fluorescence compensation, and also the analysis of higher amount of acquired data by using dimensionality-reduction algorithms.

Another key technology for immune monitoring, the bead-based multiplexing assays, emerged in the 1990s (174). Initially, this technique was based on the use of differentially sized magnetic microparticles, which allowed to distinguish and isolate several analytes, such as individual proteins, mRNA and mammalian cells, among others. In 1994 Jack Kettman, a flow cytometer expert, came up with the idea of measuring biomolecules with a flow cytometer using different sized beads. This was the start of the Luminex Corporation, which was founded in 1995 in Austin, Texas (174). Luminex technology uses microspheres - beads - with a unique spectral ratio, coupled to antibodies allowing the capture of the analyte of interest. Subsequent detection is made with a biotinylated detection antibody and a fluorescent reporter, and analysed in a Luminex instrument (175). The use of several unique beads in a single reaction allows Luminex to detect up to 500 analytes at the same time, generating more data with reduced sample quantity and analysis time (175). Nowadays,

principal applications of the Luminex technology in KT are HLA class I and class II gene typing and the detection of HLA antibodies in serum of KT recipients. However, different manufacturers and kit-strategies used for HLA antibody detection may provide differing results (176, 177).

1.5. Modulation of the immune response with immunosuppressive treatments after kidney transplantation

Since the first KTs were performed, it became evident that without immunosuppression, the recipient's immune system rapidly rejected the graft (29, 30, 178, 179). Thenceforth, several immunosuppressive drugs have been used in SOT (52). First efforts involved total body irradiation, together or not with corticosteroids and 6-mercaptopurine (180-182). These first attempts made KT clinically feasible, with very limited graft survival. Importantly, in 1963, Dr. Thomas Starzl reported higher success rates upon the use of a derivate from 6-mercaptopurine - termed azathioprine - together with prednisolone (52, 183). Three years later, in 1966, Dr. Starzl added antilymphocyte globulins to the combination of prednisolone and azathioprine, increasing the efficacy of the immunosuppressive therapy (184). Due to these achievements, azathioprine became the first widespread used immunosuppressive drug in transplantation (39). However, a major step towards successful KTs happened in 1978 with the introduction of cyclosporine A (CsA) (185). From the early 1980s, the use of the CsA, a calcineurin inhibitor (CNI), became common and significantly improved graft survival after KT (186). However, in 1989 Starzl *et al.* revealed that rejection episodes resistant to CsA treatment could be reversed with a more potent CNI, named Tacrolimus (52, 187). This drug was approved in 1997 for KT, and nowadays, is part of the standard of care immunosuppressive treatment in most SOT recipients (188). However, CNIs are involved in the development of several side effects, including PTDM, hypercholesterolemia, cardiovascular complications and nephrotoxicity (**Table 2**), which may limit long-term graft survival. These drawbacks boost the search for new immunosuppressive drugs, and in this regard, the development of inhibitors of mTOR (mTORi) promised to be a reliable alternative to CNIs. Nevertheless, due to controversial results regarding its efficacy (189-192), CNI and mTORi are now prescripted together (193). Current immunosuppressive therapy starts in the perioperative induction period, in order to limit increased inflammation from surgery, ischaemia reperfusion injury and the initial antigen exposure. Next, a maintenance immunosuppression combination is administered, with the goal of achieving stable and low immune responses against the graft. Currently, the common maintenance regimen is a triple strategy consisting of a CNI, steroids and an antiproliferative agent or mTORi (51, 179, 193) (**Table 2**).

***Table 2**. In-use immunosuppressive drugs. The table summarizes the principal immunosuppressive drugs in use in KT, their mechanism of action and the most reported side-effects during treatment. FKBP12: FK506 binding protein; IL-2: interleukin-2; mTOR: serine/threonine protein kinase mechanistic target of rapamycin; mTORc1: mTOR complex 1; mTORc2: mTOR complex 2; NFAT: nuclear factor of activated T cells; PTDM: post-transplant diabetes mellitus.*

Agent	Mechanism of action	Most relevant side-effects
Calcineurin inhibitors: Tacrolimus Cyclosporine A	Blocking activation of the enzyme calcineurin, which leads to the nuclear translocation of the NFAT. Reduction of IL-2 mediated T cell activation and proliferation.	Hypertension and hyperlipidaemia (194, 195) Neurotoxicity (196) PTDM (197) Nephrotoxicity (198)
Antiproliferative agents: Azathioprine Mycophenolate mofetil	Inhibition of *de novo* nucleotide synthesis, preventing proliferation of T and B cells.	Nausea, vomiting and diarrhoea (199)
mTOR inhibitors: Sirolimus Everolimus	Binding to the FKBP12 protein to inhibit mTORc1 and mTORc2 complexes. Inhibition of T and B cell proliferation and differentiation, and antibody production. Inhibition of non-immune cell proliferation.	Hypercholesterolemia (200) Dyslipidaemia (201) Proteinuria (200, 202, 203) Wound-healing complications (204) Anaemia (200)
Corticosteroids: Prednisone Methyl-prednisone	Prevent transcription of interleukin-1 and TNF-α by APCs, causing low MHC expression and decreasing inflammatory reaction at KT site.	Fractures (205) Reduced growth rate in children (206)
Depleting antibodies: Antithymocyte globulin (ATG) Alemtuzumab	Blocking T-cell membrane proteins (CD2, CD3, CD52...) causing prolonged T cell depletion.	Thrombocytopenia (207) Leukopenia (207)
Nondepleting antibodies: Basiliximab Daclizumab	Blocking of IL-2 receptor-α chain (CD25), inhibiting IL-2 induced T cell activation.	Hypersensitivity reactions (uncommon) (39)

2. HYPOTHESES

2. Hypotheses

Kidney transplantation (KT) is the treatment of choice for ESRD, given the improvement in life expectancy and quality of life comparing with long-term dialysis treatment. The use of different immunosuppressive drug combinations after KT is useful for preventing allograft rejection. Unfortunately, these treatments are associated with the development of multiple side-effects, such as infection, hypertension, nephrotoxicity, or neoplasia. In order to overcome these side effects, the use of different immunosuppressive medications or conversion from one drug to another are common in the clinical practice. From the early 2000s onwards, the detection of HLA donor-specific antibodies (HLA-DSA) has been extensively proven to be associated with the production of ABMR. However, it should be noted that histological ABMR cases without evidence of circulating HLA-DSA have already been described, suggesting a potential role for non-HLA antibodies in this type of damage. In this regard, HLA epitope mismatch analysis has been proposed as a better strategy than classical HLA antigen mismatch for prevention of HLA-DSA development, although its clinical relevance remains under discussion. Furthermore, in the last few years, an increasing number of reports have unravelled the implication of immune cells in both kidney allograft rejection and tolerance.

To this day, the impact of immunosuppression treatment modifications on peripheral blood immune cell subsets and the development of HLA-DSA is still unclear. Hereby, a better understanding of the impact of immunosuppression changes in immunological biomarkers may be important for improving allograft outcomes. Moreover, there is increasing evidence that the prevalence of non-HLA antibodies in serum of KT recipients is high, and the heterogeneity of post-KT clinical courses included in previous studies may hamper the correct identification of deleterious non-HLA antibodies.

Considering all previous knowledge, we hypothesize that:

1. Changes in the immunosuppression treatment after KT may modulate: a) the polarization of immune cell populations, and b) the development of HLA-DSA.
2. The analysis of pre- and post-KT HLA and non-HLA antibodies might be helpful for better delineating the risk of ABMR in KT recipients.
3. The immune monitoring of KT recipients - comprising HLA and non-HLA antibodies, HLA epitope mismatch analysis and immune cell subpopulations – may help to better tailor their immunological risk of rejecting the graft.

3. OBJECTIVES

3. Objectives

Main objective:

To evaluate whether immune monitoring - comprising HLA and non-HLA antibodies, HLA epitope mismatch analysis and peripheral blood immune cell subpopulations - may be helpful to better stratify the immunological risk of KT recipients.

Secondary objective 1:

To analyse the long-term influence of two immunosuppression treatment modifications: steroid withdrawal and conversion from a CNI to a mTORi, on HLA-DSA development.

Secondary objective 2:

To assess putative changes on peripheral blood immune cell subpopulations (T, B and NK cells) after immunosuppression treatment modifications; and to determine if they are transient or long-term maintained.

Secondary objective 3:

To evaluate the role of pre- and post-KT HLA and non-HLA antibodies in the development of histological antibody-mediated rejection.

Secondary objective 4:

To assess the usefulness of HLA epitope mismatch analysis for the prediction of:

a) *de novo* HLA-DSA, in comparison with classical HLA antigen mismatch analysis.
b) histological antibody-mediated rejection development in KT recipients.

4. ACCEPTED ARTICLES

Peripheral blood lymphocyte subsets change after steroid withdrawal in renal allograft recipients: a prospective study

Laura Llinàs-Mallol[1,2]; Dolores Redondo-Pachón[1,2]; María José Pérez-Sáez[1,2]
Dàlia Raïch-Regué[1,2]; Marisa Mir[1]; José Yélamos[2,3]; Miguel López-Botet[2,3]
Julio Pascual[1,2]; Marta Crespo[1,2]

[1] Department of Nephrology, Hospital del Mar, Barcelona, Spain
[2] Institute Hospital del Mar for Medical Research, Barcelona, Spain
[3] Department of Immunology, Hospital del Mar, Barcelona, Spain

Scientific Reports 2019; 9, 7453.
https://doi.org/10.1038/s41598-019-42913-4

SCIENTIFIC REPORTS

OPEN

Peripheral blood lymphocyte subsets change after steroid withdrawal in renal allograft recipients: a prospective study

Received: 18 October 2018
Accepted: 10 April 2019
Published online: 15 May 2019

Laura Llinàs-Mallol[1,2], Dolores Redondo-Pachón[1,2], María José Pérez-Sáez[1,2], Dàlia Raïch-Regué[1,2], Marisa Mir[1], José Yélamos[2,3], Miguel López-Botet[2,3], Julio Pascual[1,2] & Marta Crespo[1,2]

Several studies have assessed clinical outcomes after steroid withdrawal (SW) in kidney transplant (KT) recipients, but little is known about its potential impact on lymphocyte subpopulations. We designed a prospective study to evaluate the long-term impact of SW in 19 KT recipients compared to 16 KT recipients without changes in immunosuppression (steroid maintenance, SM). We assessed renal function, presence of HLA antibodies and peripheral blood lymphocyte subsets at time of inclusion, and 3, 12 and 24 months later. The immunophenotype of 20 healthy subjects was also analyzed. Serum creatinine and proteinuria remained stable in SW and SM patients. SW did not associate with generation of *de novo* donor-specific antibodies. SW patients showed decreases in T-lymphocytes ($p < 0.001$), and in the $CD4^+$ T cell subpopulation ($p = 0.046$). The proportion of B-lymphocytes ($p = 0.017$), and both naïve and transitional B cells increased compared to SM patients ($p < 0.001$). Changes in B cell subsets were detected 3 months after SW and persisted for 24 months. No changes were observed in NK cells related to steroid withdrawal. SW patients displayed significant changes in peripheral T and B cell subsets, transitioning to the phenotype detected in healthy subjects. This may be considered as a maintained positive effect of SW previously unnoticed.

Kidney transplantation is the best therapeutic option for patients with end-stage renal disease, given the improvement in quantity and quality of life compared with long-term dialysis[1–4]. Short-term graft-survival rate is over 95% one year after KT, but long-term survival is still limited, being the half-life around 10 years for kidney grafts from deceased donors[5,6]. Graft failure may occur due to loss of graft function or recipient death with a functioning graft[7]. Acute rejection in low-immunological risk KT recipients is below 15% with current immunosuppression, based on calcineurin inhibitors (CNI), antiproliferative agents such as mycophenolic acid (MPA) and steroids, and rarely produces graft-loss[8,9]. However, chronic rejection associated with donor-specific antibodies (DSA), also known as antibody-mediated rejection (AMR), has arisen as a major cause of graft loss, despite this immunosuppression strategy[7,10–12]. In the other hand, cardiovascular disease and cancer account for an excess of mortality in the transplant population[13–15]. Alternative drugs oriented to avoid CNI toxicity, such as mTOR inhibitors[9,16] or CD80/CD86 blocker Belatacept™[17], or minimization/withdrawal of some drugs have not achieved enough agreement to replace current immunosuppression.

Steroids are effective in reducing the incidence of acute rejection, but also a very frequent cause of undesirable effects and morbidity, with potential impact on graft survival[18,19]. Steroid withdrawal (SW) has been attempted for years by many groups, and the overall outcomes suggest that acute rejection rates increase, without clear advantages in morbidity and mortality[20–24]. Therefore, although SW is not generalized, it is applied to selected immunological low-risk KT recipients[25] or in children, where advantage in growth is of utmost importance[26]. The lack of both long-term follow-up studies and evaluation of this treatment strategy on the development of chronic

[1]Department of Nephrology, Hospital del Mar, Barcelona, Spain. [2]Institute Hospital del Mar for Medical Research, Barcelona, Spain. [3]Department of Immunology, Hospital del Mar, Barcelona, Spain. Julio Pascual and Marta Crespo jointly supervised this work. Correspondence and requests for materials should be addressed to J.P. (email: jpascual@psmar.cat) or M.C. (email: MCrespo@parcdesalutmar.cat)

	Steroid withdrawal (N = 19)	Steroid maintenance (N = 16)	p - value
Recipient age (years) [mean (SD)]	53.0 (11.9)	52.4 (13.9)	0.89
Recipient gender (female) (n, %)	8 (42%)	3 (19%)	0.14
Race (caucasian) (n, %)	18 (95%)	14 (88%)	0.58
Type of donor (deceased) (n, %)	15 (79%)	15 (94%)	0.35
Donor age (years) [mean (SD)]	50 (12)	47 (16)	0.50
Time after KT (months) [median (p25–p75)]	19.15 (11.37–34.73)	17.03 (3.04–48.77)	0.73
Induction immunosuppression (antilymphocyte antibodies) (n, %)	3 (16%)	0	0.23
Delayed graft function (n, %)	4 (21%)	4 (25%)	1.00
Pre-inclusion acute rejection (n, %)	0	1 (6%)	0.46
Sensitizing events before KT			
Blood transfusions (n, %)	7 (37%)	2 (13%)	0.14
Previous pregnancies (n, %)	5/8 (63%)	1/3 (33%)	0.55
Retransplantation (n, %)	0	1 (6%)	0.46
HLA mismatches			
HLA mismatch Class I (A/B) [mean (SD)]	3 (1)	3 (1)	0.96
HLA mismatch Class II (DR) [mean (SD)]	1 (1)	1 (1)	0.59
Panel reactive antibodies (PRA)			
Pre-KT PRA CDC > 5% (n, %)	0	0	NA
Peak PRA CDC > 5% (n, %)	4 (21%)	1 (6%)	0.61
Antibodies			
Anti-HLA DSA antibodies (n, %)	0	0	NA
Anti-HLA no DSA antibodies (n, %)	1 (5%)	0	1.00
CMV status			
D^+/R^+	11 (58%)	12 (75%)	0.48
D^+/R^-	5 (26%)	1 (6%)	0.19
D^-/R^+	2 (11%)	3 (19%)	0.64
D^-/R^-	1 (5%)	0	1.00
CMV infection (n, %)	4 (21%)	2 (13%)	0.67

Table 1. Baseline characteristics of the patients. Baseline characteristics of included patients. The table summarizes the baseline characteristics in patients who withdrew steroids (SW) and patients who maintained steroids (SM). *SD: Standard deviation. KT: Kidney transplantation. PRA: panel reactive antibodies. DSA: donor-specific antibodies. D: donor. R: recipient.*

rejection are current limitations when assessing the interest of SW. In this setting, using immunological biomarkers as surrogates of immunological responses may be useful.

Although the role of DSA in promoting AMR and consequent graft loss has been well reported[27,28], the influence of long-term SW in the development of anti-HLA DSA and AMR still remains unclear. Several studies have analyzed different peripheral blood lymphocyte (PBL) subpopulations in patients both before and after KT, finding some correlations with AMR. Higher percentages of T-effector memory lymphocytes correlate with increased risk of allograft dysfunction in stable KT recipients[29], whereas patients with AMR show relevant presence of $CD8^+$ $CD28^-$T lymphocytes[30]. In a recent study, we identified a significant increase of NK cells expressing CD94/NKG2A receptor in patients with HLA DSA, especially in those with AMR[31]. Nevertheless, changes in immunosuppression may modulate the polarization of immune cell populations[32]. Few studies have evaluated the influence of SW on PBL, focused on SW short-term after KT. One study reported a decrease of NK cells under steroid maintenance compared to steroid avoidance[33]. Another report found no differences in peripheral blood lymphocytes between steroid withdrawal and steroid avoidance, in patients who received thymoglobulin as induction therapy[34]. A recent report that studies the association between potential tolerance biomarkers and immunosuppression, included the short-term results of a small cohort of patients who underwent steroid withdrawal early after KT for clinical reasons. The authors showed a significant increase in transitional B cells and no changes in T cells 3–6 months after steroid withdrawal[32]. However, there is no information regarding the influence of long-term SW on PBL subsets.

Based on these data, we designed an exploratory prospective study to analyze the long-term influence of SW on immunological biomarkers.

Results

Study population and clinical follow-up. Thirty-five patients with stable kidney function and without DSA were included in a prospective observational study: 19 withdrew steroids a median time of 19 months after transplantation (steroid withdrawal group, SW) and 16 maintained their baseline treatment, as a control group (steroid maintenance group, SM). Twenty healthy individuals were also immunophenotyped. All patients were

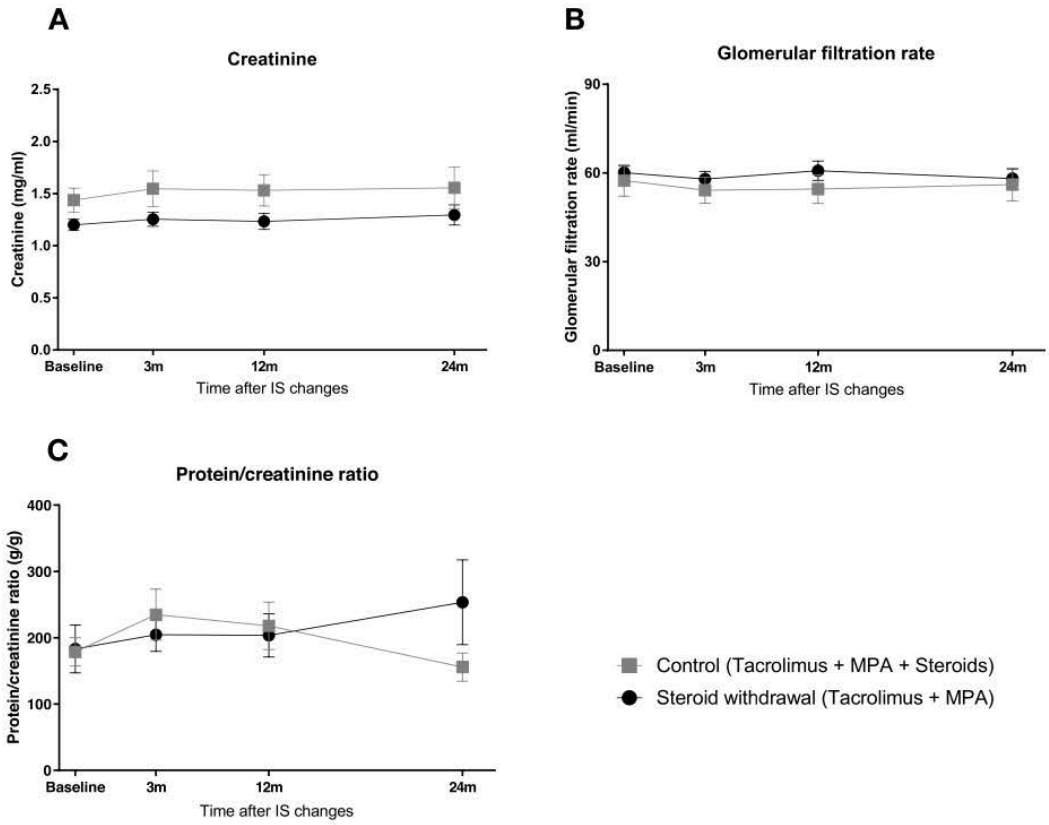

Figure 1. Both groups of the study maintained stable renal function along the follow-up, in terms of serum creatinine, eGFR and proteinuria. Plots show mean and standard error (SEM) for each study group.

followed up to 24 months. Main baseline characteristics of SW and SM patients are included in Table 1. Groups were similar in age, race, donor type, donor age, HLA mismatches, or previous acute rejection rates. The time post transplantation is detailed in Supplementary Fig. 1. A comparison between all lymphocyte subsets at baseline is shown in Supplementary Table 1. Patients in both groups showed stable renal function with no significant changes in serum creatinine, estimated glomerular filtration rate (eGFR) and proteinuria, measured as urinary protein/creatinine ratio during the study period (Fig. 1A–C). There was no graft loss or death along the study in any group. Furthermore, no patient in the steroid withdrawal group needed to reintroduce steroids.

Steroid withdrawal did not promote the development of *de novo* DSA. No patient developed *de novo* DSA during the 24 months of sequential evaluation. Four patients developed *de novo* HLA no-DSA: two in the SW group (11%) and two in the SM group (13%) ($p = 1.00$). One patient in the SW group had HLA no-DSA class I and class II prior to SW and maintained these antibodies along the study.

T cells decrease whereas B cells increase after steroid withdrawal. Patients who underwent SW showed a significant decrease in the percentage of circulating T cells during the first year of the study, followed by stabilization during the second year (baseline: $79.3 \pm 9.6\%$, 12 months: $72.4 \pm 12.6\%$, 24 months: $73.6 \pm 11.4\%$; $p < 0.001$) (Fig. 2A). On the contrary, the SM group showed no changes in T cells along the study ($p = 0.24$). Evolution of T cells between the two groups was significantly different ($p < 0.001$). T cells from SW patients reached similar levels to those of healthy subjects, in contrast to the SM group (Fig. 2A). This effect was also observed when measuring absolute numbers (Fig. 2A, SW $p = 0.027$; SM $p = 0.24$; between groups $p = 0.038$).

The proportion of B cells increased during the follow-up in SW patients (baseline: $5.7 \pm 3.9\%$, 24 months: $7.8 \pm 4.8\%$, $p = 0.005$) (Fig. 2B), but not in SM group ($p = 0.41$). Evolution of B cells between groups was significantly different ($p = 0.017$). Twenty-four months after SW, the proportion of B cells reached the level of healthy subjects, but the SM group did not (Fig. 2B). Absolute numbers of B cells behaved similarly (SW $p = 0.023$; SM $p = 0.86$) (Fig. 2B).

The NK cell percentage increased significantly within the first year and stabilized afterwards in both groups (SW $p = 0.002$; SM $p < 0.001$, Fig. 2C). The evolution was different between groups, with the highest peak reached by the SM group at three months ($p < 0.001$, Fig. 2C). No differences due to SW could be identified in NK cell subsets considering the expression of $NKG2A^+$, $NKG2C^+$ (Supplementary Fig. 2A,B), $ILT2^+$, KIR^+ and $CD161^+$ (data not shown).

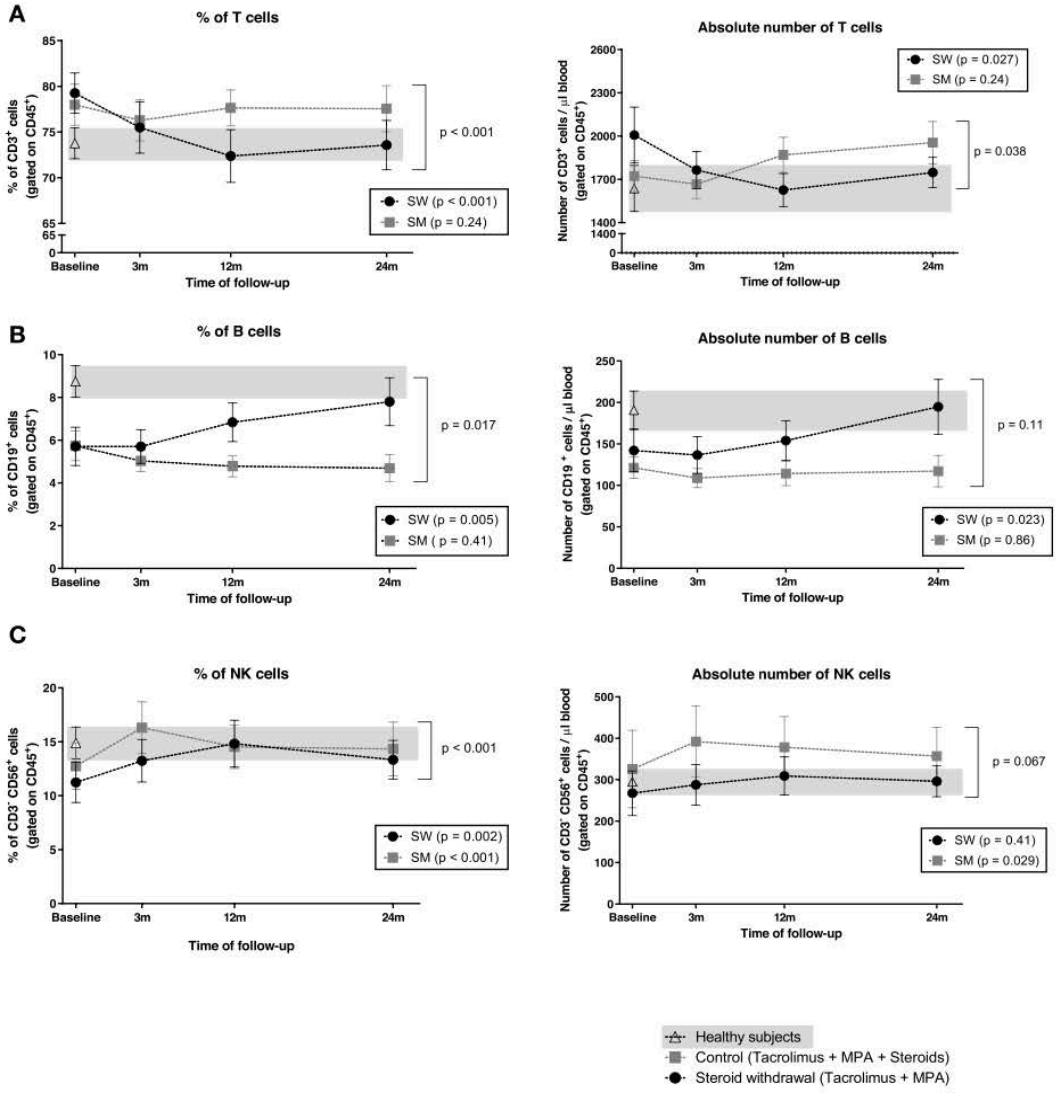

Figure 2. Evolution of T cells, B cells and NK cells percentages and absolute numbers after SW. Immunophenotyping analysis of (**A**) T cells ($CD3^+$), (**B**) B cells ($CD19^+$) and (**C**) NK cells ($CD3^-$ $CD56^+$) in patients before and after SW (black dots) and patients maintaining steroids (grey squares). HS data is depicted with white triangles and HS range is highlighted with a grey background. Dots show mean and SEM for each time point.

Steroid withdrawal promotes a decrease of $CD4^+$ T cells. In order to understand differences observed on T cells, we analyzed T cell subsets. $CD4^+$ T cells decreased significantly during the first year after SW (baseline: $53.2 \pm 17.2\%$, 12 months: $48.4 \pm 16.3\%$) and returned to baseline thereafter (24 months $52.7 \pm 15.6\%$) (Fig. 3A). The two groups of treatment had a different evolution of both proportion ($p = 0.046$) and absolute numbers ($p = 0.023$) of $CD4^+$ T cells (Fig. 3A). $CD8^+$ T cells did not display significant changes (Fig. 3B).

Higher percentages of B cells after steroid withdrawal are mainly due to an increase of naïve B cells. Analysis of B cell subsets identified a significant increase of naïve B cells after SW (baseline: $62.5 \pm 13.2\%$, 24 months: $75.1 \pm 12.9\%$; $p < 0.001$), in contrast to SM group ($p = 0.35$), being the evolution significantly different between groups ($p < 0.001$) (Fig. 4A). Absolute numbers of naïve B cells behaved similarly (SW $p = 0.002$; SM $p = 0.74$; between groups $p = 0.015$) (Fig. 4A). In SW patients, but not in SM patients, both percentages and absolute numbers of naïve B cells reached similar levels to those of healthy subjects (Fig. 4A).

Regarding memory B cells, SW patients showed significantly lower percentages along the follow-up ($p < 0.001$) compared to SM patients ($p = 0.35$) (between groups $p = 0.002$) (Fig. 4B). Absolute numbers did not reflect these changes (SW $p = 0.14$; SM $p = 0.47$; between groups $p = 0.24$) (Fig. 4B). The analysis of naïve and memory B cell subsets using a different immunophenotyping strategy based on CD38 and IgD expression[35] showed results consistent with the CD27 and IgD staining (data not shown).

Steroid withdrawal promotes an increase of circulating transitional B cells. We found a significant increase in the percentage of transitional B cells three months after SW (baseline: $2.6 \pm 1.9\%$, 24 months:

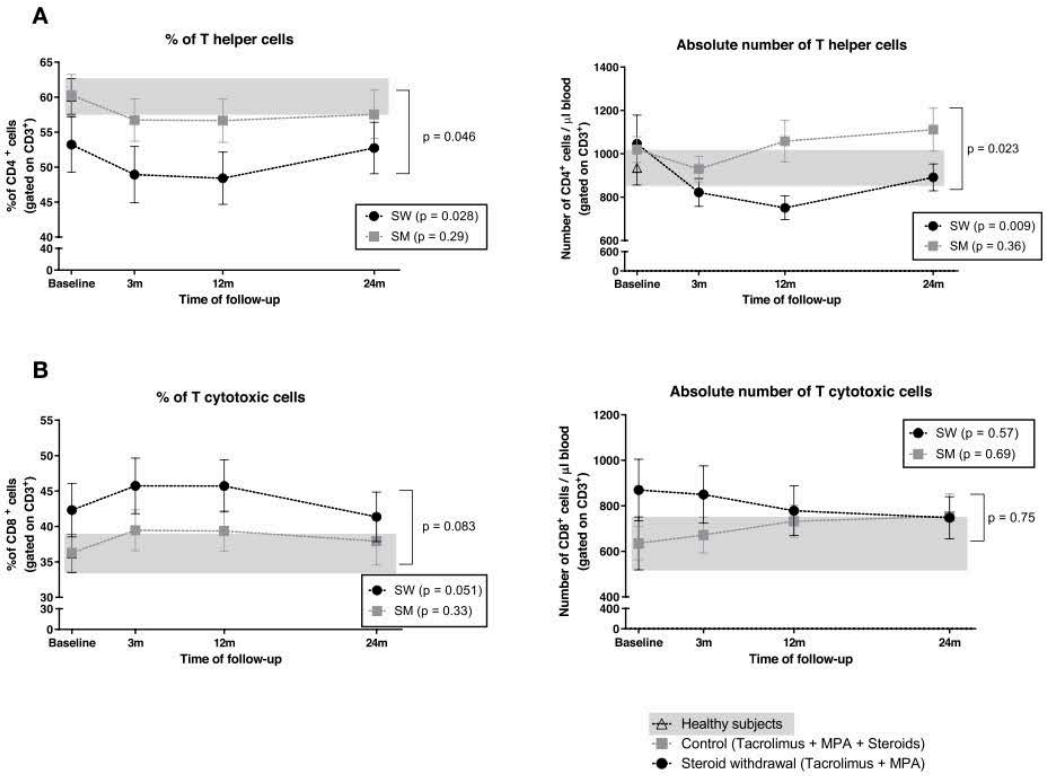

Figure 3. Lower percentage and absolute numbers of $CD4^+$ T cells after SW. Immunophenotyping analysis of percentage and absolute numbers of (**A**) $CD4^+$ T cells ($CD3^+CD4^+$) and (**B**) $CD8^+$ T cells ($CD3^+CD8^+$) in patients before and after SW (black dots) and maintaining steroids (grey squares). HS data is depicted with white triangles and HS range is highlighted with a grey background. Dots show mean and SEM for each time point.

4.5 ± 2.2; $p < 0.001$) compared to SM group ($p = 0.72$) (Fig. 4C). Absolute numbers showed similar results (Fig. 4C). SW and SM patients displayed a significantly different evolution in both the proportion and absolute numbers of transitional B cells (both $p < 0.001$). Moreover, SW patients presented similar numbers to those of healthy subjects (Fig. 4C). In order to conform the bibliographic evidence[36–39] supporting current transitional B cell characterization as $CD19^+CD24^{hi}CD38^{hi}$ cells, we assessed the percentage and absolute numbers of transitional B cells from 20 KT recipients including a CD24 immunophenotyping strategy (Fig. 5A). Our results indicate a strong correlation between $CD19^+CD38^{hi}IgD^+$ and $CD19^+CD24^{hi}CD38^{hi}$ populations (Pearson correlation value 0.947, $p < 0.001$) (Fig. 5B).

Discussion

Although steroid avoidance has been associated with increased risk of acute rejection[20–24], there is growing evidence that SW may offer several advantages in low risk KT patients[22]. Our results support that SW can be accomplished in immunologically low-risk KT patients without compromising renal function the first two years after steroid withdrawal. Interestingly, Haller *et al.* have recently suggested that SW may be safe if it is undertaken beyond 18 months after KT[24]. The median post-KT time of our cohort at SW was 19 months. We found that patients who underwent steroid withdrawal maintained stable renal function without appearance of *de novo* anti-HLA DSA and they showed decreased circulating T $CD4^+$ cells and increased naïve and transitional B cells, a similar immunophenotype profile to the one of healthy subjects.

Steroid withdrawal in low-risk KT recipients has been shown to be safe in terms of *de novo* DSA development[40–42]. Two studies showed no influence of early or mid-term SW on *de novo* DSA development[40,41]. In agreement, our long-term SW patients did not show *de novo* HLA DSA antibody during two years of follow-up.

The effect of SW on peripheral T cells is less clear in the literature. A recent study[32] reported no significant differences when comparing T cell percentages before and 3–6 months after SW. However, the fact of doing a longer follow-up allowed us to see a significant decrease in total T and $CD4^+$ T subset in the SW group. This decrease seems to be restricted to the first year after SW, although $CD4^+$ T cell numbers did not recover baseline levels. In contrast, the percentage and absolute numbers of $CD8^+$ T cells did not significantly change in the SW group, although dynamics of $CD8^+$ T cells percentages reflected the changes of $CD4^+$ T cells percentages. This supports the hypothesis that SW promotes a reduction of circulating $CD4^+$ T cells in KT patients. The role of glucocorticoids on the thymopoiesis remains uncertain, but there is evidence supporting that steroids promote the differentiation of Th2 over Th1 cells[43]. Further studies considering specific $CD4^+$ T cell subpopulations may clarify this particular issue.

To our knowledge, few studies have explored B cell dynamics before and after SW[32–34]. These studies did not describe results of naïve and memory B cell subsets. Our cohort of KT recipients after long-term SW experienced a

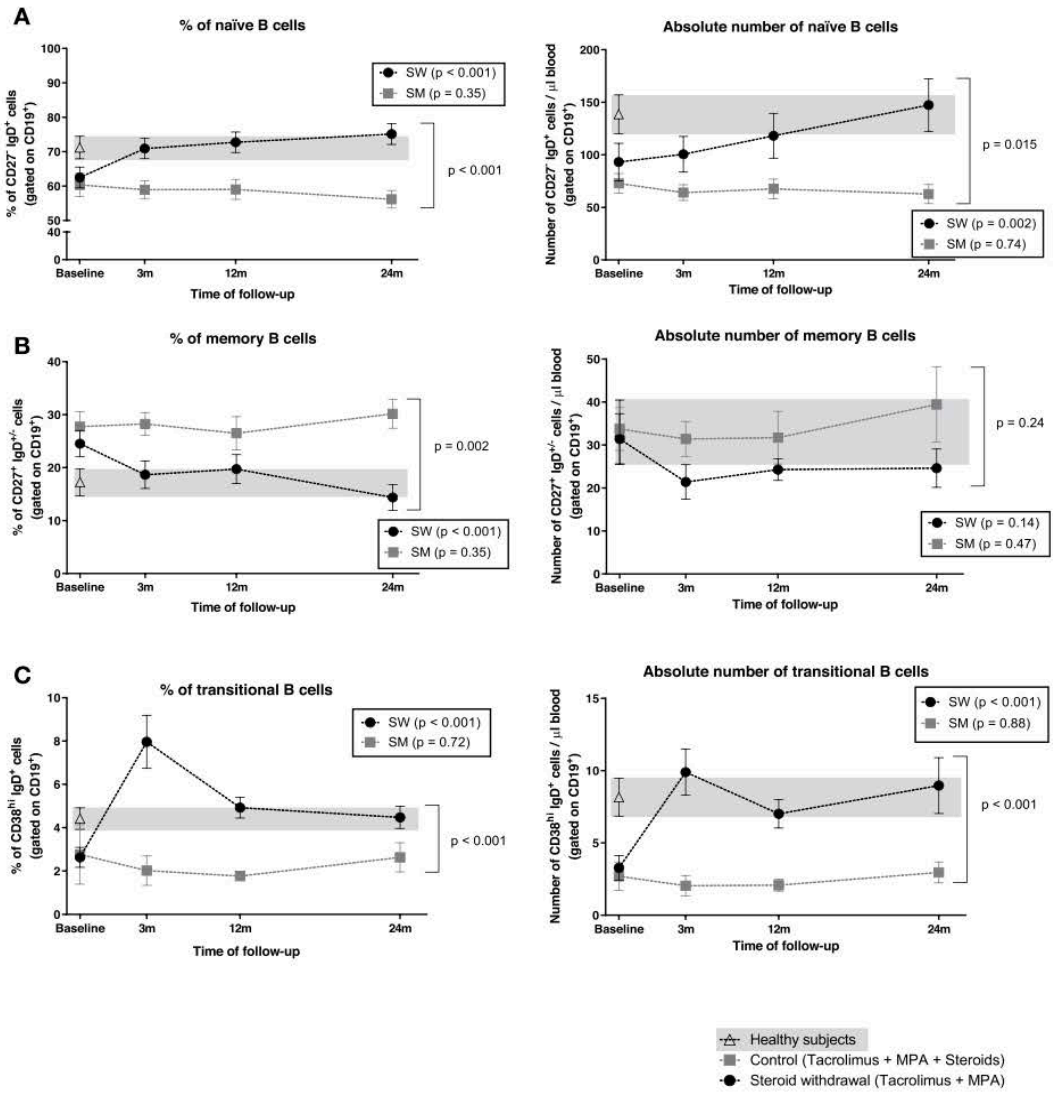

Figure 4. Evolution of B cell subsets percentages and absolute numbers after SW. Immunophenotyping analysis of percentage and absolute numbers of (**A**) naïve B cells ($CD19^+$ $CD27^-$ IgD^+), (**B**) memory B cells ($CD19^+$ $CD27^+$ $IgD^{+/-}$) and (**C**) transitional B cells ($CD19^+$ $CD38^{hi}$ IgD^+) in patients before and after SW (black dots) and patients maintaining steroids (grey squares). HS data is depicted with white triangles and grey background corresponds to HS range. Plots show mean and SEM for each time point.

significant increase in total B cells without an increase in memory B cells, potentially related to antibody production. The B cell increase was attributable to a greater number of circulating naïve B cells, which do not secrete immunoglobulins[44] and are more sensitive to glucocorticoid-induced apoptosis[43,45]. This may explain the observed relative reduction in the proportion of memory B cells but not their absolute numbers. Our results demonstrate for the first time that SW promotes a gradual increase in naïve B cells in KT recipients along the follow up, reaching the levels of healthy subjects. Regarding transitional B cells, patients under conventional immunosuppression (based on CNI, mycophenolic acid and steroids) have shown significantly lower levels than healthy subjects[46]. On the other hand, Rebollo-Mesa *et al.* found that the percentage of transitional B cells increased in KT recipients 3 to 6 months after SW[32]. Remarkably, we have also found a striking increase of transitional B cells 3 months after SW, reaching similar levels to heathy subjects after one year, whereas SM patients maintained stable numbers during the follow-up. This effect may also be due to the sensitivity of early mature B cells to glucocorticoid-induced apoptosis[43,45].

The expansion of B cells and differential expression of B cell-related genes have been described as biomarkers of tolerance in previous studies[47–49]. Our results show that the B cell immunophenotype 1-year after SW resembles the one expressed by healthy subjects. These results suggest that steroid treatment in KT recipients is disrupting normal B cell subset distribution[45]. Immunosuppression has been related not only to a distinct lymphocyte distribution but also to their gene expression in peripheral blood[32]. The estimated genetic probability of tolerance increased after SW, independently of the peripheral expansion of transitional B cells. The important function that transitional B cells may play in transplantation tolerance arises as an open question, connected to their potential

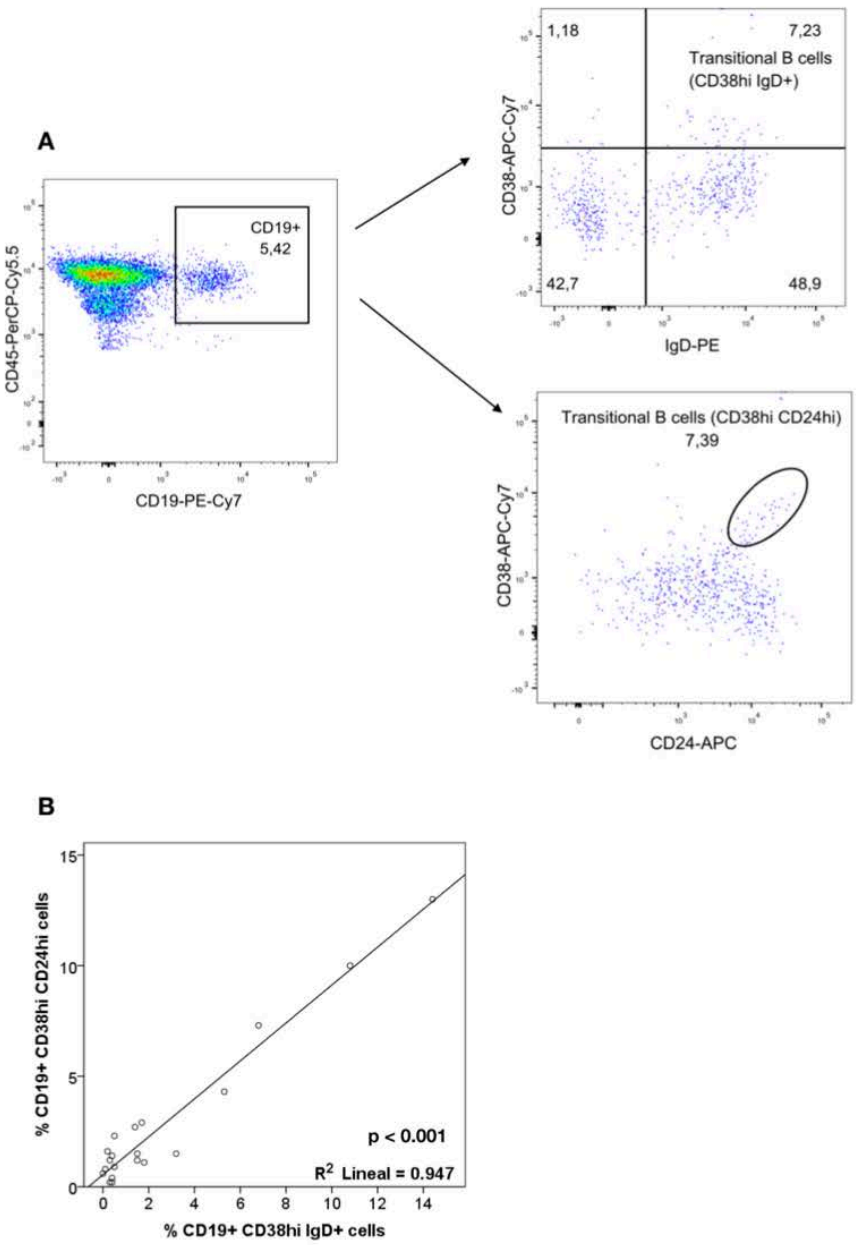

Figure 5. Characterization of transitional B cells. (**A**) Representative flow cytometry plots from the same patient, including or not CD24 staining. (**B**) Correlation graph and Pearson correlation value for the $CD19^{+}CD38^{hi}IgD^{+}$ and $CD19^{+}CD24^{hi}CD38^{hi}$ populations.

anti-inflammatory function. "Operational tolerance" has been hypothesized to be an immunological state of not recognizing the graft as "foreign", independently of the immunosuppression treatment, rather than a condition in which inflammatory processes are controlled by steroids or other immunosuppressive treatments[50].

The small sample size and the absence of graft-biopsies do not permit to evaluate whether the observed changes in PBL correlate with either tolerance or subclinical damage, and therefore the influence on graft outcomes. However, we present the results of the first exploratory study with PBL analysis long-term after SW in a homogeneous clinically stable KT group of patients. Functional studies could be of interest to understand the pathophysiological consequences of SW on the peripheral distribution of T and B cell subsets.

In summary, our study confirms that SW in low-risk KT recipients is safe in terms of renal function and does not associate with the generation of *de novo* DSA. SW leads to a distinct peripheral lymphocyte distribution in KT patients, which resembles that of healthy subjects. These changes are similar to those described in tolerant KT patients and opposite to the profile found in KT patients with chronic rejection.

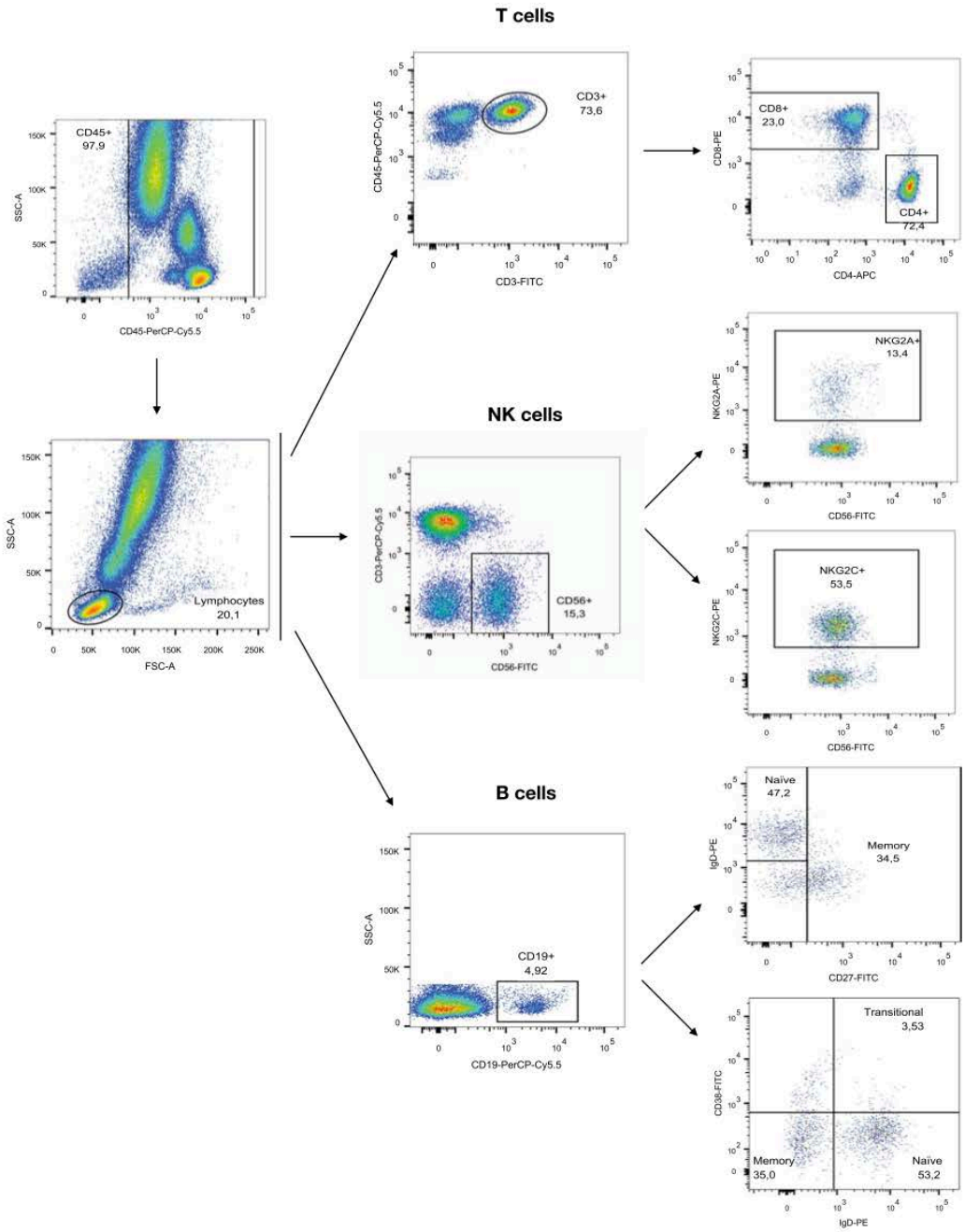

Figure 6. Gating strategy for studying PBL subpopulations. Representative flow cytometry plots from the same patient, illustrating the gating strategy used for the study. (**Above**) Gating strategy for T cell tube. (**Middle**) Gating strategy for NK cell tube. (**Below**) Gating strategy for B cell tube.

Materials and Methods

Study design and population. This is a prospective observational study where we evaluated the potential influence of immunosuppression changes in the development of HLA antibodies and the distribution of PBL subsets in 35 patients who underwent kidney transplantation at Hospital del Mar, Spain. KT recipients with stable renal function, without proteinuria and DSA who withdrew steroids (SW) per clinical practice at the out-patient clinic were included from June 2011 to September 2015. On the other hand, a parallel group of patients who maintained steroids (SM) were included as a control group.

Initially, all patients received 5 mg of prednisone/day, together with tacrolimus (mean trough blood level 7.7 ng/mL) and mycophenolate acid (mean daily dose 570 mg). At last follow-up tacrolimus trough blood levels were similar in both groups (SW 6.8 ng/mL and SM 6.49 ng/mL) and mycophenolate acid dose stayed stable (SW 508 mg/day and SM 566 mg/day). Clinical evaluation (serum creatinine, eGFR and proteinuria), HLA antibody analysis and PBL immunophenotyping were performed before and 3, 12 and 24 months after SW or inclusion. In addition, PBL subsets of 20 healthy subjects were analyzed. The study was approved by the CEIC Parc de Salut

Mar Ethical Research Board (2011/4385/I) and all patients signed informed consents. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul and the Declaration of Helsinki. No organs were procured from prisoners.

Determination of HLA antibodies. Serum samples were collected and stored at −80 °C until analysis. Screening for anti-HLA antibodies was performed with Luminex Lifecodes LifeScreen Deluxe assay (*Gen-probe®*, Stanford), and anti-HLA alloantibody identification was performed using Lifecodes LSA Class-I (93 beads) and/or Class-II (84 beads) assays (*Gen-probe®*, Stanford), as previously described[51]. Donor HLA antibody specificity was ascribed following the results of single antigen assay, considering donor HLA typing or linkage disequilibrium for C or DQ antigens when typing was not available. A reaction with mean immunofluorescence intensity over 1000 was considered positive.

Immunophenotyping analysis. Immunophenotyping was performed by flow cytometry on fresh peripheral blood samples, obtained by venous puncture in EDTA tubes. Samples were pretreated with saturating concentrations of human aggregated immunoglobulins to block FcγR and then labeled with different antibody combinations to define T and B lymphocytes, and NK-cell subsets in separated tubs (Supplementary Table 2 and Fig. 6). Samples were acquired by a FACS Canto II cytometer and data analyzed by FACS Diva and FlowJo softwares (BD Biosciences™). T lymphocytes were characterized as $CD3^+$ cells, T helper as $CD3^+CD4^+$cells and T-cytotoxic as $CD3^+CD8^+$ cells. B lymphocytes were characterized as $CD19^+$ cells and subpopulations were analyzed considering IgD and either CD27 or CD38 expression[35]: naïve ($CD27^-IgD^+$ or $CD38^-IgD^+$), memory ($CD27^+IgD^{+/-}$ or $CD38^-IgD^-$) and transitional B cells ($CD38^{high}IgD^+$)[35]. NK cells were identified as $CD3^-CD56^+$ cells and subsets were defined by expression of NKG2A, NKG2C, ILT2, KIR or CD161. Absolute numbers of cells were calculated from parallel blood counts and percentages of subsets referred to total $CD3^+$, $CD19^+$ or $CD3^-CD56^+$ cells respectively, which in turn are referred to total lymphocytes. Validation of transitional B cell immunophenotype was performed comparing the $CD19^+CD38^{hi}IgD^+$ and $CD19^+CD24^{hi}CD38^{hi}$ gating strategies in 20 KT recipients from another cohort (Fig. 5A,B).

Statistical analysis. Comparisons between normally distributed variables were carried out by using Student's t-test and non-parametric variables were analyzed with U Mann-Whitney test. Normal distribution of continuous variables was tested with Kolgorov-Smirnoff and Shapiro-Wilk tests. Chi-squared or Fisher's exact tests were used for dichotomous variables. Generalized Estimating Equations (GEE) population-averaged model was used for analyzing changes in PBL subpopulations, including an interaction term in order to check differences between study groups. Two p-values were obtained, one for each study group and PBL subpopulation evolution (therefore representing the comparison between baseline and 3, 12 and 24 months data) and another one evaluating the differences in the evolution of each PBL subpopulation between the two groups of study. A p value < 0.05 was considered statistically significant. We have further performed an alternative analysis with a repeated measures ANOVA test for PBL subpopulations (Supplementary Table 3). Statistical analysis was performed using SPSS® v.22.0 (IBM Corp, New York, USA) and Stata® v.15 (STATA Corp, Texas, USA) for clinical and cellular data respectively.

Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available because they have not been uploaded in a public database but are available from the corresponding author on reasonable request.

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Acknowledgements

We are indebted to Dulce Soto, Carme García, Sara Álvarez, Anna Faura, Judit Rigol and Rosa Causadias for their technical support. Xavier Duran (Department of Methodological Advice in Biomedical Research, Institute Mar for Medical Research, Barcelona, Spain) was of great help in statistical analyses. L LL-M did this study as part of her doctoral thesis program at the Department of Medicine from the Universitat Autonoma of Barcelona (UAB). This study was performed with funding from projects PI10/01370, PI13/00598 and PI16/00617 (Spanish Ministry of Health ISCIII FIS-FEDER), RD12/0021/0024 and RD16/0009/0013 (ISCIII FEDER RedinRen) and a grant from the Fundació Catalana de Trasplantament (to L LL-M).

Author Contributions

L.L.M. performed the study, analyzed data and wrote the paper; D.R.P., designed and performed the study, analyzed data and wrote the paper; M.J.P.S. collected data and wrote the paper; D.R.R. analyzed data and wrote the paper; M.M. collected data; J.Y. and M.L.B. analyzed data and supervised the study; J.P. and M.C. designed and performed the study, analyzed data, supervised the study and wrote the paper. All authors reviewed the manuscript draft and approved the final version.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-42913-4.

Competing Interests: The authors declare no competing interests.

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Supplementary Information

Peripheral blood lymphocyte subsets change after steroid withdrawal in renal allograft recipients: a prospective study

Laura Llinàs-Mallol[1,2] MSc, Dolores Redondo-Pachón[1,2] MD PhD, María José Pérez-Sáez[1,2] MD PhD, Dàlia Raïch-Regué[1,2] PhD, Marisa Mir[1] MD, José Yélamos[2,3] PhD, Miguel López-Botet[2,3] PhD, Julio Pascual[1,2 # *] MD PhD and Marta Crespo[1,2 #] MD PhD.

[1]Department of Nephrology, Hospital del Mar, Barcelona, Spain.

[2]Institute Hospital del Mar for Medical Research, Barcelona, Spain.

[3]Department of Immunology, Hospital del Mar, Barcelona, Spain.

* Corresponding author. E-mail: jpascual@psmar.cat

Senior authors

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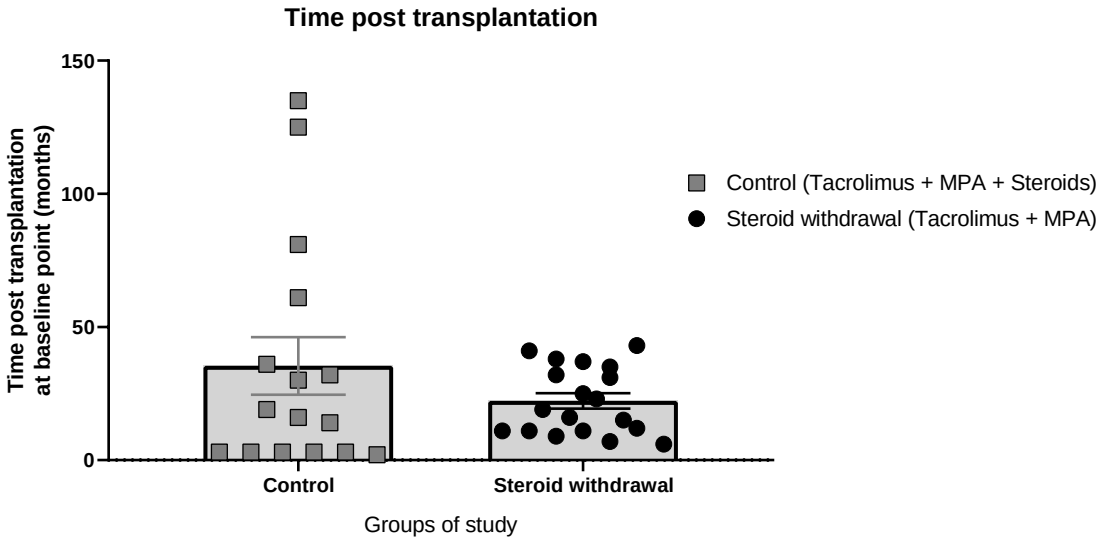

Supplementary Figure 1. Time post transplantation for each of the 35 patients at the baseline point of the study. Black dots represent patients who withdrew steroids and grey squares patients that maintained steroids.

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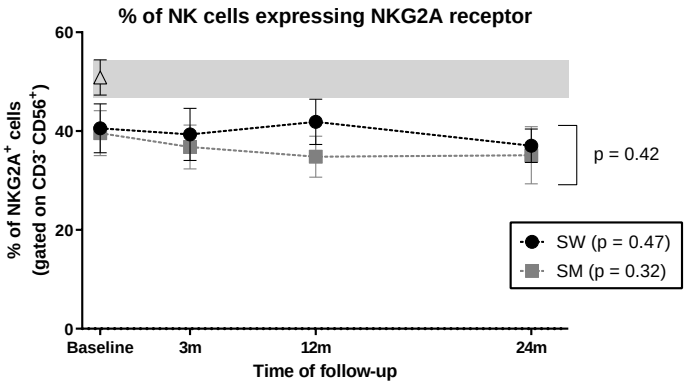

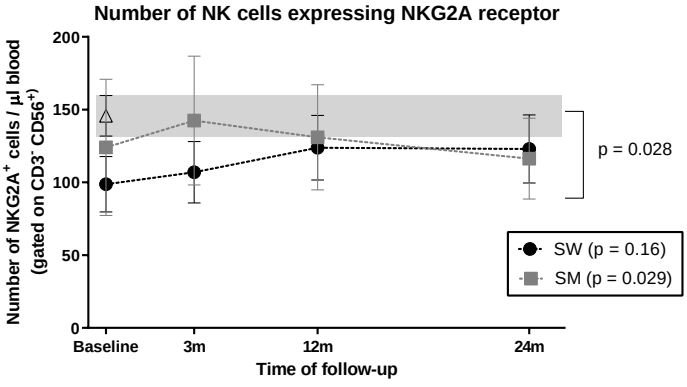

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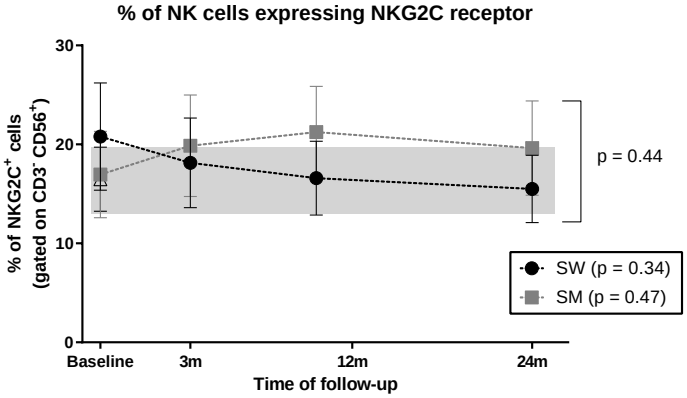
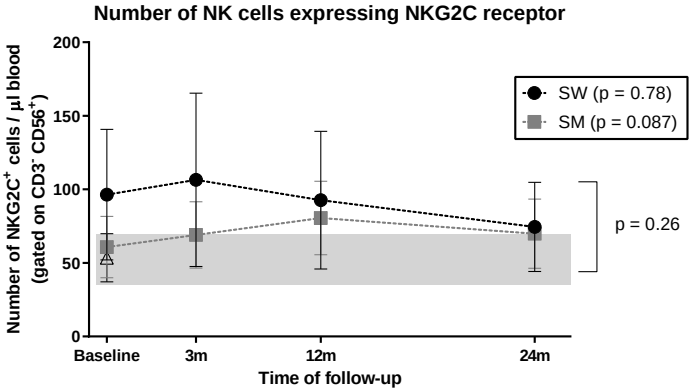
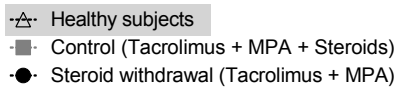






Supplementary Figure 2. Evolution of NK cell subsets NKG2A+ and NKG2C+ percentages and absolute numbers after SW. Immunophenotyping analysis of percentage and absolute numbers of **(A)** NKG2A+ NK cells (CD3- CD56+ NKG2A+) and **(B)** NKG2C+ cells (CD3- CD56+ NKG2C+) in patients before and after SW (black dots) and patients maintaining steroids (grey squares). HS data is depicted with white triangles and grey background corresponds to HS range. Plots show mean and SEM for each time point.

Supplementary Table 1. Values of lymphocyte subsets at baseline time. Values of each lymphocyte subset are expressed in mean and standard error of mean (SEM) and p-values are obtained from a Mann-Whitney-U-test.

	Steroid withdrawal (N = 19)	**Steroid maintenance (N = 16)**	**p - value**
Total lymphocytes	2564 (267)	2246 (181)	0.40
% T cells	79.3 (2.2)	78.0 (2.3)	0.71
Absolute number of T cells	2007 (194)	1723 (107)	0.27
% T helper cells	53.2 (4.0)	60.3 (3.0)	0.19
Absolute number of T helper cells	1046 (133)	1020 (59)	0.59
% T cytotoxic cells	42.3 (3.8)	36.3 (2.7)	0.29
Absolute number of T cytotoxic cells	870 (135)	636 (73)	0.27
% B cells	5.7 (0.9)	5.7 (0.7)	0.59
Absolute number of B cells	142 (25)	121 (13)	0.94
% of naïve B cells	62.5 (3.0)	60.3 (3.4)	0.71
Absolute number of naïve B cells	93 (18)	73 (9)	0.91
% of memory B cells	24.5 (2.5)	27.7 (2.8)	0.22
Absolute number of memory B cells	31 (6)	34 (5)	0.40
% of transitional B cells	2.6 (0.5)	2.8 (1.4)	0.10
Absolute number of transitional B cells	3 (1)	3 (1)	0.24
% NK cells	12.4 (2.1)	12.8 (2.2)	0.94
Absolute number of NK cells	369 (114)	326 (94)	1.00
% of $NKG2A^+$ NK cells	39.4 (4.8)	39.6 (4.6)	0.94
Absolute number of $NKG2A^+$ NK cells	115 (24)	124 (47)	0.94
% of $NKG2C^+$ NK cells	21.7 (5.2)	17.0 (4.4)	0.78
Absolute number of $NKG2C^+$ NK cells	96 (44)	61 (21)	0.78

Supplementary Table 2. Antibodies used in the study. The table summarizes antigen, clone, fluorochrome and company for each antibody used in the study.

Antigen	Clone	Fluorochrome	Company
CD3	SK7	FITC / APC / PerCP-Cy5.5	BD Biosciences™
CD4	SK3	APC / PerCP-Cy5.5	BD Biosciences™
CD8	SK1	PE	BD Biosciences™
CD19	SJ25C1	PerCP-Cy5.5	BD Biosciences™
CD27	L128	FITC	BD Biosciences™
CD38	HB-7	FITC	BD Biosciences™
CD45	2D1	PE / APC / PerCP-Cy5.5	BD Biosciences™
CD56	NCAM 16.2	FITC	BD Biosciences™
CD161	HP-3G10	PE	Generated in our lab
IgD	IA6-2	PE	BD Biosciences™
NKG2A	Z199	PE	Provided by Dr. A. Moretta
NKG2C	MAB1381	PE	R&D systems™
ILT2	HP-F1	PE	Generated in our lab
KIR	5.133, CH-L, DX9, HP-3E4	PE	Provided by Drs. M. Colonna; S. Ferrini and L. L. Lanier. HP-3E4 was generated in our lab
F(ab')2 goat anti-mouse IgG + IgM		PE	Jackson ImmunoResearch™

Supplementary Table 3. Results from repeated measures ANOVA test. Table depicts the results from an alternative analysis with a repeated measures ANOVA test for PBL subpopulations.

	Steroid maintenance	Steroid withdrawal	Joint p-value
Total lymphocytes	0.20	0.33	0.23
% T cells	0.60	< 0.001	< 0.001
Absolute number of T cells	0.10	0.006	0.006
% T helper cells	0.23	0.06	0.08
Absolute number of T helper cells	0.32	0.003	0.008
% T cytotoxic cells	0.38	0.15	0.21
Absolute number of T cytotoxic cells	0.41	0.33	0.39
% B cells	0.08	< 0.001	< 0.001
Absolute number of B cells	0.84	< 0.001	0.002
% of naïve B cells	0.28	< 0.001	< 0.001
Absolute number of naïve B cells	0.68	< 0.001	< 0.001
% of memory B cells	0.58	< 0.001	0.002
Absolute number of memory B cells	0.54	0.22	0.36
% of transitional B cells	0.76	< 0.001	< 0.001
Absolute number of transitional B cells	0.88	< 0.001	< 0.001
% NK cells	0.01	0.005	< 0.001
Absolute number of NK cells	0.21	0.62	0.39
% of $NKG2A^+$ NK cells	0.08	0.50	0.16
Absolute number of $NKG2A^+$ NK cells	0.40	0.34	0.39
% of $NKG2C^+$ NK cells	0.26	0.10	0.12
Absolute number of $NKG2C^+$ NK cells	0.36	0.74	0.61

Long-term redistribution of peripheral lymphocyte subpopulations after switching from calcineurin to mTOR inhibitors in kidney transplant recipients

Laura Llinàs-Mallol[1,2]; Dolores Redondo-Pachón[1,2]; Dàlia Raïch-Regué[1,2]
María José Pérez-Sáez[1,2]; José Yélamos[3]; Xavier Duran[4]; Anna Faura[1,2]
Miguel López-Botet[3,5,6]; Julio Pascual[1,2] and Marta Crespo[1,2]

[1] Department of Nephrology, Hospital del Mar, Barcelona, Spain
[2] Nephropathies Research Group, H. del Mar Medical Research Institute, Barcelona, Spain
[3] Department of Immunology, Hospital del Mar, Barcelona, Spain
[4] Methodology & Biostatistics Support Unit, IMIM, Barcelona, Spain
[5] Immunity and Infection Research Group, IMIM, Barcelona, Spain
[6] University Pompeu Fabra, Barcelona, Spain

Journal of Clinical Medicine 2020; 9, 1088.
https://doi.org/10.3390/jcm9041088

Journal of
Clinical Medicine

MDPI

Article

Long-Term Redistribution of Peripheral Lymphocyte Subpopulations after Switching from Calcineurin to mTOR Inhibitors in Kidney Transplant Recipients

Laura Llinàs-Mallol [1,2], Dolores Redondo-Pachón [1,2], Dàlia Raïch-Regué [1,2], María José Pérez-Sáez [1,2], José Yélamos [3], Xavier Duran [4], Anna Faura [1,2], Miguel López-Botet [3,5,6], Julio Pascual [1,2,*] and Marta Crespo [1,2,*]

1 Department of Nephrology; Hospital del Mar, 08003 Barcelona, Spain
2 Nephropathies Research Group, Hospital del Mar Medical Research Institute (IMIM), 08003 Barcelona, Spain
3 Department of Immunology, Hospital del Mar, 08003 Barcelona, Spain
4 Methodology & Biostatistics Support Unit, IMIM, 08003 Barcelona, Spain
5 Immunity and Infection Research Group, IMIM, 08003 Barcelona, Spain
6 University Pompeu Fabra, 08003 Barcelona, Spain
* Correspondence: jpascual@psmar.cat (J.P.); mcrespo@psmar.cat (M.C.); Tel.: +34-932-4832-74 (J.P.); +34-932-4831-62 (M.C.)

Received: 6 March 2020; Accepted: 8 April 2020; Published: 11 April 2020

Abstract: Classical immunosuppression based on steroids, calcineurin inhibitors, and mycophenolate results in several unwanted effects and unsatisfactory long-term outcomes in kidney transplantation (KT). New immunosuppressors search for fewer adverse events and increased graft survival but may have a distinct impact on graft function and immunological biomarkers according to their mechanism of action. This prospective study evaluates the immunological effect of tacrolimus to serine/threonine protein kinase mechanistic target of rapamycin inhibitors (mTORi) conversion in 29 KT recipients compared with 16 controls maintained on tacrolimus. We evaluated renal function, human leukocyte antigen (HLA) antibodies and peripheral blood lymphocyte subsets at inclusion and at 3, 12, and 24 months later. Twenty immunophenotyped healthy subjects served as reference. Renal function remained stable in both groups with no significant change in proteinuria. Two patients in the mTORi group developed HLA donor-specific antibodies and none in the control group (7% vs. 0%, $p = 0.53$). Both groups showed a progressive increase in regulatory T cells, more prominent in patients converted to mTORi within the first 18 months post-KT ($p < 0.001$). All patients showed a decrease in naïve B cells ($p < 0.001$), excepting those converted to mTORi without receiving steroids ($p = 0.31$). Transitional B cells significantly decreased in mTORi patients ($p < 0.001$), independently of concomitant steroid treatment. Finally, $CD56^{bright}$ and CD94/NK group 2 member A receptor positive ($NKG2A^{+}$) Natural Killer (NK) cell subsets increased in mTORi-compared to tacrolimus-treated patients (both $p < 0.001$). Patients switched to mTORi displayed a significant redistribution of peripheral blood lymphocyte subpopulations proposed to be associated with graft outcomes. The administration of steroids modified some of these changes.

Keywords: donor-specific antibody; immunophenotype; kidney transplantation; mTOR inhibitors; T regulatory cells; transitional B cells; NK cells

1. Introduction

Kidney transplantation (KT) is the treatment of choice for end-stage renal disease, given the improvement in life expectancy and quality comparing with long-term dialysis [1–4]. Despite the good results in short-term graft survival rates, half-life of renal allografts is around 10 years

under immunosuppression treatment mainly based on steroids, calcineurin inhibitors (CNI), and antiproliferative agents [5–7]. Regardless of the use of this immunosuppressive strategy, development of antibody-mediated rejection (ABMR), a form of rejection associated with donor-specific antibodies (DSA), is a major cause of graft loss [8–12]. Death accounts for around half of graft-losses, mainly due to cardiovascular disease, cancer, or infections [13,14]. These results reflect an unmet need of new immunosuppressive therapeutic regimens more efficient for improving long-term survival rates.

The introduction of the serine/threonine protein kinase mechanistic target of rapamycin (mTOR) inhibitors in transplantation pursued the challenge of reducing nephrotoxicity related to the use of CNI [15], so as to increase the life of the graft and to decrease the risk of cancer [16] or infections [17]. The intracellular mTOR complex has an important role in the modulation of both innate and adaptive immune responses [18,19]. Inhibition of mTOR displays systemic effects in cell proliferation and has different immunomodulatory effects on antigen-presenting cells, T cells, B cells, Natural Killer (NK) cells, neutrophils, and mast cells [18,19]. It has been reported that mTOR inhibitors (mTORi) stimulate the proliferation of T regulatory (Treg) cells, both in vitro [20,21] and in vivo [22–26]. Currently, there is controversial data regarding the effect of mTORi conversion in other peripheral blood T cell subsets [20,27–29]. Moreover, scarce and controversial data are available regarding peripheral blood B cells and NK cells and the use of mTORi [27,30–34].

The development of de novo DSA (dnDSA) has been recognized as a risk factor for graft loss, associated with the development of ABMR [35]. Contradictory data exist regarding the influence of mTORi on the development of dnDSA [36–41]. These controversial results may be due, among other variables, to differences in time post-transplant and on treatment; in dosage and type of mTORi, as well as to concomitant immunosuppressive drugs employed.

Here, we aimed to perform a case-control study of the influence of mTORi conversion on graft function, development of dnDSA, and peripheral blood lymphocytes (PBL) with an extensive prospective follow-up of 2 years.

2. Experimental Section

2.1. Study Design and Population

We designed a prospective observational study to evaluate the potential impact of mTORi treatment on the development of human leukocyte antigen (HLA) antibodies in kidney transplant recipients (KTR) and the distribution of PBL subsets. From April 2011 to December 2015, we recruited KTR who switched from tacrolimus to either everolimus or rapamycin (11 within clinical trials and 18 for clinical reasons, 13 of them due to previous malignancies) and a contemporaneous group of patients maintained on tacrolimus, and who were followed until February 2018. At inclusion, all 45 patients but one—on cyclosporine—received immunosuppression with tacrolimus (mean trough blood level 7.9 ng/mL) with or without concomitant mycophenolate acid (n = 39, mean dose 598 mg/day) and prednisone (n = 35, 5 mg/day). Clinical evaluation (serum creatinine, estimated glomerular filtration rate (eGFR) by Modification of Diet in Renal Disease Study equation (MDRD-4) and proteinuria measured as protein/creatinine in mg/g urine), HLA antibody analysis, and fresh blood immunophenotyping were performed before and 3, 12, and 24 months after mTORi conversion or inclusion. In addition, PBL subsets of 20 healthy subjects (HS) were also analyzed. The study was approved by the Parc de Salut Mar Ethical Research Board (2011/4385/I), and all patients gave written informed consents. Clinical and research activities being reported herein are consistent with the Principles of the Declaration of Istanbul and the Declaration of Helsinki. No organs were procured from prisoners.

2.2. Determination of HLA Antibodies

Serum samples were collected and stored at −80 °C until analysis. Screening for anti-HLA antibodies was performed with Luminex Lifecodes LifeScreen Deluxe assay (Gen-probe®, Stamford, CT, USA), and anti-HLA alloantibody identification was performed using Lifecodes LSA Class-I (93 beads)

and/or Class-II (84 beads) assays (Gen-probe®, Stamford, CT, USA), as previously described [42]. Donor HLA antibody specificity was ascribed following the results of single antigen assays, considering donor HLA typing or linkage disequilibrium for HLA-C or HLA-DQ antigens when typing was not fully available. A reaction with mean immunofluorescence intensity over 1000 was considered positive.

2.3. Immunophenotyping Analysis

Immunophenotyping was performed by flow cytometry on fresh peripheral blood samples, obtained by venous puncture in ethylenediamine tetraacetic acid (EDTA) tubes. Samples were pretreated with saturating concentrations of human-aggregated immunoglobulins to block antibody constant region heavy chain γ receptor (FcγR) and then labelled with different antibody combinations to define T, B and NK-cell subsets in separated tubs as described in Reference [43] (Table S1 and Figure S1). Samples were acquired by a FACS Canto II cytometer, and data were analyzed by FACS Diva v.7 and FlowJo v.10 softwares (BD Biosciences™, Franklin Lakes, NJ, USA), as described [43]. $CD3^+$ T lymphocytes including $CD4^+$ and $CD8^+$ subsets were identified. B lymphocytes were characterized as $CD19^+$ cells, and subpopulations were analyzed considering IgD and either CD27 or CD38 expression [44]. For this study, $CD3^-CD56^+$ NK cell subsets were defined according to CD56 fluorescence intensity ($CD56^{bright}$ and $CD56^{dim}$) and to CD94/NK group 2 member A receptor (NKG2A) and CD94/NK group 2 member C receptor (NKG2C) expression (Figure S1). Absolute cell numbers were calculated from parallel blood counts. Validation of the transitional B cell immunophenotype was performed as previously designated [43] (Figure S2).

2.4. Statistical Analysis

We performed an on-treatment analysis considering data of patients at each study point if they stayed on the intended treatment. Comparisons between normally distributed variables were carried out by using Student's t-test, and nonparametric variables were analyzed with U Mann–Whitney test. Normal distribution of continuous variables was tested with Kolgorov–Smirnoff and Shapiro–Wilk tests. Chi-squared or Fisher's exact tests were used for dichotomous variables. Generalized Estimating Equations (GEE) population-averaged model was used for analyzing changes in PBL subpopulations, including an interaction term in order to check differences between study groups. Two p-values were obtained, one for each study group and PBL subpopulation evolution (therefore representing the comparison between baseline and 3-, 12-, and 24-month data) and another one evaluating the differences between the two groups of study in the evolution of each PBL subpopulation. Analysis of T regulatory cells was also adjusted by time after KT, sex, age, and delayed graft function. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS® v.22.0 (IBM Corp, New York, NY, USA) and Stata® v.15 (STATA Corp, College Station, TX, USA).

2.5. Data Availability

The datasets generated and analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

3. Results

3.1. Study Population and Clinical Follow-Up

Forty-five patients with stable renal function were included in the study: 29 switched from CNI to mTORi (25 everolimus and 4 rapamycin, mTORi group), and 16 maintained treatment with tacrolimus, steroids, and mycophenolic acid (Tacrolimus group). Twenty-two converted patients and all 16 tacrolimus patients finalized the 24-month study period on treatment. Seven recipients did not complete 24 months on mTORi: six reintroduced tacrolimus between 12 and 24 months due to surgery (three cases), ABMR (two cases), and proteinuria; and one patient died 19 months after conversion for metastatic prostate carcinoma. Baseline and 3- and 12-month data of these patients were included in the

analysis. No graft loss was observed during the study period. Main characteristics of both groups are included in Table 1. All tacrolimus and 19 of 29 mTORi patients received prednisone during the study. At 24 months, tacrolimus trough blood levels remained stable in the tacrolimus group (6.5 ng/mL), mTORi trough blood levels were 6.8 ng/mL in the mTORi group, and MPA dose was similar in both groups (566 mg in the tacrolimus group and 725 mg in the mTORi group). Clinical follow-up showed stable renal function and a non-significant increase in proteinuria in the mTORi group (Table 1).

Table 1. Baseline and clinical follow-up characteristics of included patients: The table summarizes baseline characteristics and the clinical follow-up in patients switching from tacrolimus to serine/threonine protein kinase mechanistic target of rapamycin inhibitors (mTORi group) and patients maintaining tacrolimus (Tacrolimus group).

	Tacrolimus Group (*n* = 16)	**mTORi Group (*n* = 29)**	***p*-Value**
Recipient age (years) (mean (SD))	52.4 (13.9)	52.5 (15.9)	0.990
Recipient sex (female) (*n*, %)	3 (19%)	9 (31%)	0.491
Race (caucasian) (*n*, %)	14 (88%)	25 (86%)	1.000
Type of donor (deceased) (*n*, %)	15 (94%)	25 (86%)	0.641
Donor age (years) (mean (SD))	46.9 (15.7)	43.2 (12.4)	0.385
HLA mismatch class I (A/B)/class II (DR) (mean (SD))	3 (1)/1 (1)	3 (1)/1 (1)	0.794/0.922
Sensitizing events before KT (yes) (*n*, %)	3 (19%)	9 (31%)	0.491
Induction immunosuppression (antilymphocyte antibodies) (*n*, %)	0 (0%)	2 (7%)	0.531
Delayed graft function (*n*, %)	4 (25%)	5 (17%)	0.700
Acute rejection pre inclusion (*n*, %)	1 (6%)	0	0.356
Anti-HLA DSA/no DSA antibodies prior to the study (*n*, %)	0 (0%)/0 (0%)	2 (6.9%)/1 (3%)	0.531/1.000
Time after KT (months) (median (p25-p75))	17.0 (3.0–48.8)	15.6 (3.3–50.1)	0.827
Immunosuppression treatment at inclusion			
CNI (*n*, %)	16 (100%)	29 (100%)	
MPA (*n*, %)	16 (100%)	23 (79%)	
Steroids (*n*, %)	16 (100%)	19 (66%)	
Immunosuppression treatment at the end of study *			
CNI (*n*, %)	16 (100%)	6 (21%)	
mTORi (*n*, %)	0 (0%)	23 (82%)	
MPA (*n*, %)	16 (100%)	21 (75%)	
Steroids (*n*, %)	16 (100%)	25 (89%)	
Renal function and proteinuria			
Creatinine at the start of study (mg/dL) (mean (SD))	1.4 (0.5)	1.3 (0.4)	0.286
eGFR at the start of study (mL/min/1.73 m^2) (mean (SD))	57 (21)	59 (14)	0.763
pCOR < 500 mg/g at the start of study (yes) (*n*, %)	16 (100%)	29 (100%)	NA
Creatinine at the end of study (mg/dL) (mean (SD))	1.6 (0.8)	1.3 (0.5) **	0.246
eGFR at the end of study (mL/min/1.73 m^2) (mean (SD))	56 (22)	61 (16) **	0.424
pCOR < 500 mg/g at the end of study (yes) (*n*, %)	16 (100%)	17 (77%) **	0.067

CNI: calcineurin inhibitor; pCOR: ratio protein/creatinine in urine; DSA: donor-specific antibodies; eGFR: estimated glomerular filtration rate; HLA: human leukocyte antigen; KT: kidney transplantation; MPA: mycophenolate acid; mTORi: mTOR inhibitor; NA: not applicable; SD: standard deviation * One patient associated tacrolimus to everolimus due to subclinical antibody-mediated rejection (ABMR). One patient died in everolimus treatment before the end of study and is not included in this count. ** From 22 patients at 24 months.

3.2. Conversion from Tacrolimus to mTOR Inhibitor was not Associated with a Significant Development of de novo Donor Specific Antibodies

During the study, 7 patients showed de novo HLA antibodies: five in the mTORi group, two of them HLA DSA (one class I and one class II, 6.9%) and three HLA no DSA (10%), and two patients in the tacrolimus group had de novo HLA class II no DSA (13%). Rates of HLA no DSA (mTORi: 10% vs. tacrolimus: 13%, $p = 1.00$) and anti-HLA dnDSA (mTORi: 7% vs. tacrolimus: 0% $p = 0.53$) were statistically similar.

3.3. Peripheral Blood T Cell Numbers were not Affected by the mTOR Inhibitor Conversion

Patients from both groups showed similar proportions and absolute numbers of circulating T cells during the study (Figure 1A). The proportions of T cells were greater in both groups at 24 months

compared to HS (KT patients 78% ± 9.7% vs. HS 73.8% ± 7.6%, $p = 0.035$) (Figure 1A). Further analysis showed similar proportions of $CD4^+$ and $CD8^+$ T cells in both groups during the follow-up with no differences compared to HS (Figure 1B).

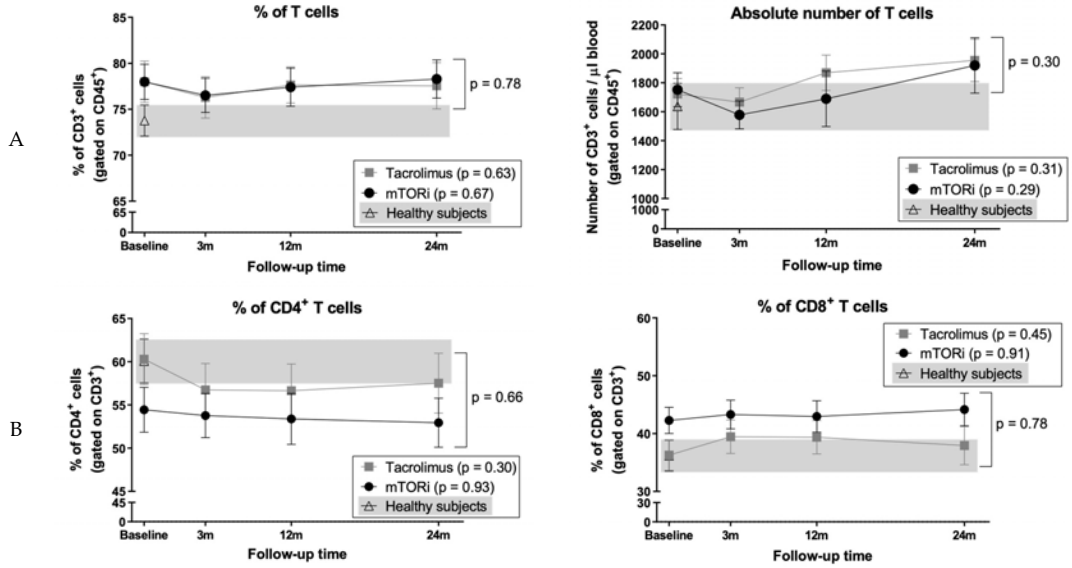


Figure 1. Evolution of T cells after switching from tacrolimus to serine/threonine protein kinase mechanistic target of rapamycin inhibitors (mTORi). Immunophenotyping of (**A**) total T cells and (**B**) $CD4^+$ and $CD8^+$ T cell subpopulations was carried out in patients before and after switching to mTORi (black dots) and in patients maintaining tacrolimus (grey squares). Healthy subjects (HS) data is depicted with white triangles, and the grey background corresponds to range. Plots show mean and standard error of the mean (SEM) for each time point.

3.4. mTOR Inhibitors and Time Posttransplantation Promote Expansion of T Regulatory Cells

During follow-up, both groups showed a significant increase in the relative and absolute numbers of Tregs (mTORi baseline: 2.6% ± 1.5% and 49 ± 44 cells/µl, 24 months: 5.9% ± 4.3% and 111 ± 93 cells/µl, $p < 0.001$ for both; Tacrolimus baseline: 2.7% ± 1.3% and 47 ± 23 cells/µl, 24 months: 4.8% ± 2.4% and 95 ± 65 cells/µl, $p = 0.001$ and $p = 0.01$) (Figure 2A). The increase was lesser and delayed in the tacrolimus group ($p < 0.001$). A multivariable analysis assessing typical confounding variables such as recipient sex, age, delayed graft function, and time post-transplantation was performed. Results confirmed a significant influence of posttransplant time ($p = 0.039$) and sex ($p = 0.050$) but no influence of recipient age ($p = 0.28$) and delayed graft function ($p = 0.90$) in the increase of Treg cells (Table 2). As median time post-KT for inclusion was 18 months, we performed a sub-analysis considering patients enrolled "early" (<18 months) and "late" (>18 months) after KT (Figure 2B,C). Patients switched early to mTORi showed a significant increase in Tregs during the study (baseline: 2.5% ± 1.8%, 24 months: 5.1% ± 3.2%, $p < 0.001$), in contrast with no significant changes in early tacrolimus patients (baseline: 2.2% ± 1.2%, 24 months: 3.4% ± 1.7%, $p = 0.27$). Evolution between groups was significantly different ($p < 0.001$): proportions of Tregs in the mTORi but not in the tacrolimus group at 24 months were comparable to those of HS (5.1% ± 3.2% vs. 4.9% ± 1.3% $p = 0.50$, 3.4% ± 1.7% vs. 4.9% ± 1.3% $p = 0.016$) (Figure 2B). Patients switched late to mTORi showed an increase during the 24-month follow-up similar to those maintained on tacrolimus (tacrolimus baseline: 3.2% ± 1.2%, 24 months: 4.9% ± 1.6%, $p < 0.001$; mTORi baseline: 2.7% ± 1.2%, 24 months: 3.9% ± 2.2%, $p < 0.001$) (Figure 2C). Absolute numbers behaved similarly, and both groups showed comparable percentages to those of HS at 24 months (mTORi $p = 0.08$ and tacrolimus $p = 0.94$).

Table 2. Multivariable adjustment in the Generalized Estimating Equations (GEE) model for Treg cell numbers: The GEE population-averaged model analysis was also adjusted by time after KT, sex, age, and delayed graft function. Table shows the β and 95% confidence interval (CI) values and the *p*-value corresponding to each variable in the adjustment.

Adjusting Variable	β (95% CI)	*p*-value
Time Point of the Study	1.46 (0.57; 2.34)	0.001
Group of the Study		
Tacrolimus Group	0	0.901
mTORi Group	1.32 (−19.63; 22.28)	
Interaction Group and Time Point of the Study	0.10 (−1.04; 1.23)	0.868
Time After KT	2.97 (0.15; 5.80)	0.039
Recipient Sex		
Male	0	0.050
Female	20.01 (0.01; 40.01)	
Recipient Age	−0.35 (−0.99; 0.29)	0.281
Delayed Graft Function		
No	0	0.902
Yes	1.62 (−24.13; 27.38)	

CI: confidence interval; GEE: Generalized Estimating Equations; KT: kidney transplantation; mTORi: mTOR inhibitor.

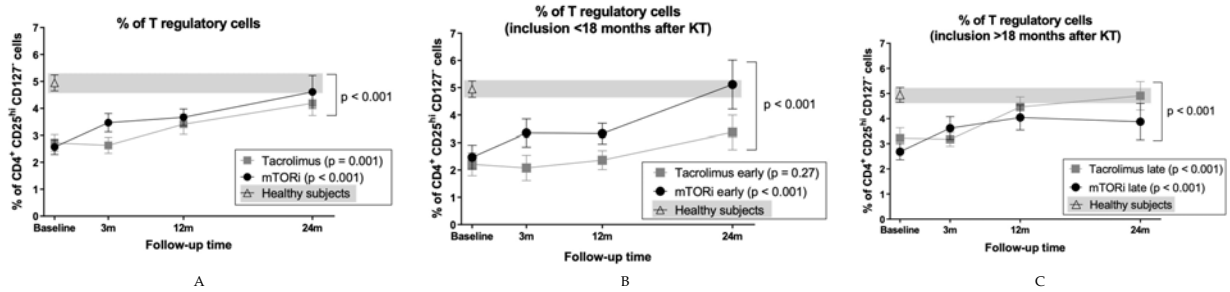
3.5. B Cells and Naïve B Cells Decrease After Conversion to mTOR Inhibitors

The proportions of B cells changed over time in the mTORi group, with a slight increase at 3 months followed by a continued decrease up to 24 months (baseline: 7.8% ± 4.9%, 24 months: 6.5% ± 2.5%, $p = 0.002$), whereas they remained stable in the tacrolimus group ($p = 0.44$), determining significant differences between groups ($p = 0.006$) (Figure 3A). No significant differences were observed in absolute numbers of B cells. Compared to HS, mTORi patients had similar proportions and numbers of B cells 3 months after conversion (8.6% ± 5% vs. 8.8% ± 3.3%) in contrast to tacrolimus patients (5.1% ± 2%), but at 24 months both groups depicted significantly lower percentages of B cells compared to HS (KT patients: 5.8% ± 2.6% vs. HS 8.8% ± 3.3%, $p = 0.002$) (Figure 3A).

Patients on mTORi showed a significant decrease of naïve B cells (baseline: 63.1% ± 17.5%; 24 months: 56.6% ± 19.4%, $p < 0.001$), but tacrolimus patients did not ($p = 0.50$), resulting in a different evolution during follow-up between groups ($p = 0.002$) (Figure 3B). Absolute numbers mirrored these changes (mTORi group $p = 0.009$, tacrolimus group $p = 0.86$, between groups $p = 0.06$). Memory B cells did not display significant changes (Figure 3B). Compared to HS, both groups of KT patients displayed lower percentages of naïve B cells (KT patients 56.4% ± 16% vs. HS 71.2% ± 14.6%, $p = 0.001$) and higher percentages of memory B cells at 24 months (KT patients 27.9% ± 12.8% vs. HS 17.3% ± 11.4%, $p = 0.003$) (Figure 3B).

3.6. Conversion to mTOR Inhibitors Promotes a Decrease in Circulating Transitional B Cells

We observed a significant decrease in the percentages of transitional B cells 3 months after mTORi conversion that persisted afterwards (baseline: 3.9% ± 2.9%, 24 months: 2.5% ± 3.2%, $p < 0.001$) compared to tacrolimus group ($p = 0.53$) (Figure 3B), confirmed by absolute numbers. Evolution of the two groups was different ($p < 0.001$), both in relative and absolute numbers (Figure 3B). Compared to HS, lower percentages and absolute numbers of transitional B cells in all KT patients were found at 24 months (KT patients: 2.1% ± 2.2% vs. HS 4.4% ± 2.2%, $p < 0.001$; 3 ± 3 cells vs. 8 ± 6 cells, $p < 0.001$) (Figure 3B). Further analysis of this subset revealed significant differences in the percentage of transitional B cell subset between both KTR groups at baseline (mTORi group 3.9% ± 2.9%, tacrolimus group 2.8% ± 5.5%, $p = 0.004$), probably mainly due to the absence of steroids in the treatment scheme of many patients in the mTORi group, as we revealed in a prior publication [43] (discussed next in Section 3.7).



A B C

Figure 2. Evolution of Tregs after switching from tacrolimus to mTORi in all cases and according to time of inclusion in the study. Immunophenotyping of (**A**) total Tregs, (**B**) Tregs in patients included in the study during the first 18 months after transplantation, and (**C**) Tregs in patients included in the study after 18 months posttransplant. Patients before and after switching to mTORi are depicted with black dots, and patients maintaining tacrolimus are depicted with grey squares. HS data is depicted with white triangles, and the grey background corresponds to range. Plots show mean and SEM for each time point.

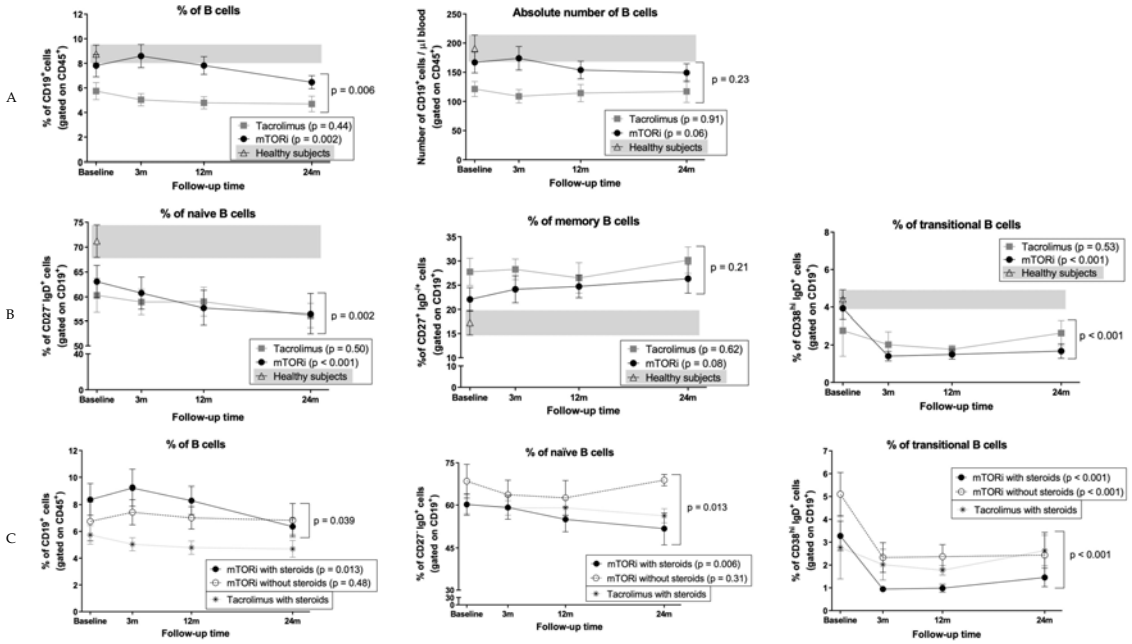


Figure 3. Evolution of B cells after switching from tacrolimus to mTORi. Immunophenotyping of (**A**) total B cells and (**B**) naïve, memory, and transitional B cells in patients before and after switching to mTORi (black dots) and patients maintaining tacrolimus (grey squares). HS data is depicted with white triangles, and the grey background corresponds to range. Plots show mean and SEM for each time point. Immunophenotyping analysis of (**C**) total, naïve, and transitional B cells in patients switching from tacrolimus to mTORi with (black dots) and without (white dots) concomitant steroid treatment. Patients who maintained tacrolimus and steroids are marked with (*) as a reference.

3.7. Impact of mTOR Inhibitors on B Cells in Patients who did not Receive Steroids

Our group recently showed that steroid withdrawal has a remarkable impact on B cell subpopulations [43]. Here, we stratified patients that changed to mTORi in two subgroups according to steroid treatment before starting the study (yes: $n = 19$; no: $n = 10$). The proportions of total B cells were significantly reduced at follow-up in patients on steroids (baseline: 8.4% ± 5.1%, 24 months: 6.4% ± 2.5%, $p = 0.013$) but not in steroid-free cases ($p = 0.48$); evolution between groups was different ($p = 0.039$) (Figure 3C). Steroid-free patients had higher percentages of naïve B cells at baseline and during follow-up in comparison with patients on steroids ($p = 0.013$), who showed significantly reduced naïve B cell proportions during follow-up (baseline: 60.2% ± 16.5%, 24 months: 51.6% ± 21.5%, $p = 0.006$) (Figure 3C); no differences in memory B cells were noticed. Finally, steroid-free patients displayed greater relative and absolute numbers of transitional B cells during follow-up compared to patients with steroids (between groups $p < 0.001$), though both subgroups showed a decrease in transitional B cells after conversion (both $p < 0.001$) (Figure 3C).

3.8. Conversion to mTOR Inhibitor Induces an Increase in the Proportion of $CD56^{bright}$ Cells and $NKG2A^+$ NK Cell Subsets

Proportions and absolute numbers of total NK cells remained stable in both groups, and their evolution was comparable (Figure 4A). By contrast, proportions of $CD56^{bright}$ NK cells increased in mTORi patients (baseline: 5.5% ± 5.7%; 24 months 13.8% ± 7.6%, $p < 0.001$) with a reciprocal reduction of the $CD56^{dim}$ subset (baseline: 94.5% ± 5.5%; 24 months 86.2% ± 7.7%, $p < 0.001$) (Figure 4B). Conversely, NK cell subsets remained stable in the tacrolimus group ($p = 0.98$), resulting in significant differences between groups (both $p < 0.001$) (Figure 4B); calculation of absolute NK cell numbers confirmed these findings. A significant increase in the proportions of $NKG2A^+$ NK cells occurred following mTORi conversion with levels similar to HS (baseline: 41.2% ± 20.6%; 24% months: 52.1% ± 18%, $p < 0.001$). By contrast, this parameter remained stable in the tacrolimus group ($p = 0.46$), accounting for differences between groups ($p < 0.001$) (Figure 4C); absolute numbers of $NKG2A^+$ cells also increased in the mTORi group ($p = 0.009$; between groups $p = 0.025$). By contrast, no differences of NKG2C expression levels were observed. Since the majority of $CD56^{bright}$ NK cells are $NKG2A^+$, we compared the expression of this receptor in both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets. The numbers of $CD56^{bright}$ $NKG2A^+$ NK cells increased in the mTORi group (baseline 8 ± 6 cells/µl, 24 months 22 ± 14 cells/µl, $p < 0.001$), although the proportions varied during the study period ($p = 0.06$); and no differences were observed in the $CD56^{dim}$ $NKG2A^+$ NK cell subset (Figure 5A,B). Finally, considering previous results from our group [45], we analyzed these subsets excluding patients who developed HLA antibodies. No significant influence of HLA antibodies development was found.

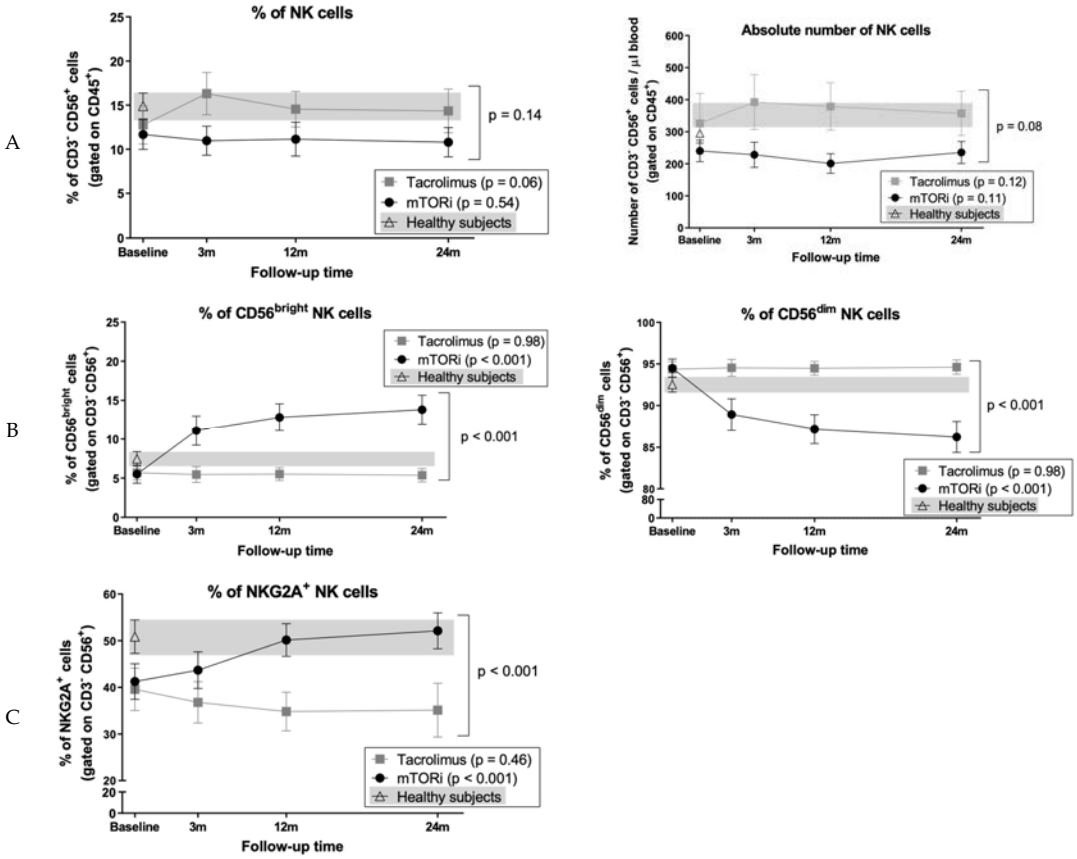


Figure 4. Evolution of Natural Killer (NK) cells after switching from tacrolimus to mTORi. Immunophenotyping of (**A**) total NK cells, (**B**) $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets, and (**C**) NK cells expressing the CD94/NK group 2 member A (NKG2A) receptor. Patients before and after switching to mTORi are depicted with black dots, and patients maintaining tacrolimus are depicted with grey squares. HS data is depicted with white triangles, and the grey background corresponds to range. Plots show mean and SEM for each time point.

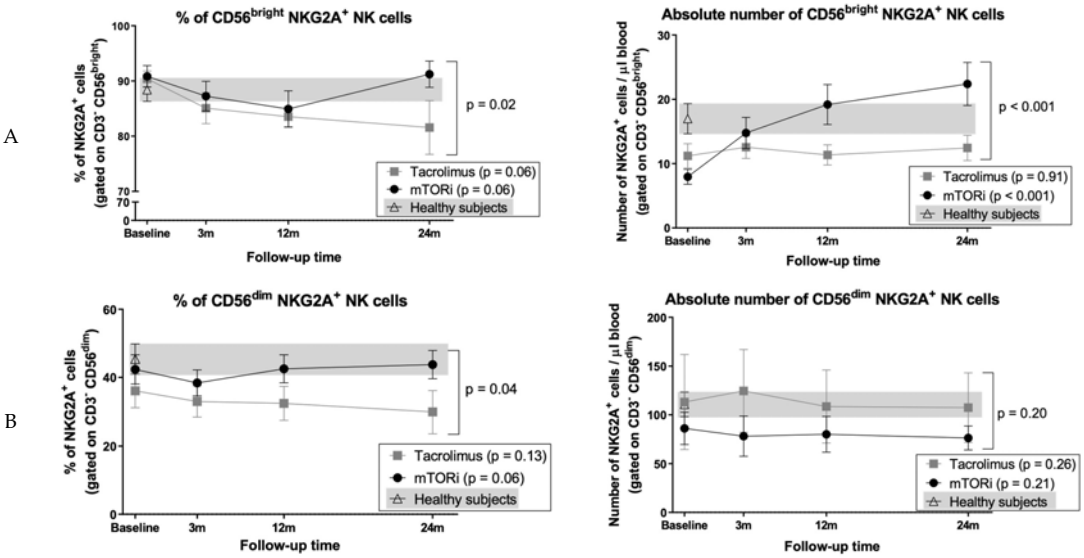


Figure 5. Evolution of CD94/NKG2A expression in NK cell subsets. Analysis of NKG2A expression was carried out gating (**A**) $CD56^{bright}$ and (**B**) $CD56^{dim}$ NK cells. Patients before and after switching to mTORi are depicted with black dots, and patients maintaining tacrolimus are depicted with grey squares. HS data is depicted with white triangles, and the grey background corresponds to range. Plots show mean and SEM for each time point.

4. Discussion

We report here that, after conversion to mTORi in KTR, graft function remained stable and there was no increase of dnDSA rate. However, there was a significant redistribution of PBL consisting of an increase in the proportion of peripheral regulatory T cells, $CD56^{bright}$ and $NKG2A^{+}$ NK cells, and a decrease in the proportion of transitional B cells.

Although conversion to mTORi has been associated with improvement in eGFR [46,47], we found no significant advantage in eGFR during two years of follow-up. Certainly, our study was not powered to assess graft function evolution. Proteinuria increased slightly and non-significantly in mTORi patients with no clinical impact, as earlier described [48].

Early conversion to mTORi without steroids may induce an under-immunosuppression state and an increased risk of dnDSA development [37]. These dnDSA are associated with the development of ABMR and worse graft survival [12]. In this study, we found no increased rates of dnDSA in late mTORi conversion for two years of follow-up, in agreement with several studies [40,41,49–51]. On the contrary, other studies have shown higher rates of dnDSA after conversion to everolimus [38,39,52]. Most data suggest that early (<1 year) mTORi monotherapy conversion may provide inadequate immunosuppression [36] and is a potential risk factor for dnDSA emergence, especially associated with steroid withdrawal [46,53]. In our study, only two cases developed dnDSA on mTORi, one converted early (3 months) after KT, and the other one not receiving steroids at the time of conversion.

Regarding the T cell immunophenotype, we found no influence of mTORi conversion on main T cell subsets, as previously reported in a study comparing de novo treatment with mTORi and cyclosporine A (CsA) after KT with a 2-year follow-up time [27]. In agreement with previous publications, we found a significant increase in regulatory T cells in the mTORi group [24,25]. Interestingly, patients recruited >18 months after KT and maintained on tacrolimus for the following two years also showed an increase of Tregs but not those included within the first 18 months after KT. Although multiple reasons can be linked to this phenomenon, lower dosage and trough levels of tacrolimus later after KT might explain the increase in Tregs. To our knowledge, this is the first study to show no difference in Treg expansion between tacrolimus and mTORi conversion >18 months after transplantation.

Analysis of the B cell compartment showed that only transitional B cells were affected by mTORi conversion, resulting in a striking reduction of their relative and absolute numbers only 3 months after conversion. The analysis of this B cell subset has gained interest during the last years as they potentially include regulatory B cells, which secrete interleukin-10 (IL-10) and show immunoregulatory properties [54,55]. Cherukuri et al. [56] suggested a clear implication of transitional B cells in renal allograft outcomes. In this study, transitional B cells were classified into T1 and T2 subsets (being T1 subset the one which expresses a higher ratio of IL-10 to tumor necrosis factor-alpha (TNF-alpha)) and they reported that a low T1/T2 ratio was independently associated with allograft dysfunction. On the other hand, regarding regulatory B cells, a study reported a similar reduction in mTORi and CNI patients [26] and another found no influence of sirolimus or tacrolimus in the proportion of these cells [23]. Regarding other B cell subsets, a study comparing the use of sirolimus and CsA de novo after KT reported an expansion of memory B cells and a reduction of naïve B cells [32]. In our study, other differences in B cell subsets, such as the reduction of total B cells and naïve B cells in mTORi patients, were related to the use of concomitant prednisone treatment. A previous study from our group showed that steroid withdrawal clearly modified B cell subsets, increasing the proportions of total, naïve, and transitional B cells [43]. In agreement with these results, we report here that steroid-free patients showed stable percentages of total B cells and naïve B cells after mTORi conversion whereas these cell populations decreased in steroid-treated patients. Naïve B cells are more sensitive to glucocorticoid-induced apoptosis [57,58], and mTORi may contribute to this overall effect. In contrast with CNIs, direct effects on B cells of mTORi have been reported [53]. In vitro cultures of human B cells with mTORi showed a profound attenuation of B-cell activation and IgG production [30,31]. Moreover, sirolimus but not tacrolimus was able to inhibit the proliferation of B cells and their differentiation into plasma cells [59].

Patients displayed a significant redistribution of NK cell subsets after switching to an mTORi, in agreement with reported data sustaining that the NK cell repertoire may be modulated by different immunosuppressive drugs [33]. Data from experimental studies in vitro and in mice [60,61] suggest different effects of sirolimus and everolimus in the inhibition of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) that may be critical in terms of NK cell development and functionality and should be taken into account in human studies. Jin et al. [60] showed that everolimus was more efficient than sirolimus in inhibiting mTORC2 formation and mTORC2-dependent signaling in vitro. Data in mice suggest that mTORC1 sustains mTORC2 activity whereas mTORC2 acts as a repressor of mTORC1 to control NK cell effector functions [61]. In humans, the effects of mTORi in NK cells have been evaluated in vitro [33,34] and in vivo in combination with tacrolimus [33,34]. In agreement with a previous report [27], we did not find significant alterations in total NK cells. However, the expression of the CD94/NKG2A inhibitory receptor increased after mTORi conversion when compared to the tacrolimus group. Neudoerfl et al. [33] found that tacrolimus treatment was associated with slightly increased NKG2A expression in NK cells when compared to tacrolimus and sirolimus treatment, although it did not reach statistical significance. To our knowledge, the present study is the first to assess CD94/NKG2A expression in KTR after mTORi conversion without tacrolimus and to report a follow-up of two years. Interestingly, mTORi patients and HS displayed comparable proportions of $NKG2A^+$ NK cells. We previously described an association of greater proportions of $NKG2A^+$ cells with the presence of HLA DSA in KTR, both in cases treated with tacrolimus and in patients treated with mTORi without CNI compared to patients without HLA DSA [45]. In the present study, the low number of patients with HLA DSA precludes this analysis. We also observed a significant increase of $CD56^{bright}$ NK cells, which produce cytokines but display low cytotoxic activity, and conventionally considered to represent an early maturation stage [62]. This minor subset lacks FcγR-IIIA (CD16), which triggers antibody-dependent cellular cytotoxicity (ADCC) and displays the CD94/NKG2A inhibitory receptor [34,62], which recognizes HLA-E [63], in the absence of inhibitory killer immunoglobulin-like receptors (KIR) specific for HLA class I molecules [64]. This phenotypic profile in mTORi patients was encompassed by a reciprocal reduction of the major $CD56^{dim}$ NK

cell subset, which mediates cytotoxicity and pro-inflammatory cytokine secretion [34]. Moreover, a significant increase of the proportions of NKG2A$^+$ CD56^{dim} NK cells, conventionally considered to differentiate from the CD56^{bright} subset, was also detected. Altogether, these data support that conversion to mTORi therapy promotes a redistribution of the NK cell compartment characterized by the presence of greater numbers of NK cell subsets at early differentiation stages. Implications of this phenomenon remain unclear and deserve further assessment in prospective studies.

Our study has some limitations: First, the restricted number of patients included in the study who conversely underwent an extensive follow-up for two years. This fact, in addition to the high number of statistical tests performed in the results section, implies a weakness in terms of taking conclusions of individual results and increases the chance of type 1 error. Second, the absence of protocol graft biopsies precluding the analyses of potential correlations between peripheral lymphocytes and intragraft infiltrations. Finally, the absence of additional markers in the immunophenotypic analysis (e.g., CD16 and Foxp3), which would have allowed to more precisely characterize the influence of mTORi conversion on the lymphocyte subsets redistribution.

The strength of the present study relies on the long follow-up of KT recipients which permits to confirm that some of the changes observed in peripheral blood lymphocyte subsets after switching to an mTORi remain stable for two years. To our knowledge, this study is the first to evaluate significant immune markers such as NKG2A—previously linked to DSA detection [45]—in a cohort of KT patients treated with mTORi without concomitant tacrolimus, assessing in addition the influence of steroid treatment in peripheral blood lymphocyte subsets of KT recipients.

In summary, our study supports that mTORi conversion is safe in terms of renal function and dnDSA generation and influences the distribution of peripheral blood lymphocyte subsets, alone or in combination with steroid withdrawal. More studies analyzing the function and relevance of transitional B lymphocytes and NKG2A$^+$ NK cells are necessary in order to better understand the immune and clinical implications of immunosuppressive treatment with mTORi.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/4/1088/s1. Figure S1: Gating strategy for flow cytometry analysis of PBL populations: Representative flow cytometry plots from the same patient illustrate the gating strategy used for the study; Figure S2: Characterization of transitional B cells; Table S1: Antibodies used in the study: The table summarizes antigen, clone, fluorochrome, and company for each antibody used in the study.

Author Contributions: L.L.-M., J.P., and M.C. designed the study, analyzed the results, and drafted the manuscript. D.R.-P., D.R.-R., M.J.P.-S., J.Y., and M.L.-B. analyzed the results and revised the manuscript. L.L.-M. and A.F. coordinated sample drawing and storage, logistics, and lab procedures. X.D. contributed with the statistical assessment. All authors have read and agreed to the published version of the manuscript.

Funding: This study was performed with funding from projects PI10/01370, PI13/00598, and PI16/00617 (Spanish Ministry of Health ISCIII FIS-FEDER); from RD12/0021/0024 and RD16/0009/0013 (ISCIII FEDER RedinRen); 201822-10 (Fundació la Marató de TV3); and a grant from the Fundació Catalana de Trasplantament (to L.Ll.-M.). D.R.-R. was supported by a Sara Borrell Fellowship from ISCIII.

Acknowledgments: We are indebted to Dulce Soto, Judit Rigol, Sara Álvarez, and Rosa Causadias for their technical support. L.Ll-M. did this study as part of her doctoral thesis program at the Department of Medicine from the Universitat Autonoma of Barcelona (UAB).

Conflicts of Interest: The authors of this manuscript declare no competing interests. The results presented in this paper have not been published previously in whole or part, except in abstract format. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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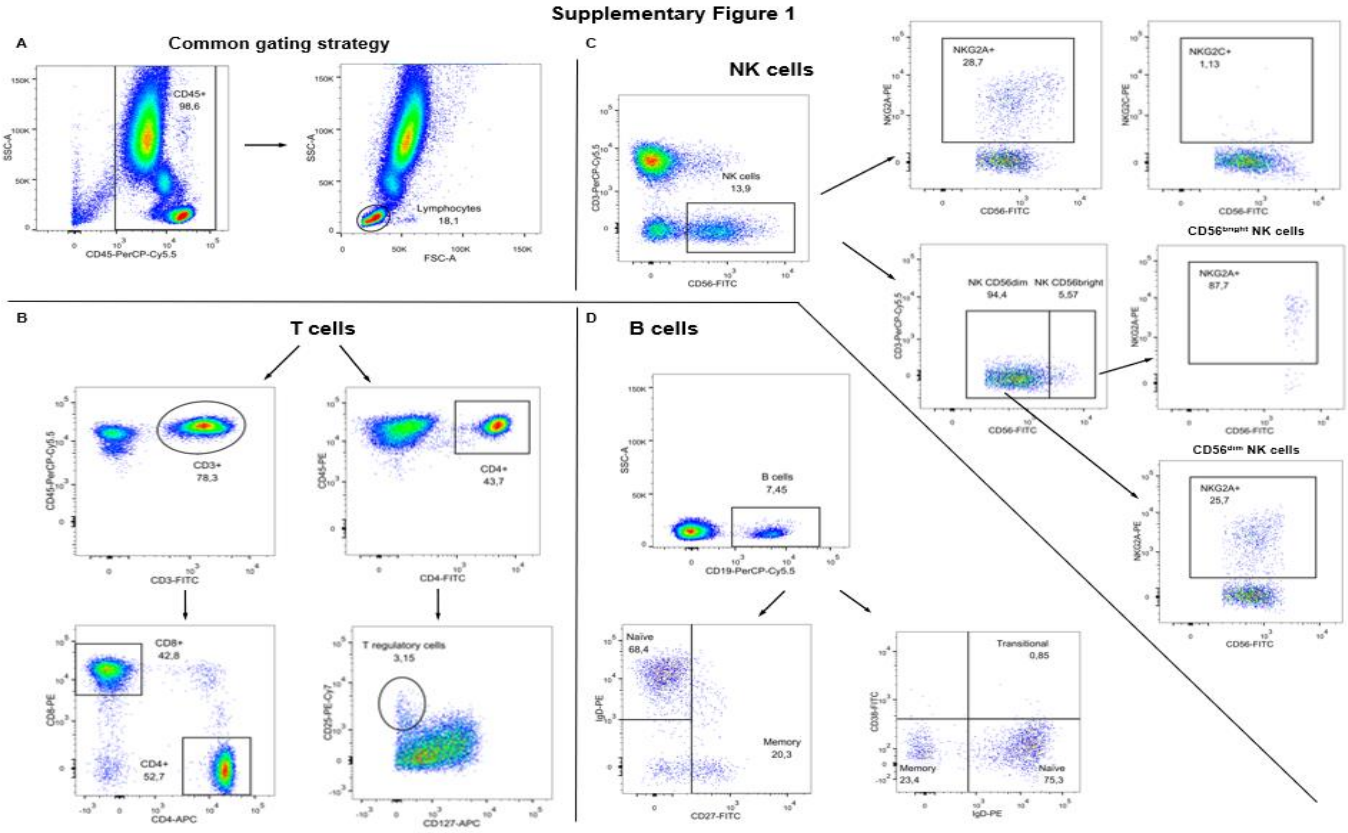
Supplementary file

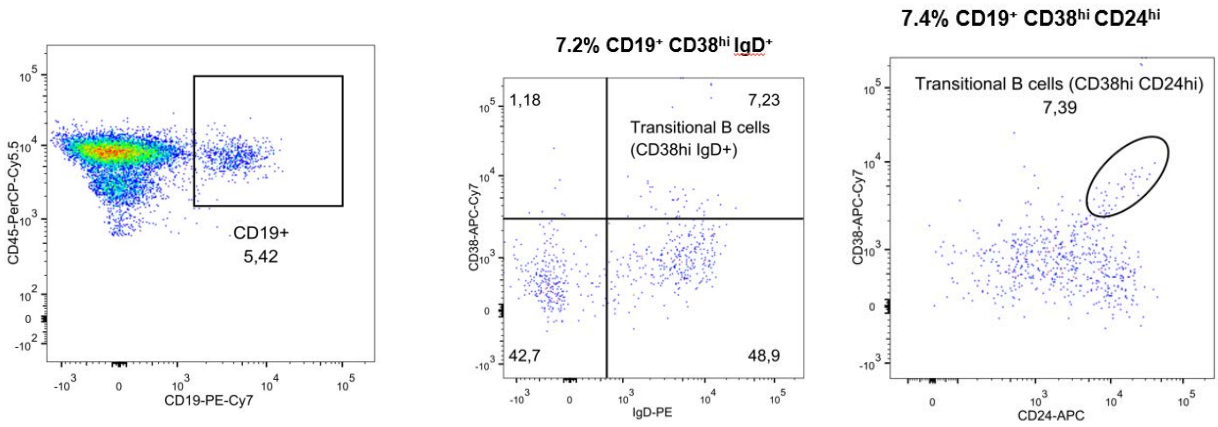
Supplementary Table 1. Antibodies used in the study. The table summarizes antigen, clone, fluorochrome and company for each antibody used in the study. NK-cell subsets $NKG2A^+$ and $NKG2C^+$ were identified by indirect immunofluorescence staining using a PE-conjugated F(ab')2 goat anti-mouse IgG + IgM secondary antibody.

Antigen	Clone	Fluorochrome	Company
CD3	SK7	FITC / APC / PerCP-Cy5.5	BD Biosciences™
CD4	SK3	APC / PerCP-Cy5.5	BD Biosciences™
CD8	SK1	PE	BD Biosciences™
CD19	SJ25C1	PerCP-Cy5.5	BD Biosciences™
CD27	L128	FITC	BD Biosciences™
CD38	HB-7	FITC	BD Biosciences™
CD45	2D1	PE / APC / PerCP-Cy5.5	BD Biosciences™
CD56	NCAM 16.2	FITC	BD Biosciences™
IgD	IA6-2	PE	BD Biosciences™
CD4/CD25/CD127 Regulatory T cell kit		FITC/PE-Cy7/APC	BD Biosciences™
NKG2A	Z199		Provided by Dr. A. Moretta
NKG2C	MAB1381		R&D systems™
F(ab')2 goat anti-mouse IgG + IgM		PE	Jackson ImmunoResearch™

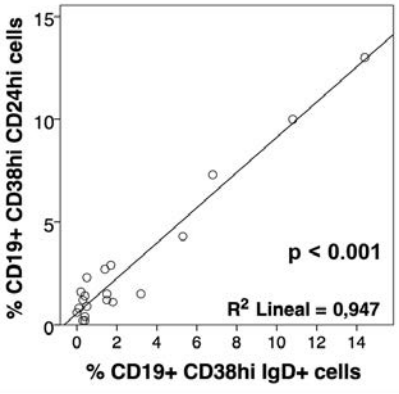


Supplementary Figure 1. Gating strategy for flow cytometry analysis of PBL populations. Representative flow cytometry plots from the same patient illustrate the gating strategy used for the study. (A) $CD45^+$ and lymphocyte gates were common into all analysis. (B) Gating strategy of T cells, (C) NK cells and (D) B cells.

A



B



Supplementary Figure 2. Characterization of transitional B cells. (A) Representative flow cytometry plots from the same patient, including or not CD24 staining. (B) Correlation graph and Pearson correlation value for the $CD19^{+}CD38^{hi}IgD^{+}$ and $CD19^{+}CD24^{hi}CD38^{hi}$ populations.

Non-HLA antibodies and epitope mismatches in kidney transplant recipients with histological antibody-mediated rejection

Marta Crespo[1]; Laura Llinàs-Mallol[1*]; Dolores Redondo-Pachón[1]*
Carrie Butler[2]; Javier Gimeno[3]; María José Pérez-Sáez[1]; Carla Burballa[1]
Anna Buxeda[1]; Carlos Arias[1]; Montserrat Folgueiras[1]; Sara Sanz-Ureña[1]
*Nicole M. Valenzuela[2]; Elaine F. Reed[2**] and Julio Pascual[1**]*

[1] Department of Nephrology, Hospital del Mar, Barcelona, Spain; Hospital del Mar Medical Research Institute, Barcelona, Spain

[2] UCLA Immunogenetics Center, University of California Los Angeles, Los Angeles, CA, USA; Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA

[3] Department of Pathology, Hospital del Mar, Barcelona, Spain: Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain

* *Share first authorship*

** *Share senior authorship*

Frontiers in Immunology 2021; volume 12, article 703457.
Accepted: 15 Jun 2021
doi: 10.3389/fimmu.2021.703457

frontiers in Immunology

ORIGINAL RESEARCH
published: 02 July 2021
doi: 10.3389/fimmu.2021.703457

Non-HLA Antibodies and Epitope Mismatches in Kidney Transplant Recipients With Histological Antibody-Mediated Rejection

Marta Crespo[1,2†], Laura Llinàs-Mallol[1,2†], Dolores Redondo-Pachón[1,2], Carrie Butler[3,4], Javier Gimeno[2,5], María José Pérez-Sáez[1,2], Carla Burballa[1,2], Anna Buxeda[1,2], Carlos Arias-Cabrales[1,2], Montserrat Folgueiras[1,2], Sara Sanz-Ureña[1,2], Nicole M. Valenzuela[3,4], Elaine F. Reed[3,4‡] and Julio Pascual[1,2‡]*

[1] Department of Nephrology, Hospital del Mar, Barcelona, Spain, [2] Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain, [3] UCLA Immunogenetics Center, University of California Los Angeles, Los Angeles, CA, United States, [4] Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, United States, [5] Department of Pathology, Hospital del Mar, Barcelona, Spain

Background: Correlation between antibody-mediated rejection (ABMR) and circulating HLA donor-specific antibodies (HLA-DSA) is strong but imperfect in kidney transplant (KT) recipients, raising the possibility of undetected HLA-DSA or non-HLA antibodies contributing to ABMR. Detailed evaluation of the degree of HLA matching together with the identification of non-HLA antibodies in KT may help to decipher the antibody involved.

Methods: We retrospectively assessed patients with transplant biopsies scored following Banff'15 classification. Pre- and post-transplant serum samples were checked for HLA and non-HLA antibodies [MICA-Ab, angiotensin-II type-1-receptor (AT_1R)-Ab, endothelin-1 type-A-receptor (ETAR)-Ab and crossmatches with primary aortic endothelial cells (EC-XM)]. We also analyzed HLA epitope mismatches (HLA-EM) between donors and recipients to explore their role in ABMR histology ($ABMR_h$) with and without HLA-DSA.

Results: One-hundred eighteen patients with normal histology (n = 19), $ABMR_h$ (n = 52) or IFTA (n = 47) were studied. $ABMR_h$ patients were HLA-DSA_{pos} (n = 38, 73%) or HLA-DSA_{neg} (n = 14, 27%). Pre-transplant HLA-DSA and AT_1R-Ab were more frequent in $ABMR_h$ compared with IFTA and normal histology cases (p = 0.006 and 0.003), without differences in other non-HLA antibodies. Only three $ABMR_hDSA_{neg}$ cases showed non-HLA antibodies. $ABMR_hDSA_{neg}$ and $ABMR_hDSA_{pos}$ cases showed similar biopsy changes and graft-survival. Both total class II and DRB1 HLA-EM were associated with $ABMR_hDSA_{pos}$ but not with $ABMR_hDSA_{neg}$. Multivariate analysis showed that pre-transplant HLA-DSA (OR: 3.69 [1.31–10.37], p = 0.013) and AT_1R-Ab (OR: 5.47 [1.78–16.76], p = 0.003) were independent predictors of $ABMR_hDSA_{pos}$.

Conclusions: In conclusion, pre-transplant AT_1R-Ab is frequently found in $ABMR_hDSA_{pos}$ patients. However, AT_1R-Ab, MICA-Ab, ETAR-Ab or EC-XM^+ are rarely

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Edited by:
Frans H. J. Claas,
Leiden University, Netherlands

Reviewed by:
Vincenzo Cantaluppi,
Università del Piemonte Orientale, Italy
Gonca Emel Karahan,
Leiden University Medical Center, Netherlands

***Correspondence:**
Marta Crespo
mcrespo@psmar.cat

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share senior authorship

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: 30 April 2021
Accepted: 15 June 2021
Published: 02 July 2021

Citation:
Crespo M, Llinàs-Mallol L, Redondo-Pachón D, Butler C, Gimeno J, Pérez-Sáez MJ, Burballa C, Buxeda A, Arias-Cabrales C, Folgueiras M, Sanz-Ureña S, Valenzuela NM, Reed EF and Pascual J (2021) Non-HLA Antibodies and Epitope Mismatches in Kidney Transplant Recipients With Histological Antibody-Mediated Rejection. Front. Immunol. 12:703457. doi: 10.3389/fimmu.2021.703457

found among $ABMR_hDSA_{neg}$ patients. Pre-transplant AT_1R-Ab may act synergistically with preformed or *de novo* HLA-DSA to produce $ABMR_hDSA_{pos}$ but not $ABMR_hDSA_{neg}$. HLA epitope mismatch associates with $ABMR_hDSA_{pos}$ compared with $ABMR_hDSA_{neg}$, suggesting factors other than HLA are responsible for the damage.

Keywords: kidney transplantation, antibody-mediated rejection, HLA antibodies, non-HLA antibodies, HLA epitope mismatch, AT_1R antibodies

INTRODUCTION

Correlation between the detection of HLA donor-specific antibodies (HLA-DSA) and antibody-mediated rejection (ABMR) is strong but imperfect in kidney transplant (KT) recipients (1–7). Not all patients with pre- or post-transplant HLA-DSA have ABMR damage in their biopsies (8). Different groups have tried to identify characteristics of HLA-DSA that may predict ABMR (9–12). There is also an active search for other invasive or non-invasive biomarkers for ABMR diagnosis (13–15). In the other hand, some patients have biopsies with histological findings suggestive of ABMR ($ABMR_h$) without circulating HLA-DSA (16), generating the concept of the existence of $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$ cases. There is still limited literature describing the incidence of this type of ABMR without HLA-DSA, evaluating if these cases collectively show different clinical or histological characteristics or if non-HLA antibodies may explain the damage. Besides, controversial results in outcomes comparing $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$ cases have been reported (17, 18).

Based on the hypothesis that other antibodies may play a lead role in the case of ABMR histological damage with or without HLA-DSA, some groups have evaluated non-HLA antibodies in KT recipients (19, 20). Although first reports connecting non-HLA antibodies and graft outcomes were published in 2005 (19, 21), evidence is still weak and debated. Antibodies against specific alloantigens such as MICA (MICA-Ab) or MICB, or against autoantigens like angiotensin II type 1 receptor (AT_1R-Ab), endothelin-1 type A receptor (ETAR-Ab), perlecan, agrin or vimentin, among others, have been reviewed recently (22). Some groups focused into the analysis of pathogenic antibodies directed against endothelial cells—which express some of those but also other antigens—with endothelial cell crossmatches (23–25). The increased evidence that the prevalence of non-HLA antibodies in KT recipients is high (26), together with the heterogeneous post-KT clinical course of patients included in these studies (25) hamper the correct identification of deleterious non-HLA antibodies. On the other hand, HLA epitope mismatch (HLA-EM) assessment has gained interest as an added immune monitoring tool to provide a more precise evaluation of HLA matching (27–29). HLA-EM has been previously associated with the development of *de novo* HLA-DSA (30) and ABMR (31). The clinical relevance of HLA-EM analysis remains under discussion and its application is not generalized yet.

Here, we systematically explored pre- and post-KT serum samples for HLA and different types of non-HLA antibodies: MICA-Ab, AT_1R-Ab and ETAR-Ab, and other non-HLA antibodies performing crossmatches with primary aortic endothelial cells (EC-XM). Additionally, we evaluated pre-KT HLA-EM load. We focused on KT patients with biopsies with Banff category 2 diagnosis and compared them with two other Banff diagnosis: category 1 or no abnormalities (normal), as a usual control group, and category 5 or interstitial fibrosis and tubular atrophy (IFTA), damage with not clear pathogenicity to evaluate the potential role of non-HLA antibodies in this case (32).

MATERIALS AND METHODS

Study Population and Design

Prospective observational study performed in KT patients active at our transplant program in Hospital del Mar. A total of 234 consecutive clinical and surveillance renal biopsies were performed in ABO compatible KT after a negative CDC crossmatch (February 2011–June 2015). Ninety-two biopsies fulfilling Banff 2015 categories 3, 4 and 6 were excluded and 142 biopsies achieving categories 1, 2 or 5 were selected. From these 142 biopsies, we selected only one biopsy per patient according to these criteria: the first biopsy obtained after 3 months post-transplantation, unless a biopsy with category 2 diagnosis was available. Five biopsies were excluded due to unsuitable serum samples. Finally, 118 biopsies corresponding to 118 patients were included in the study (**Supplementary Figure 1**). Demographical and clinical data were collected as previously described (33), and follow-up was done until graft-loss, death, 96 months post biopsy or July/2020. The study was approved by the Parc de Salut Mar Ethical Research Board (2010/3904/I) and all patients signed informed consents. All clinical

Abbreviations: ABMR, antibody-mediated rejection; $ABMR_h$, antibody-mediated rejection histology; $ABMR_hDSA_{pos}$, antibody-mediated rejection histology with HLA-DSA; $ABMR_hDSA_{neg}$, antibody-mediated rejection histology without HLA-DSA; AT_1R-Ab, antibodies against angiotensin II type 1 receptor; CDC, complement-dependent cytotoxicity; CTG, chronic transplant glomerulopathy; ECs, primary human aortic endothelial cells; EC-XM, crossmatch with primary human aortic endothelial cells; EM, electron microscopy; ETAR-Ab, antibodies against endothelin-1 type A receptor; GFR, glomerular filtration rate; HLA-DSA, HLA donor-specific antibodies; HLA-AM, HLA antigen mismatches; HLA-EM, HLA epitope mismatches; IFTA, interstitial fibrosis and tubular atrophy; IQR, interquartile range; KT, kidney transplant; MICA-Ab, antibodies against major histocompatibility complex class I related chain A; PRA, panel-reactive antibody; PTCML, peritubular capillary multilayering; SAB, Single Antigen Bead assays; SD, standard deviation.

and research activities reported are consistent with the Declarations of Istanbul and Helsinki.

Histological Scoring and Classification of the Biopsies

Biopsies were performed for indication or follow-up (including HLA-DSA detection without graft dysfunction). Processing was undertaken as previously described (33). All biopsies were scored by a pathologist following Banff 2015 classification and assigned to any of the six Banff categories (33). Category 2 included biopsies that met the first two Banff 2015–2019 criteria for ABMR histology, fulfilling the suspicious or full diagnosis of ABMR in Banff 2015 classification.

Sera Collection and Detection of HLA and Non-HLA Antibodies

One-hundred one available pre-KT and 118 post-KT serum samples collected contemporaneously to biopsies were retrospectively analyzed. HLA antibody testing (HLA-A, B, C, DRB and DQB) was performed as previously described (34) using Luminex HLA Single Antigen Bead assays (LABScreen, One Lambda, Canoga Park, CA). Antibodies against MICA antigens (*001, *002, *004, *007, *009, *012, *017, *018, *019, *027) were determined using LABScreen assay by Luminex Technology, according to the manufacturer's specifications (One Lambda, CA). MICA-Ab were considered positive if mean fluorescence intensity >1,000. MICA typing for donors and recipients was not available. AT_1R-Ab and ETAR-Ab were measured using enzyme-linked immunosorbent-based assays (35) (One Lambda, CA), diluted 1:100, tested in duplicate and read on an Epoch Microplate Spectrophotometer (Bio-Tek, Winooski, VT). Samples with AT_1R-Ab or ETAR ≥10 U/ml were considered positive based on previous studies and our receiver operating curve analysis.

Endothelial Cell Crossmatches

Primary human aortic endothelial cells (ECs) were isolated from aortic rings of explanted donor hearts (36). EC were cultured in M199 medium supplemented with 20% (vol/vol) FBS, penicillin–streptomycin (100 U/ml and 100 ug/ml; Invitrogen Life Technologies), sodium pyruvate (1 mM), heparin (90 ug/ml; Sigma-Aldrich) and EC growth supplement (20 mcg/ml; Fisher Scientific). ECs from passages 7–8 were frozen and used in the EC-XM. Two different ECs (phenotyped as follows, donor CAR: HLA A2, A68, B60, B65; and donor Y126: HLA A1, A11, B35, B37) were employed avoiding for each KT recipient any HLA class I match with the kidney graft which could yield a reaction towards donor-specific HLA antigens. A total of 2×10^5 ECs were incubated 30 min with 100 ul patient serum on ice. ECs were washed three times and incubated with 50 mc of 1:400 diluted FITC-AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG Fc fragment (Jackson ImmunoResearch Laboratories) for 30 min on ice. After three washes, cell fluorescence was analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Gates for forward and side scatter measurements were set on EC, and a minimum of 10,000 events was acquired. Positive EC-XM threshold was set at two standard deviations (50 Median Channel Shift) above the mean of negative control serum tests. EC-XM were only performed in 83 pre and 103 post-KT cases due to insufficient sample.

HLA Epitope Mismatch Characterization

HLAMatchmaker software according to the July 2020 update (ABC and DRDQDP Eplet Matching Program V3.1, http://www.epitopes.net) was used to define potential HLA-EM between donors and recipients (37). High-resolution typing for all donors and recipients was performed or inferred using the HaploStats tool (www.haplostats.org) selecting the most likely high-resolution typing for HLA-A, B, C, DR and DQ according to three-five highest haplotype frequencies in the population of each one (Caucasian, African American, Asian or Hispanic).

Statistics

Data are presented as mean (± standard deviation), median, interquartile range, or number (percentage) based on data distribution. Comparisons between clinical variables were carried out using Student's T test for parametric continuous variables and U Mann–Whitney or Kruskal–Wallis test for non-parametric data. Chi-squared or Fisher's exact tests were used to test categorical variables. Survival analyses were performed using the Kaplan–Meier method using the log-rank test. Logistic regression analysis was used to estimate the odds ratio (OR) for $ABMR_hDSA_{pos}$ development. All variables with a p-value <0.10 in the univariate analysis were included in the multivariate analysis. Statistical analysis was performed using SPSS v.27.0 (IBM Corp., Armonk, NY, USA) and p-values <0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Graft Survival

The selected 118 patients were grouped according to Banff diagnostic categories: category 1 or normal biopsy (n = 19), category 2 or ABMR histology ($ABMR_h$, n = 52) and category 5 or IFTA (n = 47). Twenty-nine patients (24.6%) lost their grafts and 13 died with a functioning graft (11%) during the study period. Death-censored graft survival 68 months after the biopsy [IQR 48–80] was worse in $ABMR_h$ cases than in those with IFTA or normal biopsies (**Supplementary Figure 2**). Baseline characteristics showed that normal histology patients were more frequently males, whereas $ABMR_h$ patients received grafts from younger donors and were more frequently retransplanted. $ABMR_h$ biopsies were less frequently surveillance biopsies and were performed later post-KT. At biopsy time, $ABMR_h$ patients had worse glomerular filtration rate (GFR) and higher proteinuria. Finally, IFTA patients were more frequently receiving calcineurin inhibitors and less on mTOR inhibitors (**Table 1**).

TABLE 1 | Demographics and clinical characteristics of all included patients.

	Normal (n = 19)	$ABMR_h$ (n = 52)	IFTA (n = 47)	p-value
Recipient age (years) [mean (SD)]	47.9 (12.9)	47.4 (15.2)	53.1 (14.9)	0.14
Recipient gender (female) (n, %)	3 (15.8)	27 (51.9)	20 (42.6)	**0.024**
Recipient race (caucasian) (n, %)	15 (78.9)	46 (88.5)	43 (91.5)	0.38
Type of donor (deceased) (n, %)	15 (78.9)	46 (88.5)	45 (95.7)	0.11
Donor age (years) [mean (SD)]	50.0 (13.4)	45.8 (17.5)	54.4 (16.2)	**0.039**
Underlying renal disease				
– Glomerular disease (n, %)	2 (10.5)	11 (21.2)	10 (21.3)	
– SLE and other autoimmune disease (n, %)	0 (0)	2 (3.8)	2 (4.3)	0.33
– Diabetes (n, %)	1 (5.3)	1 (1.9)	6 (12.8)	
– Other (n, %)	16 (84.2)	38 (73.1)	29 (61.7)	
Retransplantation (n, %)	2 (10.5)	16 (30.8)	5 (10.6)	**0.028**
Peak CDC cPRA (%) [mean (SD)]	3.2 (5.8)	10.6 (23.1)	6.4 (16.5)	0.29
Pretransplant HLA antibodies (SAB) (yes) (n, %)*	15 (78.9)	28 (71.8)	31 (72.1)	0.82
HLA mismatch Class I (A/B) [mean (SD)]	3.1 (0.9)	2.8 (1.0)	2.9 (1.3)	0.59
HLA mismatch Class I (C) [mean (SD)]	1.5 (0.7)	1.3 (0.7)	1.3 (0.7)	0.56
HLA mismatch Class II (DR) [mean (SD)]	1.3 (0.8)	1.2 (0.6)	1.2 (0.7)	0.65
HLA mismatch Class II (DQ) [mean (SD)]	0.7 (0.7)	0.9 (0.7)	0.8 (0.6)	0.82
Antilymphocyte induction (n, %)	0 (0)	12 (23.1)	9 (19.1)	0.10
Delayed graft function (n, %)	3 (15.8)	19 (36.5)	14 (29.8)	0.24
Acute cellular rejection < 3 months after KT (n, %)	2 (10.5)	11 (21.2)	3 (6.4)	0.15
Clinical characteristics and graft function at biopsy				
Surveillance biopsy (n, %)	13 (68.4)	7 (13.5)	25 (53.2)	**<0.001**
Biopsy time after KT (months) [median (IQR)]	13 [10–23]	45 [14–120]	13 [11–35]	**<0.001**
Time biopsy to serum (days) [median (IQR)]	0 [–56,+34]	0 [–1,+53]	–0.5 [–20,+34]	0.40
Serum creatinine (mg/dl) [mean (SD)]	1.42 (0.5)	1.92 (0.9)	1.86 (1.4)	0.23
Estimated GFR (ml/min) [mean (SD)]	65.5 (30.7)	45.1 (23.7)	50 (22.2)	**0.009**
Urine protein/creatinine ratio (mg/g) [median (IQR)]	135.6 [114–295]	549 [180–1181]	199 [133–375]	**<0.001**
Immunosuppressive treatment at biopsy				
Prednisone (n, %)	17 (89.5)	39 (75)	42 (89.4)	0.14
Calcineurin inhibitors (n, %)	15 (78.9)	39 (75)	44 (93.6)	**0.030**
Mycophenolic acid (n, %)	17 (89.5)	43 (82.7)	38 (80.9)	0.76
mTOR inhibitors (n, %)	5 (26.3)	17 (32.7)	5 (10.6)	**0.027**
Follow-up				
Graft loss (n, %)	2 (10.5)	27 (51.9)	14 (29.8)	**0.003**
Death-censored graft loss (n, %)	2 (10.5)	21 (40.4)	7 (14.9)	**0.005**
Time after biopsy (months) [median (IQR)]	74 [67–83]	59 [23–81]	68 [62–77]	**0.044**

*$ABMR_h$, antibody-mediated rejection histology; CDC, complement-dependent cytotoxicity; GFR, glomerular filtration rate; IFTA, interstitial fibrosis and tubular atrophy; IQR, interquartile range; KT, kidney transplantation; PRA, panel-reactive antibody; SAB, Single Antigen Bead assays; SD, standard deviation; SLE, systemic lupus erythematosus. *From 101 available samples pre-transplantation.*

The bold values represent those p-values that are statistically significant.

Pretransplant HLA-DSA and Non-HLA Antibodies

Pre-Transplant HLA-DSA

Pre-transplant serum samples were available for 101 patients (19 normal histology, 39 $ABMR_h$ and 43 IFTA). We found pre-transplant HLA-DSA in 18 $ABMR_h$ (46.2%), 9 IFTA (20.9%) and in two normal histology cases (10.5%) (p = 0.006) (**Figure 1A**). In $ABMR_h$ pre-transplant HLA-DSA were more frequently class I&II combined (38.9%, p = 0.087) and less isolated class I (27.8%).

Pre-Transplant AT_1R-Ab

Pre-transplant AT_1R-Ab strongly associated with $ABMR_h$ diagnosis (16/39 $ABMR_h$ (41%) *vs.* 2/19 normal histology (10.5%) and 5/43 IFTA (11.6%), p = 0.003) (**Figure 1A**). All 16 $ABMR_h$ patients with pre-transplant AT_1R-Ab developed $ABMR_hDSA_{pos}$, whereas no $ABMR_hDSA_{neg}$ patient showed pre-transplant AT_1R-Ab (p = 0.029). Detection of pre-transplant AT_1R-Ab correlated with both persistent preformed HLA-DSA (12/23, 52%) and *de novo* HLA-DSA detection (8/18, 44%), but not with preformed HLA-DSA which cleared after transplant (1/6, 17%) or no HLA-DSA (2/54, 4%, p <0.001) (**Figure 1B**). The median MFI of preformed HLA-DSA coexistent with AT_1R-Ab was not significantly different than preformed HLA-DSA without AT_1R-Ab (8898 *vs* 2874, p = 0.083).

Other Non-HLA Antibodies

Neither pre-transplant ETAR-Ab nor MICA-Ab associated with $ABMR_h$. Pre-transplant ETAR-Ab and MICA-Ab were present similarly in normal histology, $ABMR_h$ and IFTA cases (31.6, 25.6 and 18.6%, p = 0.51; 10.5, 7.7 and 9.3%, p = 1.00). Of 83 KT recipients tested with EC-XM, only 3/29 $ABMR_h$ (10.3%) and 3/39 IF/TA cases (7.7%) had a pre-transplant positive EC-XM (**Figure 1A**).

Pre-Transplant Combination of HLA-DSA and Non-HLA Antibodies

Detection of pre-transplant HLA-DSA and/or AT_1R-Ab were highly associated with $ABMR_h$ compared with IFTA and normal biopsies (66.7 *vs.* 25.6 *vs.* 21%, p <0.001, **Figure 1C**). Nine $ABMR_h$ cases presented with simultaneous HLA-DSA and AT_1R-Ab

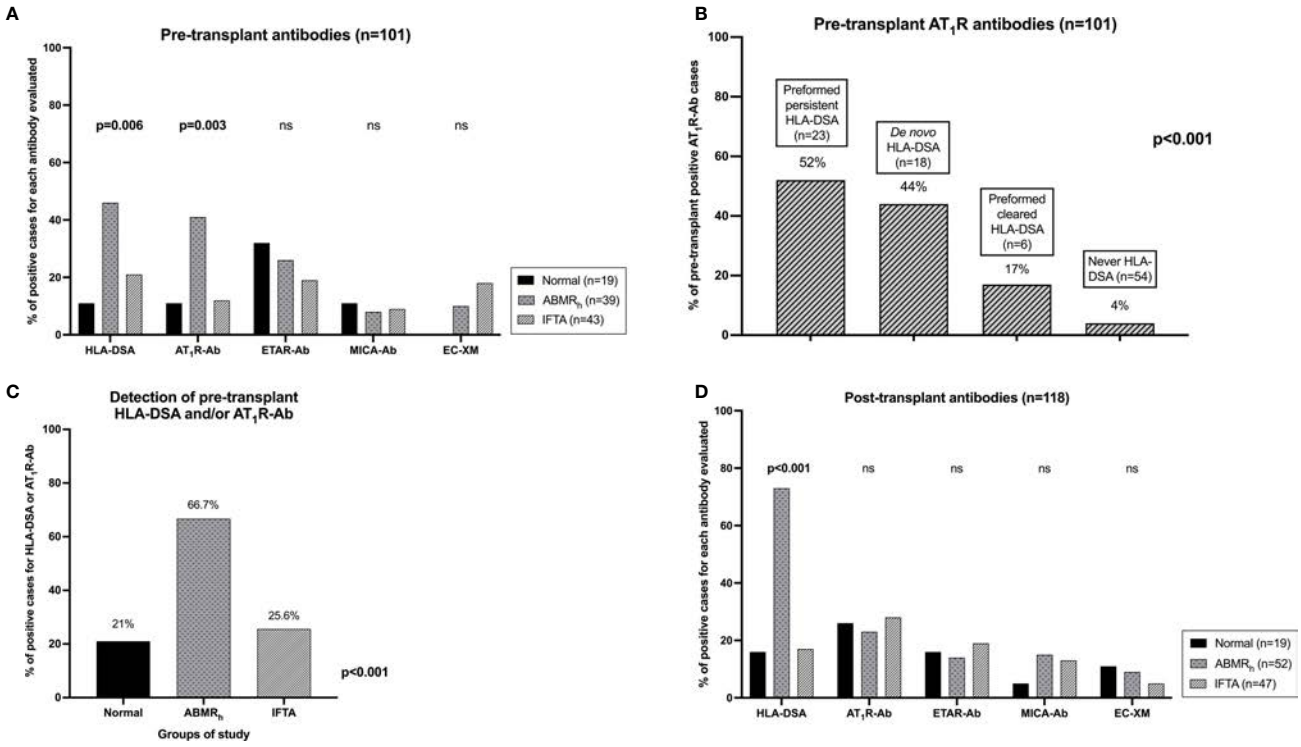

FIGURE 1 | Pre-transplant HLA and non-HLA antibodies. **(A)** HLA, AT_1R-Ab, ETAR-Ab, MICA-Ab and EC-XM before transplantation in the three groups of study. **(B)** Pre-transplant AT_1R-Ab positive and negative patients with preformed persistent HLA-DSA, preformed cleared HLA-DSA, *de novo* HLA-DSA or without HLA-DSA at any time. **(C)** Detection of pre-transplant HLA-DSA and/or AT_1R-Ab in the three study groups. **(D)** Post-transplant HLA and non-HLA antibodies. HLA, AT_1R-Ab, ETAR-Ab, MICA-Ab and EC-XM after transplantation in the three groups of study. $ABMR_h$, antibody-mediated rejection histology; AT_1R-Ab, antibodies against angiotensin II type 1 receptor; EC-XM, crossmatch with primary aortic endothelial cells; ETAR-Ab, antibodies against endothelin-1 type A receptor; IFTA, interstitial fibrosis and tubular atrophy; KT, kidney transplantation; MICA-Ab, antibodies against major histocompatibility complex class I related chain A. ns, non-significant.

(23.1%), 17 with either HLA-DSA or AT_1R-Ab (43.6%) and the remaining 13 did not present any of these antibodies (33.3%).

Post-Transplant HLA-DSA and Non-HLA Antibodies

Post-Transplant HLA-DSA

At the time of biopsy, HLA-DSA was detectable in 38/52 $ABMR_h$ patients [73.1%, 17 preformed (44.7%) and 21 *de novo* (55.3%)]. Among them, 7.7% were class I, 53.8% class II and 11.5% combined class I&II. HLA-DSA were also detected in 17% IFTA and 15.8% normal histology cases (**Figure 1D**).

Post-Transplant AT_1R-Ab

Post-transplant AT_1R-Ab showed no association with $ABMR_h$ (23.1% in $ABMR_h$ *vs.* 26.3% in normal histology and 27.7% in IFTA cases, p = 0.85, **Figure 1D**). Detection of post-transplant AT_1R-Ab did not correlate with the detection of HLA-DSA [15/49 HLA-DSA_{pos} cases had AT_1R-Ab at biopsy (30.6%) *vs.* 15/69 HLA-DSA_{neg} cases (21.7%), p = 0.28].

Other Non-HLA Antibodies

Neither post-transplant ETAR-Ab nor MICA-Ab was related with $ABMR_h$. Post-transplant ETAR-Ab were found in 3/19 normal histology (15.8%), 7/52 $ABMR_h$ (13.5%) and 9/47 IFTA cases (19.1%, p = 0.80). MICA-Ab were detectable in 1/19 normal histology (5.3%), 8/52 $ABMR_h$ (15.4%) and 6/47 IFTA cases (12.8%, p = 0.62). Two normal histology (11.1%), four $ABMR_h$ (9.3%) and two IFTA cases (4.8%) had a positive EC-XM (p = 0.70, **Figure 1D**).

Patients With $ABMR_h$ With and Without HLA-DSA

From 52 patients with $ABMR_h$ 14 (26.9%) had no peri-biopsy HLA-DSA. $ABMR_hDSA_{pos}$ cases were more frequently HLA sensitized, less well DR-matched with their donors and received more frequently a graft from a deceased donor than those $ABMR_hDSA_{neg}$. No differences were found in graft function or immunosuppression at biopsy (**Table 2**). Patients showed similar microvascular inflammation, but diffuse C4d was more frequent in $ABMR_hDSA_{pos}$ cases (27% *vs* 0%, p = 0.07, **Table 2**). Graft survival was similar between both groups (**Figure 2**). We assessed pre- and post-transplant non-HLA antibodies in $ABMR_hDSA_{neg}$ cases. Of 7 cases with pre-transplant sample, two had EC-XM^+ but none showed MICA-Ab, AT_1R-Ab or ETAR-Ab (**Table 3A**). After KT, one had coexistent MICA-Ab, AT_1R-Ab and ETAR-Ab; one had

TABLE 2 | Characteristics of patients with and without HLA-DSA.

	$ABMR_hDSA_{pos}$ (n = 38)	$ABMR_hDSA_{neg}$ (n = 14)	p-value
Recipient age (years) [mean (SD)]	47.8 (15.7)	46.4 (14.2)	0.76
Recipient gender (female) (n, %)	20 (52.6)	7 (50)	1.00
Recipient race (caucasian) (n, %)	34 (89.5)	12 (85.7)	0.46
Type of donor (deceased) (n, %)	36 (94.7)	10 (71.4)	**0.038**
Donor age (years) [mean (SD)]	45.5 (18.9)	46.9 (13.7)	0.80
Underlying renal disease			
– Glomerular disease (n, %)	5 (13.2)	6 (42.9)	0.10
– SLE and other autoimmune disease (n, %)	2 (5.3)	0 (0)	
– Diabetes (n, %)	1 (2.6)	0 (0)	
– Other (n, %)	30 (78.9)	8 (57.1)	
Retransplantation (n, %)	14 (36.8)	2 (14.3)	0.18
Peak CDC cPRA (%) [mean (SD)]	14.2 (26.2)	0.6 (2.4)	**0.003**
Pretransplant HLA antibodies (SAB) (yes) (n, %)*	25 (78.1)	3 (42.9)	0.08
HLA mismatch Class I (A/B) [mean (SD)]	2.8 (1.0)	2.6 (1.0)	0.52
HLA mismatch Class I (C) [mean (SD)]	1.3 (0.7)	1.1 (0.8)	0.25
HLA mismatch Class II (DR) [mean (SD)]	1.4 (0.5)	0.7 (0.6)	**<0.001**
HLA mismatch Class II (DQ) [mean (SD)]	0.9 (0.7)	0.7 (0.7)	0.41
Antilymphocyte induction (n, %)	9 (23.7)	3 (21.4)	0.28
Delayed graft function (n, %)	16 (42.1)	3 (21.4)	0.21
Acute cellular rejection <3 months after KT (n, %)	5 (13.2)	6 (42.9)	0.08
Clinical characteristics and graft function at biopsy			
Surveillance biopsy (n, %)	18 (47.4)	4 (28.6)	0.34
Biopsy time after KT (months) [median (IQR)]	44 [14–99]	74 [15–220]	0.22
Time biopsy to serum (days) [mean (SD)]	30 (78)	20 (61)	0.66
Serum creatinine (mg/dl) [mean (SD)]	2.01 (1.0)	1.70 (0.6)	0.30
Estimated GFR (ml/min) [mean (SD)]	44.8 (25.5)	45.8 (19.1)	0.89
Urine protein/creatinine ratio (mg/g) [median (IQR)]	413 [170–1189]	695 [406–1,174]	0.27
Immunosuppressive treatment at biopsy			
Prednisone (n, %)	30 (78.9)	9 (64.3)	0.30
Calcineurin inhibitors (n, %)	27 (71.1)	12 (85.7)	0.47
Mycophenolic acid (n, %)	32 (84.2)	11 (78.6)	0.69
mTOR inhibitors (n, %)	14 (36.8)	3 (21.4)	0.34
Follow-up			
Graft loss (n, %)	19 (50)	8 (57.1)	0.76
Death-censored graft loss (n, %)	15 (39.5)	6 (42.9)	1.00
Time after biopsy (months) [median (IQR)]	61 [21–85]	55 [27–76]	0.87
Histological features of $ABMR_h$			
Percentage of glomerulosclerosis [mean (SD)]	18.4% (17.5)	18.8% (18.4)	0.95
Glomerulitis (g ≥1) (yes, %)	30 (78.9)	12 (85.7)	0.71
g0	8 (21.1)	2 (14.3)	
g1	16 (42.1)	4 (28.6)	0.58
g2	10 (26.3)	5 (35.7)	
g3	4 (10.5)	3 (21.4)	
Peritubular capilaritis (ptc ≥1) (yes, %)	31 (81.6)	9 (64.3)	0.27
ptc0	7 (18.4)	5 (35.7)	
ptc1	21 (55.3)	5 (35.7)	0.18
ptc2	10 (26.3)	3 (21.5)	
ptc3	0 (0)	1 (7.1)	
Microvascular inflammation (g + ptc ≥2) (yes, %)	31 (81.6)	12 (85.7)	1.00
C4d positivity (yes, %)	17 (44.7)	6 (42.9)	1.00
C4d0	20 (54.1)	8 (57.1)	
C4d1	4 (10.8)	2 (14.3)	0.07
C4d2	3 (8.1)	4 (28.6)	
C4d3	10 (27.0)	0 (0)	
Chronic transplant glomerulopathy (yes, %)[#]	20 (58.9)	9 (69.2)	0.74
EM CTG or PTCML (yes, %)[#]	28 (82.4)	9 (69.2)	0.43
Arteriolar hialinosis (ah ≥1) (yes, %)	18 (47.4)	6 (42.9)	0.76
Arterial intimal fibrosis (cv ≥1) (yes, %)[#]	18 (52.9)	6 (50)	1.00
Interstitial fibrosis (ci ≥1) (yes, %)	35 (92.1)	14 (100)	0.56
Tubular atrophy (ct ≥1) (yes, %)	32 (84.2)	14 (100)	0.17
Tubulitis (t ≥1) (yes, %)	8 (21.1)	0 (0)	0.09
Interstitial inflammation (i ≥1) (yes, %)	6 (15.8)	0 (0)	0.17
Intimal arteritis (v ≥1) (yes, %)[#]	1 (3.1)	0 (0)	1.00

*$ABMR_h$, antibody-mediated rejection histology; CDC, complement-dependent cytotoxicity; CTG, chronic transplant glomerulopathy; EM, electron microscopy; GFR, glomerular filtration rate; IFTA, interstitial fibrosis and tubular atrophy; IQR, interquartile range; KT, kidney transplantation; PRA, panel-reactive antibody; PTCML, peritubular capillary multilayering; SAB, Single Antigen Bead assays; SD, standard deviation; SLE, systemic lupus erythematosus. *From 101 available samples pre-transplantation. [#]From 46/47 biopsies (34 $ABMR_hDSA_{pos}$, 12/13 $ABMR_hDSA_{neg}$). The bold values represent those p-values that are statistically significant.*

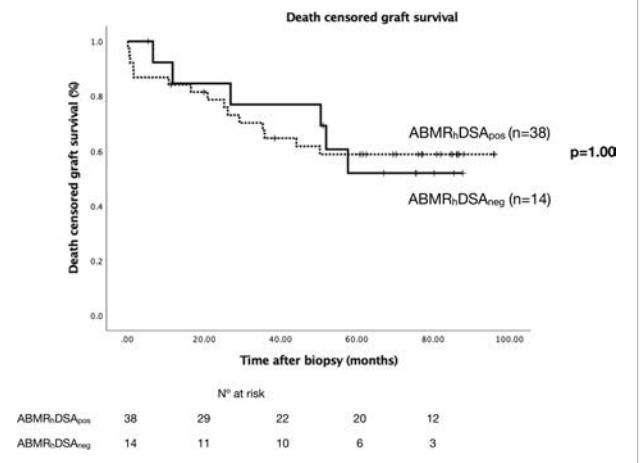

FIGURE 2 | Death censored graft survival in $ABMR_h$ patients with and without HLA-DSA. Kaplan–Meier survival curves representing death censored graft survival. $ABMR_h$, antibody-mediated rejection histology; DSA, donor-specific antibodies.

MICA-Ab and a third one AT_1R-Ab (**Supplementary Table 1A**). In 9/14 $ABMR_hDSA_{neg}$ patients (64.3%) we could not identify any of the non-HLA antibodies studied.

HLA Epitope Mismatch Characterization

The median number of class I and class II HLA-EM in our cohort were 16 (0–36) and 18 (0–46) respectively. Among them, 10 class I and 7 class II HLA-EM were antibody-verified ($HLA\text{-}EM^{ver}$). We observed similar class I and class II $HLA\text{-}EM^{ver}$ in all three groups of study (data not shown). We compared the load of $HLA\text{-}EM^{ver}$ between $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$ patients, finding similar class I but significantly higher class II and DRB $HLA\text{-}EM^{ver}$ in $ABMR_hDSA_{pos}$ cases (8 *vs* 4.5, p = 0.046; 5 *vs.* 0.5, p = 0.044, **Figure 3**). We compared HLA-EM and HLA antigen mismatch (HLA-AM) for *de novo* DSA (dnDSA) development prediction. Neither class I $HLA\text{-}EM^{ver}$ nor HLA-AM were useful tools for class I dnDSA prediction. Class II $HLA\text{-}EM^{ver}$ were significantly associated with class II dnDSA (8 *vs.* 7, p = 0.031), but not class II HLA-AM (p = 0.26). The extent of DRB $HLA\text{-}EM^{ver}$ associated with DRB dnDSA (6 *vs.* 4, p = 0.024), and the rate of DQB $HLA\text{-}EM^{ver}$ showed a weak association with DQB dnDSA (4 *vs.* 2, p = 0.077). Neither DRB nor DQB HLA-AM predicted DRB or DQB dnDSA (p = 0.27, p = 0.21).

Risk Factors for Post-Transplant $ABMR_hDSA_{pos}$ Development

$ABMR_hDSA_{pos}$ patients showed higher rates of pre-transplant HLA-DSA and AT_1R-Ab (p <0.001, **Table 3B**), but regarding post-transplant antibodies, only HLA-DSA was associated with $ABMR_hDSA_{pos}$ (p <0.001, **Supplementary Table 1B**). In order to assess the role of each factor, we adjusted a multivariate model which showed that both pre-transplant HLA-DSA (OR: 3.69 [1.31–10.37], p = 0.013) and AT_1R-Ab (OR: 5.47 [1.78–16.76], p = 0.003) were independent $ABMR_hDSA_{pos}$ predictors. DRB $HLA\text{-}EM^{ver}$ also showed a weak association with $ABMR_hDSA_{pos}$ (p = 0.071, **Table 4**).

DISCUSSION

We report here that ABMR damage in KT recipients occurs in a significant proportion of cases without the detection of HLA-DSA at biopsy. We have evaluated the role of non-HLA antibodies, such as AT_1R-Ab, ETAR-Ab, MICA-Ab or anti-EC antibodies detected with crossmatches and found they could not explain $ABMR_hDSA_{neg}$. Our results suggest a synergistic interaction between pre-transplant AT_1R-Ab and HLA-DSA to

TABLE 3A | Comparison of pre-transplant non-HLA antibodies between $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$ cases.

	$ABMR_hDSA_{pos}$ (n = 38)*	$ABMR_hDSA_{neg}$ (n = 14)*	p-value
Pre-transplant AT_1R-Ab (yes, %)	16 (50)	0 (0)	**0.029**
Pre-transplant ETAR-Ab (yes, %)	10 (31.3)	0 (0)	0.16
Pre-transplant MICA-Ab (yes, %)	3 (9.4)	0 (0)	1.00
Pre-transplant EC-XM (positive, %)#	1 (4.5)	2 (28.6)	0.14

**From 32 $ABMR_hDSA_{pos}$ and 7 $ABMR_hDSA_{neg}$ cases with pre-transplant available samples. #From 22 $ABMR_hDSA_{pos}$ and 7 $ABMR_hDSA_{neg}$ cases.*
The bold values represent those p-values that are statistically significant.

TABLE 3B | Pre-transplant HLA and non-HLA antibodies: comparison between $ABMR_hDSA_{pos}$ and non-$ABMR_hDSA_{pos}$ cases (normal histology, IFTA and $ABMR_hDSA_{neg}$ cases).

	$ABMR_hDSA_{pos}$ (n = 38)*	No $ABMR_hDSA_{pos}$ (n = 80)*	p-value
Pre-transplant HLA-DSA (yes, %)	17 (53.1)	12 (17.4)	**<0.001**
Pre-transplant AT_1R-Ab (yes, %)	16 (50)	7 (10.1)	**<0.001**
Pre-transplant ETAR-Ab (yes, %)	10 (31.2)	14 (20.3)	0.23
Pre-transplant MICA-Ab (yes, %)	3 (9.4)	6 (8.7)	1.00
Pre-transplant EC-XM (positive, %)$	1 (4.5)	5 (8.2)	1.00

**32 $ABMR_hDSA_{pos}$ cases and 69 non-$ABMR_hDSA_{pos}$ cases with pre-transplant available samples. $22 $ABMR_hDSA_{pos}$ and 61 non-$ABMR_hDSA_{pos}$ cases.*

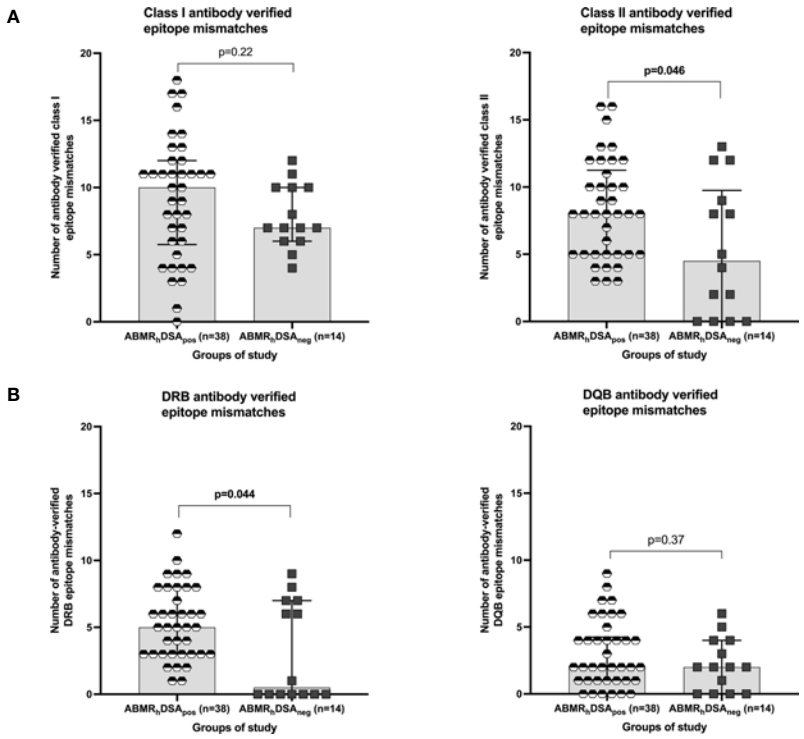

FIGURE 3 | HLA epitope mismatch analysis in $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$ cases. **(A)** Number of antibody-verified class I and class II epitope mismatches and **(B)** Number of antibody-verified DRB and DQB epitope mismatches in $ABMR_hDSA_{pos}$ (black and white hexagons) and $ABMR_hDSA_{neg}$ (black squares) cases. All plots show median and interquartile range (IQR).

TABLE 4 | Logistic regression analysis of $ABMR_hDSA_{pos}$ risk factors.

	Univariate		Multivariate	
Risk factor	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
Pre-transplant HLA-DSA	5.38 (2.12–13.68)	**<0.001**	3.69 (1.31-10.37)	**0.013**
Pre-transplant AT_1R-Ab	8.86 (3.12–25.17)	**<0.001**	5.47 (1.78-16.76)	**0.003**
Pre-transplant ETAR-Ab	1.79 (0.69–4.62)	0.23		
Pre-transplant MICA-Ab	1.09 (0.25–4.65)	0.91		
Pre-transplant positive EC-XM	0.53 (0.06–4.84)	0.58		
Class I HLA-EM^{ver}	0.99 (0.90–1.08)	0.79		
DRB HLA-EM^{ver}	1.21 (1.04–1.40)	**0.011**	1.18 (0.99-1.41)	0.071
DQB HLA-EM^{ver}	1.10 (0.94–1.29)	0.23		

AT_1R-Ab, antibodies against angiotensin II type 1 receptor; EC-XM, crossmatch with primary aortic endothelial cells; ETAR-Ab, antibodies against endothelin-1 type A receptor; HLA-DSA, HLA donor-specific antibodies; HLA-EM^{ver}, antibody-verified HLA epitope mismatches; MICA-Ab, antibodies against major histocompatibility complex class I related chain A.
The bold values represent those p-values that are statistically significant.

produce $ABMR_hDSA_{pos}$ or facilitate *de novo* appearance of HLA-DSA, but not to induce $ABMR_hDSA_{neg}$. Interestingly, it appears more strongly associated with $ABMR_h$ than incompatibility evaluated through HLA-EM analysis.

The relationship between $ABMR_h$ and HLA-DSA has been described in KT recipients for over 20 years (1, 2). However, there is increased evidence that ABMR compatible histological lesions may be present in the graft without detectable circulating HLA-DSA (18, 38). Up to 27% of our $ABMR_h$ patients did not show circulating HLA-DSA at the time of biopsy. This could be attributed to the inability of current techniques to detect these HLA antibodies or due to the participation of a different set of antibodies in graft damage. $ABMR_hDSA_{neg}$ patients presented significantly lower class II and DRB HLA-EM compared with $ABMR_hDSA_{pos}$ cases. This finding strengthens the hypothesis of the participation of other mechanisms of damage in these cases rather than non-detected HLA-DSA. However, neither AT_1R-Ab, ETAR-Ab, MICA-Ab nor antibodies identified with EC-XM before or after KT were able to explain the $ABMR_hDSA_{neg}$ cases in our study. We describe here that $ABMR_h$ patients without

HLA-DSA showed similar graft function, immunosuppressive treatment, histological features at biopsy and graft survival at the end of follow-up compared with $ABMR_hDSA_{pos}$ cases. Like us, Sablik et al. (17) reported a similar histological phenotype in $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$ patients, but a larger study by Senev et al. (18) found that $ABMR_hDSA_{pos}$ biopsies were more frequently C4d positive compared with $ABMR_hDSA_{neg}$ cases, as the unique histological difference between the groups. In our series, although C4d positivity was similar between both groups, C4d intensity was higher in the $ABMR_hDSA_{pos}$ group. In our cohort, graft survival was similar between both groups, in agreement with results reported by Sablik et al. (17) but in contrast with the study from Senev et al. (18), which included mostly active ABMR cases without chronicity, unlike our cohort.

KT recipients may produce immune responses through indirect recognition against foreign proteins or even against own proteins expressed by the donor graft acting as autoantigens due to different factors that induce graft damage during the transplant process. These antibodies may then react against polymorphic alloantigens, like HLA related MICA or MICB, or against autoantigens like AT_1R, ETAR, agrin, vimentin, perlecan, K-tubulin, etc. (39–41) which may be prevalent in KT recipients. Some of these autoantibodies and new ones recently validated (42) have not been evaluated in our cohort yet. They might explain some $ABMR_hDSA_{neg}$ cases. Some groups have evaluated the relationship between antibodies directed against ECs—the barrier between donor and recipient—and graft survival (43), and exploratory studies have employed array techniques in limited series with antibodies against ECs validating potential target proteins with ELISA (44, 45). Jackson and col. were able to identify four antigenic targets expressed on ECs in nine patients with $ABMR_hDSA_{neg}$ (44). They found that antibodies against these proteins in pre-transplant sera predicted $ABMR_hDSA_{pos}$. In our cohort, of seven $ABMR_hDSA_{neg}$ cases with pre-transplant samples, two had a positive EC-XM^+, but none showed MICA-Ab, AT_1R-Ab or ETAR-Ab. In line with our results, a recent report from Delville et al. (23) found that only 26% of patients with early acute $ABMR_hDSA_{neg}$ had pre-transplant AT_1R-Ab using our same threshold of 10 UI/ml. Moreover, MICA-Ab were only detected in two of these $ABMR_hDSA_{neg}$ cases. However, these cases had preformed IgG antibodies against constitutively expressed antigens of microvascular glomerular cells (23). Of note, our two cases with pre-transplant EC-XM^+ developed $ABMR_h$ within the first 12 months of KT, while the other twelve developed $ABMR_h$ later on. Unlike Lefaucheur et al. (46), despite employing the same threshold for AT_1R antibodies, the presence of these antibodies in our $ABMR_hDSA_{neg}$ cohort is negligible. Nevertheless, our overall prevalence of 25% in post-transplant AT_1R-Ab is not different from theirs. Unfortunately, these authors do not analyze the relation between pre-transplant AT_1R-Ab and ABMR.

We report here a strong and independent association between pre-transplant AT_1R-Ab and $ABMR_hDSA_{pos}$ development. AT_1R can be found in several cell types such as vascular endothelial cells and binds to angiotensin II (39, 47). First report linking AT_1R-Ab and kidney allograft rejection suggested a potential relationship between AT_1R agonistic antibodies and vascular injury (19, 39). Subsequently, pre- or post-transplant AT_1R-Ab detection have been linked to both rejection and allograft failure (19, 48). Philogene et al. (24) described higher post-transplant AT_1R-Ab levels in patients with ABMR compared with patients with cellular rejection or those without rejection, however, they provided no data regarding pre-transplant AT_1R-Ab. In another report (49), pre- and post-transplant AT_1R-Ab were strongly associated with biopsy-proven rejection, not specifically ABMR. Some reports suggest that non-HLA and HLA-DSA antibodies may function in synergy (24, 49). Taniguchi et al. (49) reported lower graft survival mainly in the presence of *de novo* AT_1R-Ab and HLA-DSA at biopsy with lesions compared with those cases with HLA-DSA alone. Here we show a strong association of pre-transplant AT_1R-Ab with post-transplant HLA-DSA, either persistent preformed or *de novo*, and with $ABMR_hDSA_{pos}$ development. This association may be of utmost importance for KT outcomes. We previously reported the strong association among persistent preformed HLA-DSA and lower ABMR free survival, only surpassed by the development of *de novo* HLA-DSA (34). Moreover, here we show that all 16 $ABMR_h$ patients with pre-transplant AT_1R-Ab had HLA-DSA at biopsy, nine of them maintained the preformed HLA-DSA and seven developed *de novo* HLA-DSA. We found no association between pre-transplant AT_1R-Ab and graft survival, in line with other reports (26, 49). In our multivariate analysis, pre-transplant HLA-DSA and AT_1R-Ab were independent predictors for $ABMR_h$. Our study may not be powered enough to assess the relationship between AT_1R-Ab and graft loss. Given the strong and already known association between ABMR and increased risk of kidney allograft loss (34, 50–52), our data supports that pre-transplant AT_1R-Ab assessment should be carefully considered in KT candidates.

In the last years, HLA-EM analysis has been proposed as a better strategy to prevent HLA-DSA development than antigen matching (29). Here we confirm that class II and DRB dnDSA development may be predicted with HLA-EM, as previously reported (30), however, only a weak association was observed with DQB dnDSA, probably due to the limited number of cases included. Interestingly, neither class II, DRB or DQB HLA-AM were able to predict dnDSA. As mentioned, the detection of lower number of class II and DRB HLA-EM in $ABMR_hDSA_{neg}$ cases may contradict the idea of undetected HLA-DSA responsible for the damage. Class II and DRB HLA-EM associated with $ABMR_hDSA_{pos}$, although the existence of preformed HLA-DSA or AT_1R-Ab are more potent predictors of $ABMR_hDSA_{pos}$ in our experience. In our study, $ABMR_hDSA_{neg}$ could not be explained by higher HLA-EM or by the non-HLA antibodies evaluated. Interestingly, an alternative mechanism to produce $ABMR_h$ termed "the missing-self hypothesis" has been proposed. According to it, the inability of graft EC to provide HLA I-mediated inhibitory signals to recipient circulating NK cells may trigger NK cell activation, resulting in endothelial damage and chronic vascular rejection (53).

The main limitation of our study is the restricted number of $ABMR_hDSA_{neg}$ cases in the whole cohort. In order to further increase its number and the significance of the study, a

multicenter trial is advisable. Besides, it is based on a mix of indication and surveillance biopsies which introduces heterogeneity in the timing and clinical picture of patients. Of note, EC-XM were performed with aortic cells which may not express the same proteins as a renal EC. Last, another limitation may be the use of inferred four-digit HLA typing for HLA-EM analysis. Despite careful estimation of second field HLA typing, we cannot rule out the possibility that some rare HLA genotypes are not correctly assigned, as recently suggested (54). However, ours is a large well characterized cohort of KT recipients, reflecting clinical practice, with thorough analysis of biopsies, including electron microscopy, crucial to detect some cases of $ABMR_h$ and with systematical study of HLA-DSA and a known set of non-HLA antibodies.

In summary, although the majority of patients with HLA-DSA at the time of biopsy show $ABMR_h$, almost 30% of $ABMR_h$ patients did not show evidence of circulating HLA-DSA. These patients were more frequently HLA unsensitized pretransplant and less HLA matched but did not show other specific characteristics at transplantation or at biopsy. Neither AT_1R-Ab, ETAR-Ab, MICA-Ab nor antibodies identified with EC-XM before or after KT were able to explain $ABMR_hDSA_{neg}$ cases. Importantly AT_1R-Ab with or without HLA-DSA before KT clearly increased the risk of $ABMR_hDSA_{pos}$, suggesting it should be included in the pre-transplant immune assessment together with HLA-DSA.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Parc de Salut Mar Ethical Research Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MC designed the study, coordinated logistics, analyzed the results, and drafted the manuscript. LL-M analyzed the results and drafted the manuscript. DR-P analyzed the results and revised the manuscript. CBut coordinated lab procedures and revised the manuscript. JG contributed with the assessment of the graft biopsies. MP-S, CBur, AB, CA-C, and SS-U revised the manuscript. MF coordinated sample drawing and storage. NV supervised HLA and non-HLA antibody interpretation. ER and JP evaluated the design of the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was performed with funding from projects PI13/00598, PI16/00617, and PI20/00090 (Spanish Ministry of Health ISCIII FIS-FEDER); RD16/0009/0013 (ISCIII FEDER REDinREN) and 201822-10 (Fundació la Marató de TV3). MC received grants of the Sociedad Española de Trasplante and Parc de Salut Mar for a research stay at UCLA Immunogenetics Center (Los Angeles, USA). ER is supported by National Institute of Allergy and Infectious Diseases Grants RO1 AI135201. One Lambda provided reagents but had no role in the design of the study or the analyses and writing of the manuscript.

ACKNOWLEDGMENTS

We are indebted to Anna Faura, Rosa Causadias and Anna Bach for their assistance with patients. We thank Duska Dragun for her personal encouragement to perform the study the way it is. We also extend our gratitude to the staff of the UCLA Immunogenetics Center for technical assistance with non-HLA antibody tests and EC-XM. Finally, the authors hereby express their thanks to the organ donors and their families for giving the gift of life and, together with the recipients, the gift of knowledge. LL-M did this study as part of her doctoral thesis program at the Department of Medicine from the Universitat Autonoma of Barcelona (UAB).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.703457/full#supplementary-material

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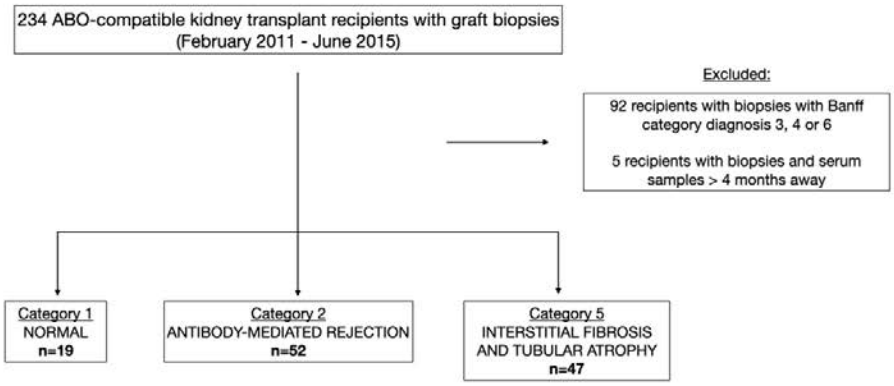
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

1 Supplementary Figures and Tables

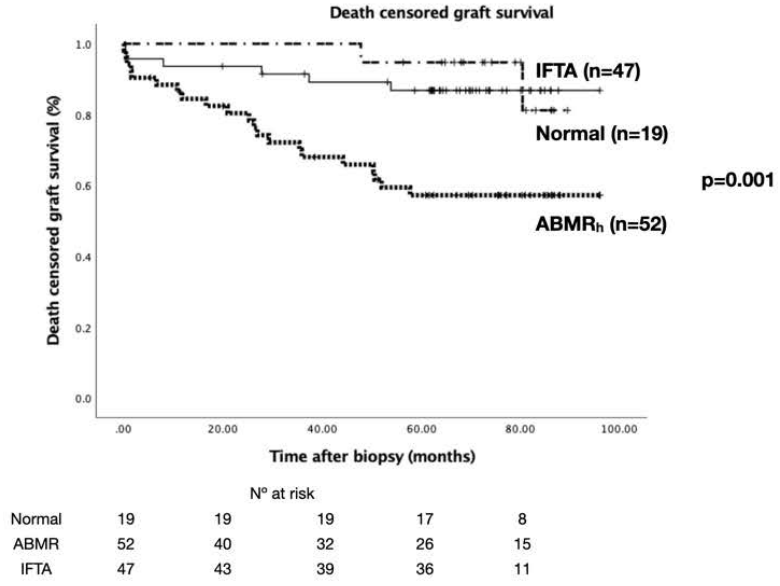
1.1 Supplementary Figures

Supplementary Figure 1



Supplementary Figure 1. Patient flow-chart.

Supplementary Figure 2



Nº at risk

Normal	19	19	19	17	8
ABMR	52	40	32	26	15
IFTA	47	43	39	36	11

Supplementary Figure 2. Death censored graft survival in patients with normal, $ABMR_h$ and IFTA biopsies. Kaplan-Meier survival curves representing death censored graft survival. $ABMR_h$: antibody-mediated rejection histology; IFTA: interstitial fibrosis and tubular atrophy.

1.2 Supplementary Tables

Supplementary Table 1.

(**A**) Comparison of post-transplant non-HLA antibodies between $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$ cases.

	$ABMR_hDSA_{pos}$ (n=38)	**$ABMR_hDSA_{neg}$ (n=14)**	**p-value**
Post-transplant AT_1R-Ab (yes, %)	10 (26.3%)	2 (14.3%)	0.48
Post-transplant ETAR-Ab (yes, %)	6 (15.8%)	1 (7.1%)	0.66
Post-transplant MICA-Ab (yes, %)	6 (15.8%)	2 (14.3%)	1.00
Post-transplant EC-XM (positive, %) [$]	4 (13.3%)	0 (0%)	0.30

[$] From 30 $ABMR_hDSA_{pos}$ and 13 $ABMR_hDSA_{neg}$ cases.

$ABMR_h$: antibody-mediated rejection histology; AT_1R-Ab: antibodies against angiotensin II type 1 receptor; EC-XM: crossmatch with primary aortic endothelial cells; ETAR-Ab: antibodies against endothelin-1 type A receptor; MICA-Ab: antibodies against major histocompatibility complex class I related chain A.

(**B**) Comparison of post-transplant HLA and non-HLA antibodies between $ABMR_hDSA_{pos}$ and non-$ABMR_hDSA_{pos}$ cases (normal histology, IFTA and $ABMR_hDSA_{neg}$ cases).

	$ABMR_hDSA_{pos}$ (n=38)	**No $ABMR_hDSA_{pos}$ (n=80)**	**p-value**
Post-transplant HLA-DSA (yes, %)	38 (100%)	11 (13.8%)	**<0.001**
Post-transplant AT_1R-Ab (yes, %)	10 (26.3%)	20 (25%)	0.88
Post-transplant ETAR-Ab (yes, %)	6 (15.8%)	13 (16.3%)	0.95
Post-transplant MICA-Ab (yes, %)	6 (15.8%)	9 (11.3%)	0.56
Post-transplant EC-XM (positive, %) [#]	4 (13.3%)	4 (5.5%)	0.23

[#]30 $ABMR_hDSA_{pos}$ and 73 non-$ABMR_hDSA_{pos}$ cases.

AT_1R-Ab: antibodies against angiotensin II type 1 receptor; EC-XM: crossmatch with primary aortic endothelial cells; ETAR-Ab: antibodies against endothelin-1 type A receptor; HLA-DSA: HLA donor-specific antibodies; MICA-Ab: antibodies against major histocompatibility complex class I related chain A.

5. RESULTS SUMMARY

5. Results summary

5.1 Neither steroid withdrawal nor conversion from tacrolimus to mTOR inhibitors were associated with a significant development of *de novo* HLA donor-specific antibodies

For the first study, 35 patients were included: 19 withdrew steroids (steroid withdrawal group, SW) and 16 maintained their baseline treatment (steroid maintenance group, SM). In the second study, 45 patients were included: 29 switched from CNI to mTORi (mTORi group) and 16 maintained their treatment with tacrolimus, steroids and mycophenolic acid (tacrolimus group). All patients were followed up for 24 months.

Regarding SW study, all patients showed stable renal function considering serum creatinine, estimated glomerular filtration rate (eGFR) and proteinuria. No graft loss or death was registered, and no patient in SW group needed to reintroduce steroids. Concerning mTORi study, clinical follow-up showed stable renal function and a non-significant increase in proteinuria in the mTORi group. Seven KT recipients converted to mTORi did not finalize the study on treatment: six reintroduced tacrolimus between 12 and 24 months and one died during the study period. With respect to the development of HLA-DSA and HLA no DSA, in the SW study no patient developed dnHLA-DSA during the 24 months of sequential evaluation, whereas in the mTORi study, two mTORi patients developed dnHLA-DSA (2/29, 6.9%). Rate of dnHLA-DSA was similar between mTORi group and tacrolimus group (7% vs. 0%, p=0.53). Four patients developed *de novo* HLA no DSA in the SW study, two in the SW (11%) and two in the SM group (13%, p=1.00). In the mTORi study, three mTORi (10%) and two tacrolimus patients presented *de novo* HLA no DSA (13%, p=1.00).

5.2 Long-term redistribution of peripheral blood lymphocyte subsets after steroid withdrawal or conversion from tacrolimus to mTOR inhibitors

After SW, patients presented a significant decrease of T cells compared to SM patients. This T-cell decrease was observed during the first year, followed by a stabilization during the second year. At the end of the follow-up, both the proportion and absolute numbers of T cells in SW patients – but not in SM patients - were similar to those displayed by healthy subjects (HS). The decrease of T cells was partially due to a significant decrease of $CD4^+$ T cell subset, without alterations in the $CD8^+$ T cell subset. Interestingly, SW patients showed a B cell increase during the follow-up, reaching the levels of HS in the 24-month point of study (**Figure 5A**). Further analysis of B cell subsets identified a significant increase of naïve B cells after SW, in contrast with SM group. Of note, SW but not SM patients reached comparable

proportion and numbers of naïve B cells to those of HS (**Figure 5B**). A remarkable reduction of memory B cell relative numbers after SW was also observed, but absolute numbers did not reflect this change (**Figure 5C**). SW patients also presented increased proportions of transitional B cells along follow-up compared to SM patients (**Figure 5D**).

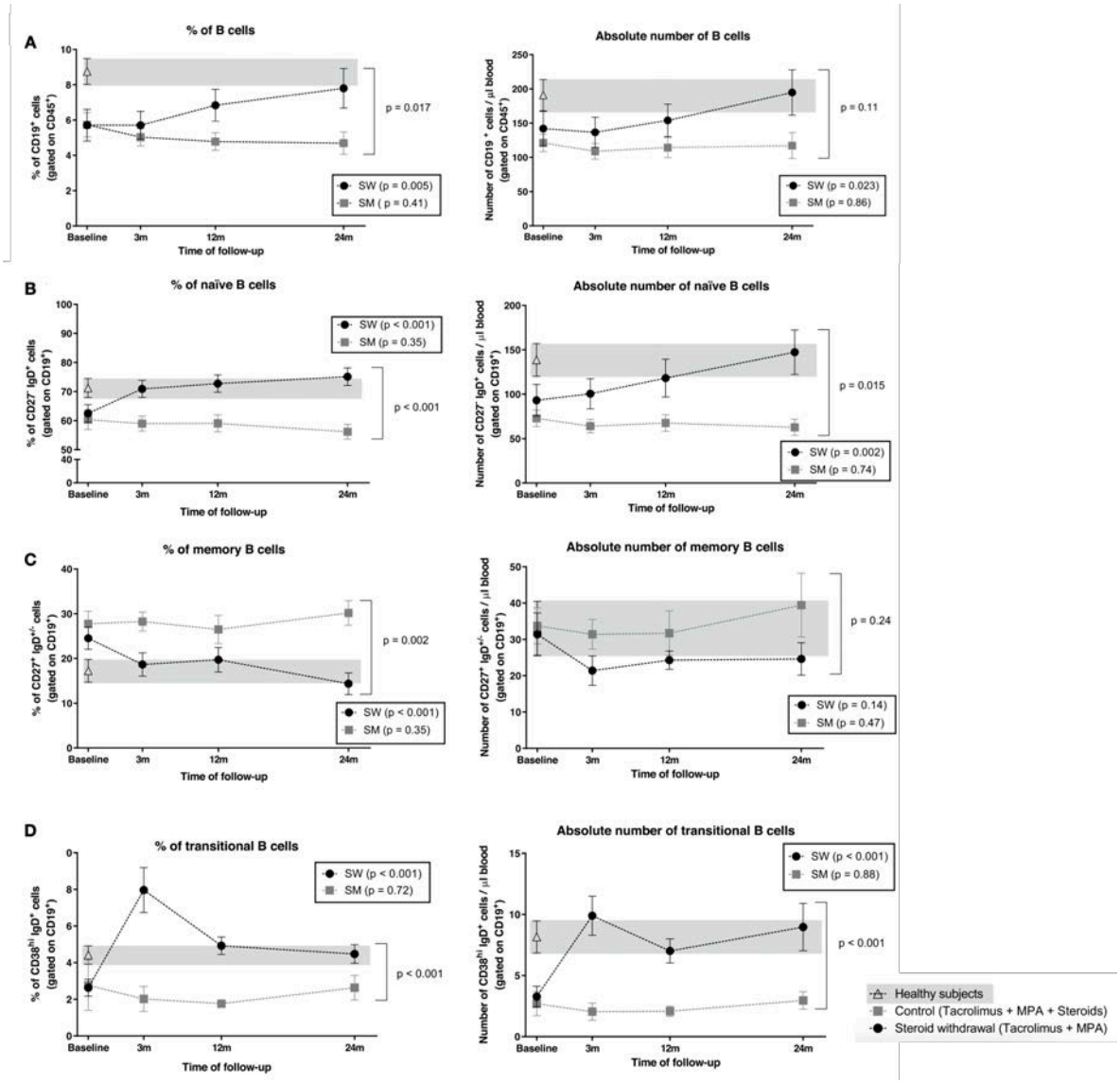


Figure 5. *Evolution of total B cell and B cell subsets percentages and numbers after SW. Immunophenotyping analysis of (**A**) total B cells, (**B**) naïve B cells, (**C**) memory B cells and (**D**) transitional B cells in patients before and after SW (black dots) and SM patients (grey squares). HS data are depicted with white triangles and range is highlighted with a grey background. Dots show mean and standard error (SEM) for each time point.*

On the other hand, conversion to mTORi promoted the expansion of T regulatory (Treg) cells. Interestingly, tacrolimus group also presented a significant increase of Treg cells, although it was of lesser magnitude and delayed in time. A multivariable analysis demonstrated that both mTORi and time post-transplant promote the expansion of Treg cells. Regarding B cell subsets, mTORi patients showed a decrease in total B cell proportions, and in naïve B cell

proportions and absolute numbers. A remarkable reduction of transitional B cells after switching to a mTORi was also observed. In agreement with the data presented in the previous publication, patients who converted to mTORi without concomitant steroid treatment presented higher proportions of naïve and transitional B cells, and stable percentage of B cells compared to patients who converted to mTORi receiving steroid treatment. Regarding NK cells, total NK cells remained stable in both mTORi and tacrolimus groups (**Figure 6A**). However, a remarkable increase in total CD56^{bright} NK cells was observed after switching from tacrolimus to mTORi, encompassed by a reduction of total CD56^{dim} NK cells (**Figure 6B**). On the other hand, NKG2A^{+} NK cell subset significantly increased in mTORi compared to tacrolimus-treated patients (**Figure 6C**).

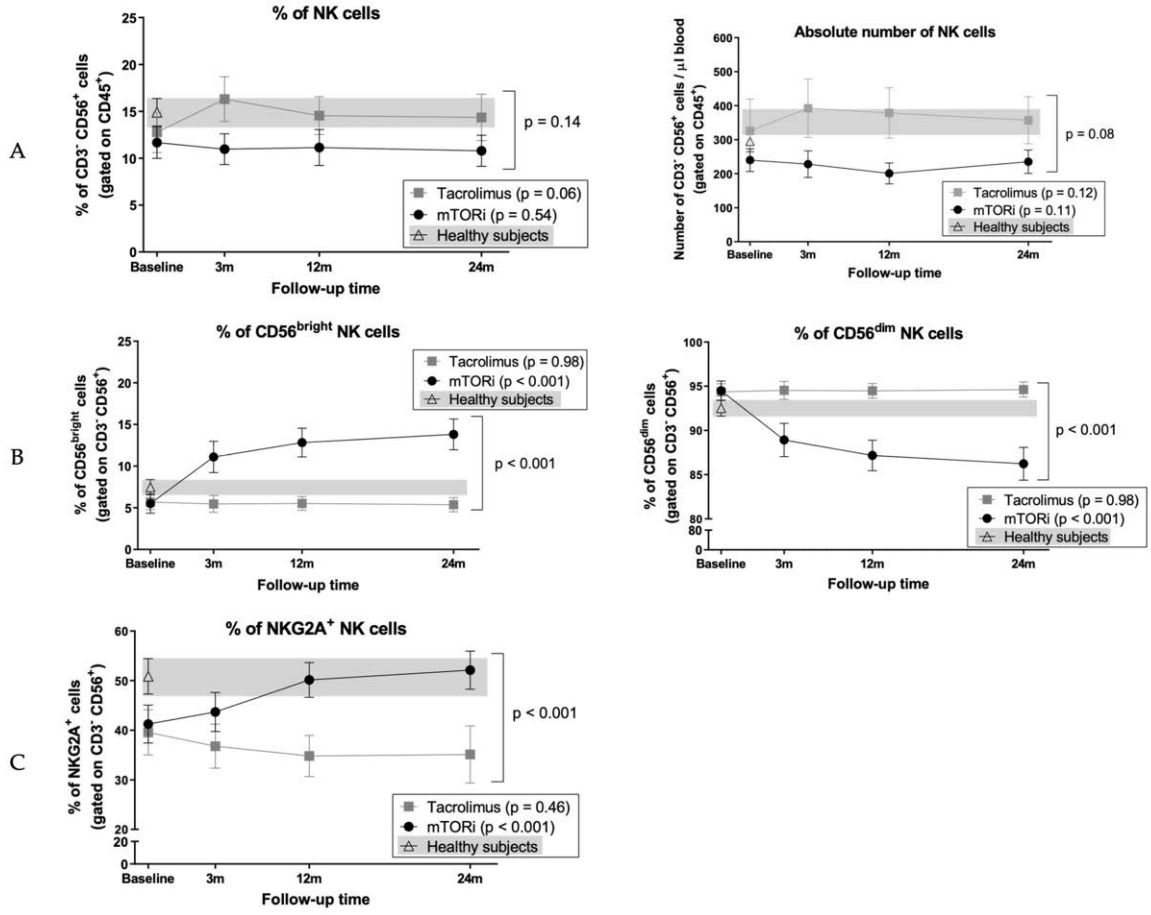


Figure 6. *Evolution of NK cells after switching from tacrolimus to mTORi. Immunophenotyping of (**A**) total NK cells, (**B**) CD56^{bright} and CD56^{dim} NK cell subsets, and (**C**) NK cells NKG2A^{+} in patients before and after switching to mTORi (black dots), and patients maintained on tacrolimus (grey squares). HS data are depicted with white triangles, and the grey background corresponds to HS range. Plots show mean and SEM for each time point.*

All in all, a significant redistribution of peripheral blood lymphocyte subsets was observed after both immunosuppressive treatment changes. Of note, SW seems to have a remarkable

impact on B cell subsets, and to a lesser extent, to total and $CD4^+$ T cell subsets. On the other hand, the conversion from tacrolimus to mTORi impacts on the distribution of T and NK cell subsets, promoting the presence of greater numbers of Treg cells and NK cell subsets at early differentiation stages.

5.3 Pre-transplant AT_1R antibodies are frequently found in patients with HLA donor-specific antibodies and histological antibody-mediated rejection

In the third study, 118 patients according to the Banff 2015 diagnostic categories of their renal biopsies (performed 2011-2015) were included: normal biopsy (n=19), ABMR histology ($ABMR_h$, n=52) and interstitial fibrosis and tubular atrophy (IFTA). Twenty-nine patients (24.6%) lost their grafts and 13 died with a functioning graft (11%). Death-censored graft survival 68 months after biopsy [48-80 months] was worse in $ABMR_h$ compared with normal and IFTA cases (p=0.001). Pre-transplant serum samples were available for 101 patients (19 normal, 39 $ABMR_h$ and 43 IFTA). The detection of both pre-transplant HLA-DSA and AT_1R antibodies (AT_1R-Ab) was associated with $ABMR_h$ cases. Pre-transplant HLA-DSA was detected in 18 $ABMR_h$ cases (46%), but also in 9 IFTA (21%) and 2 normal cases (11%, p=0.006). AT_1R-Ab were detected in 16 $ABMR_h$ (41%), 5 IFTA (12%) and 2 normal cases (11%, p=0.003) (**Figure 7**). We did not observe association between ETAR antibodies (ETAR-Ab), MICA antibodies (MICA-Ab) or a positive crossmatch with primary aortic endothelial cells (EC-XM) detection and $ABMR_h$.

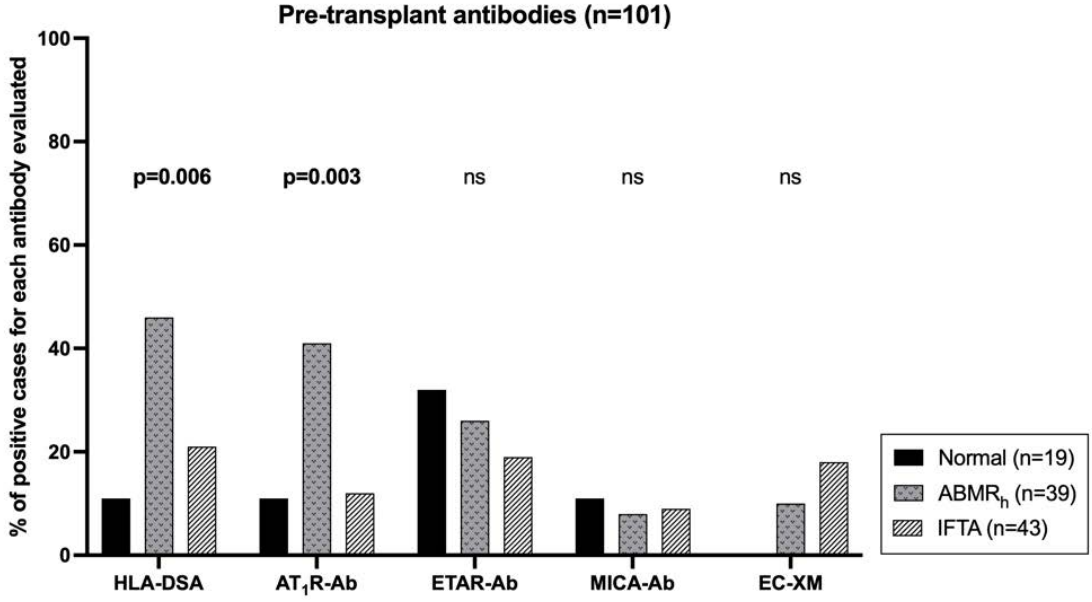


Figure 7. *Percentage of pre-transplant positive cases for HLA-DSA, AT_1R-Ab, ETAR-Ab, MICA-Ab and positive EC-XM according to the three groups of study.*

Detection of pre-transplant AT_1R-Ab correlated with the detection of both persistent preformed HLA-DSA (52%) and *de novo* HLA-DSA post-transplantation (44%), but not with preformed HLA-DSA which cleared after transplant (17%) or no HLA-DSA detection (4%, p<0.001). Post-transplant HLA-DSA was detectable in 38/52 $ABMR_h$ patients ($ABMR_hDSA_{pos}$, 73.1%), together with 17% IFTA and 16% normal cases (p<0.001), without differences in the other antibodies evaluated. From 52 $ABMR_h$, 14 (27%) had no detectable peri-biopsy HLA-DSA ($ABMR_hDSA_{neg}$). When compared to $ABMR_hDSA_{pos}$ cases, no differences were found in graft function and survival, immunosuppression, or microvascular inflammation in their biopsies. Moreover, we could identify at least one non-HLA antibody only in 5/14 $ABMR_hDSA_{neg}$ cases (36%). Next, we evaluated the presence of pre- and post-transplant HLA-DSA and non-HLA antibodies in $ABMR_hDSA_{neg}$ cases and non-$ABMR_hDSA_{pos}$ cases (normal, IFTA and $ABMR_hDSA_{neg}$ cases). Both pre-transplant HLA-DSA and AT_1R-Ab were more frequently detected among $ABMR_hDSA_{pos}$ cases (both p<0.001), but only post-transplant HLA-DSA associated with $ABMR_hDSA_{pos}$ (p<0.001). A multivariate analysis showed that both pre-transplant HLA-DSA [OR: 3.7 (1.3-10.4), p=0.013] and AT_1R-Ab [OR: 5.5 (1.8-16.8), p=0.003] were independent $ABMR_hDSA_{pos}$ predictors.

5.4 HLA epitope mismatch load did not associate with antibody-mediated rejection in the absence of HLA donor-specific antibodies and better predicted the detection of *de novo* class II HLA donor-specific antibodies

The median number of class I and class II HLA epitope mismatches (HLA-EM) in our cohort of the third study were 16 and 18. Among them, 10 class I and 7 class II HLA-EM were antibody-verified ($HLA\text{-}EM^{ver}$). We observed similar $HLA\text{-}EM^{ver}$ among the three groups of study. However, when comparing the load of $HLA\text{-}EM^{ver}$ between $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$ patients, we found significantly higher class II and DRB $HLA\text{-}EM^{ver}$ in $ABMR_hDSA_{pos}$ cases (p=0.046, p=0.044). Furthermore, we compared HLA-EM and classical HLA antigen mismatch (HLA-AM) for *de novo* DSA (dnDSA) development prediction. The rate of class II and DRB $HLA\text{-}EM^{ver}$ predicted both class II dnDSA (p=0.031) and DRB dnDSA (p=0.024); and the amount of DQB $HLA\text{-}EM^{ver}$ showed a weak association with DQB dnDSA (p=0.077). In contrast, neither class II nor DRB or DQB HLA-AM predicted class II, DRB or DQB dnDSA.

6. SUMMARY AND GENERAL DISCUSSION

6. Summary and general discussion

Kidney transplantation is the best therapeutic option for patients with ESRD, due to the significant improvement in quantity and quality of life compared to remaining on dialysis treatment (1, 4, 5). First KTs were not successful mainly due to the development of hyper-acute rejection, but the introduction of different immunosuppressive drugs was key for achieving acceptable short-term graft survival rates (52, 53). However, these treatments do often associate with the development of several side-effects, such as hypertension (194), PTDM (197) or nephrotoxicity (198). These drawbacks justify the use of different immunosuppressive drugs in clinical practice, the withdrawal of some of them or the conversion from one drug to another. Here, we report that after both SW and conversion from tacrolimus to mTORi, kidney graft function remained stable and there was no increase of dnHLA-DSA during the 24-month period of study. Interestingly, both changes in immunosuppression treatment triggered a significant redistribution of peripheral blood lymphocyte subsets. Steroid withdrawal was associated with a decrease of circulating T and $CD4^+$ T cells together with an increase of circulating B cells, naïve B cells and transitional B cells, a similar immunophenotype profile to the one presented by HS. Furthermore, conversion from tacrolimus to mTORi was related to an increase in the proportion of Treg cells, $CD56^{bright}$ and $NKG2A^+$ NK cells, and a decrease in transitional B cells.

Steroid treatment is a very frequent cause of undesirable effects and morbidity (205, 208), which in turn may impact graft survival. These weaknesses justified the use of steroid avoidance protocols after KT. However, SW seemed to associate with increased rates of short-term allograft rejection (209-213), which precluded a generalized application of SW after KT. Despite these discouraging data, Haller *et al.* proposed that SW might be safe if undertaken beyond 18 months after KT (213). Of note, in our study, the median time of SW was 19 months post-KT. Two recent reports have provided more light to this issue. Woodle *et al.* reported that SW in low- to moderate-immune risk KT recipients was not associated with an increased risk of long-term allograft failure after a median follow-up of 15.8 years after KT (214). On the other hand, Bae *et al.* assessed the relationship between panel reactive antibodies (PRA) and early SW, reporting that early SW in KT patients with higher PRA (>60) was indeed associated with increased allograft rejection, but this association was not found among PRA<60 KT recipients (215). SW might be a feasible immunosuppressive strategy in low to medium immunological risk recipients but should be carefully evaluated for high immunological risk recipients. In agreement, here we reported that SW can be accomplished in immunological low risk KT recipients without compromising renal function for the first two years after SW. Regarding HLA-DSA development, SW patients did not show *de novo* HLA-

DSA during the two years of follow-up. These results are in line with other published reports (216-218) and support SW as a safe immunosuppressive strategy in low risk KT recipients.

Conversion from CNI to mTORi pursued the reduction of the CNI-induced side effects, such as nephrotoxicity (198), but it was also supported by successful reports of secondary skin-cancer prevention in mTORi- compared to CNI-treated patients (219). Some reports have associated the conversion to mTORi with a significant improvement in eGFR (220, 221), however, here we did not find a significant advantage in eGFR during the two years of follow-up. A matter of debate after mTORi conversion has been whether it is associated or not to higher risk of dnHLA-DSA development. Contradictory data can be found in the literature: some studies (including ours) did not report a raise in the rate of dnHLA-DSA after conversion (190, 222-225), whereas some highlighted increased rates of dnHLA-DSA after switching to a mTORi (192, 226, 227). It is important to mention that dnHLA-DSA monitoring was not performed systematically in all these studies, which may explain these contradictory results. There is extended evidence that early mTORi monotherapy (<1 year) may provide inadequate immunosuppression (217), especially when associated with SW (191, 221, 228, 229). In our study, only two cases developed dnHLA-DSA, and both accomplished these risk factors: one was converted early after KT (3 months) and the other one was not receiving steroids at the time of conversion. In summary, available data suggest that both immunosuppressive strategies are safe and feasible - but not compatible -, in low- to intermediate immunological risk KT recipients, especially when performed after the first year following KT and with the appropriate drug companion.

Importantly, both immunosuppression treatment changes triggered a significant redistribution of circulating immune cell subsets. According to our data, SW mainly impacted in the B-cell compartment, whereas conversion to mTORi exerted its principal effects in the NK-cell niche. To our knowledge, few studies have explored the B cell dynamics before and after SW (116, 230, 231) without analysing the dynamics of memory and naive B cell subsets. Our cohort of long-term SW experienced a significant increase in total B cells, but of note, this increase could be attributable to greater proportion of circulating naïve B cells in peripheral blood. No significant effects of SW were observed in memory B cells, potentially related to antibody production. This could be explained considering that naïve B cells are more sensitive to glucocorticoid-induced apoptosis than memory B cells (232, 233). Transitional B cell proportion and absolute numbers also increased 3 months after SW and were maintained stable afterwards, confirming the results from Rebollo-Mesa *et al.* (116) obtained 3 to 6 months after SW. Also, our results demonstrate that, in KT recipients, SW promotes a gradual

increase in total, naïve and transitional B cells along the 24-month follow-up, reaching HS proportion. This highlights the profound impact of the steroid treatment in the B-cell niche after KT and could be of importance due to the transcendental role of B cells in antibody-mediated responses (42, 45, 50). In fact, B cell expansion and a differential expression of B cell-related genes have been proposed as biomarkers of tolerance (234-237). Nevertheless, in 2016, Rebollo-Mesa *et al.* reported that immunosuppressive therapy influenced these results (116). Besides, transitional B cells may include a subset of regulatory B cells, which are able to produce anti-inflammatory cytokines such as interleukin-10, supress pro-inflammatory lymphocytes and pathogenic T cells (238, 239) and could play a role in transplantation tolerance.

Regarding the impact of mTORi conversion on immune cell phenotype, a Treg cell increase after mTORi conversion has been reported (240, 241), and our data confirmed it. However, our tacrolimus group recruited >18 months after KT, but not those included <18 months following KT, also showed increased proportions of Treg cells. We speculate that lower dosages and through levels of tacrolimus when first months after KT are accomplished might explain these data. Importantly, our data suggests for the first time that no significant advantage in Treg expansion may be obtained from mTORi conversion when performed >18 months after transplantation compared to tacrolimus maintenance. Our mTORi-converted KT recipients presented increased $NKG2A^+$ NK cell subset proportions, and remarkably, after the first year $NKG2A^+$ proportions were comparable to those presented by HS. Previous data comparing tacrolimus to tacrolimus and sirolimus treatment showed that the first ones presented slightly increased $NKG2A^+$ proportions (242). Ours is the first study reporting $NKG2A^+$ NK cell subset analysis in mTORi patients without tacrolimus treatment. In 2015, we described greater percentages of $NKG2A^+$ cells in HLA-DSA positive KT recipients compared to those without HLA-DSA, regardless of the immunosuppression treatment, but no significant increase of $NKG2A^+$ absolute numbers was observed (112). Recent data from our group suggest that the association between HLA-DSA and $NKG2A^+$ subset in KT with ABMR may be related to a higher consumption of $NKG2A^-$ subset (243), causing increased percentages of $NKG2A^+$ cells without altering their absolute number. This suggests an alternative pathway than the one displayed by mTORi-converted patients, in which we observed an increase in both $NKG2A^+$ cells percentage and absolute number, similar to those displayed by HS. Interestingly, $NKG2A^-$ subset consumption can be also observed in ABMR cases without detectable HLA-DSA (243), which undoubtedly merits further assessment. In mTORi converted patients, we also observed a significant increase of $CD56^{bright}$ NK cells, which produce cytokines but display low cytotoxic activity, present the NKG2A receptor (244)

but lack CD16 – trigger of antibody-dependent cellular cytotoxicity (ADCC) –, and are conventionally considered to represent an early maturation stage (105, 245). This was encompassed by a reciprocal reduction of the major $CD56^{dim}$ subset, which mediates cytotoxicity and pro-inflammatory cytokine secretion (246). These results support that mTORi conversion promotes greater proportions of NK cell subsets at early differentiation stages compared with tacrolimus-treated patients. Functional studies assessing their *in vitro* cytotoxic activity, together with a more detailed immunophenotyping of these NK $CD56^{bright}$ $NKG2A^{+}$ cells are warranted to clarify if this NK-immunophenotype might be beneficial for graft outcomes, considering the similarity with the one presented by HS. Finally, given our previous data of the SW impact in the B-cell compartment, we considered the steroid treatment when analyzing the impact of mTORi conversion. We observed a reduction of total and naïve B cells only in mTORi-steroid cases, suggesting that although naïve B cells are more sensitive to glucocorticoid-induced apoptosis (232, 233), mTORi treatment may contribute to this overall effect. Transitional B cells were strikingly reduced after mTORi conversion independently of steroid treatment, which may have an impact in immunoregulation, as previously discussed.

All in all, our data show that the use of immunosuppression drugs leaves a significant trace in the immunophenotype of KT recipients. Changes in the immunosuppressive treatment significantly altered the immune cell distribution, and of note, these changes were mostly maintained after 24-month follow-up period. Previous research has highlighted the importance of assessing the immune phenotype for the prediction of several KT outcomes, such as PTDM (247), cancer development (61) or infections (63). In some cases, the immunosuppressive treatment is not reported, or mixed immunosuppression regimens are included. It is worth noting that Ducloux *et al.* reported two different immune profiles that predicted PTDM when considering ATG- or non-ATG-treated patients (247), which highlights the impact of immunosuppression treatment in their study. Moreover, Rebollo-Mesa *et al.* demonstrated that previously published tolerance signatures in peripheral blood were influenced by the immunosuppression treatment (116). We propose here that immunosuppression treatment, as well as its significant changes, should always be considered in studies aiming to characterize immune cells in KT recipients, being these from peripheral blood or intra-graft infiltrating immune cells.

Nowadays, short-term kidney allograft survival is appropriate (10), but half-life of renal allografts is around 10 years. The main cause of late allograft loss is the development of ABMR, a form of rejection classically associated with HLA-DSA (65, 66). Nevertheless,

ABMR compatible histological lesions may be present in the graft without detectable circulating HLA-DSA (131, 132, 248, 249) and contradictory results regarding graft survival and histological allograft phenotypes have been reported (131, 132). Here, nearly a third of $ABMR_h$ patients did not show circulating HLA-DSA at biopsy time, and this could be due to undetected HLA-DSA or to the participation of another antibodies in graft damage. We reported here that $ABMR_hDSA_{neg}$ patients presented significantly lower class II and DRB HLA-EM^{ver} compared with $ABMR_hDSA_{pos}$ cases. This finding strengthens the hypothesis that other mechanisms of damage are playing a role in these cases, rather than non-detected HLA-DSA. In this regard, the study of non-HLA antibodies has gained interest in KT. It is known that KT recipients may produce immune responses through indirect recognition against foreign proteins or own proteins – acting as autoantigens - expressed by the donor graft. These antibodies may then react against polymorphic alloantigens such as MICA or MICB or against autoantigens like AT_1R, ETAR, agrin or perlecan, among others (136, 250, 251), and these non-HLA antibodies may be prevalent in KT recipients (146). Of note, antibodies against endothelial cells (EC) have been related to $ABMR_hDSA_{neg}$ cases (135), and antibodies against EC (EC-XM^+) were the only ones detected in $ABMR_hDSA_{neg}$ pre-transplant samples. However, neither AT_1R-Ab, ETAR-Ab, MICA-Ab nor antibodies identified with EC-XM before or after KT were able to fully explain the $ABMR_hDSA_{neg}$ cases in our study. An alternative mechanism termed “the missing-self hypothesis” has been proposed. It suggests that the inability of graft EC to provide HLA I-mediated inhibitory signals to recipient circulating NK cells may trigger NK cell activation, resulting in endothelial damage and chronic vascular rejection (252). NK cells have been associated with ABMR damage by us (112, 243) and others (111, 113, 253) and further research in this field will provide new valuable evidence regarding their exact role in $ABMR_h$ damage.

Interestingly, we found here a strong and independent association between pre-transplant AT_1R-Ab and $ABMR_h$ development in the presence of HLA-DSA. AT_1R is an essential G-protein coupled receptor expressed at the EC surface and on immune cells (monocytes, T and B cells). Its main function is to transiently bind to angiotensin II, mediating blood pressure regulation and water-salt balance, wound healing and normal immune responses (136, 254-256). When hyper-activated, it is described to cause vasoconstriction, hypertension or vascular smooth muscle migration and proliferation (256-258). While AT_1R activation via angiotensin II binding is normally transient, if substituted by AT_1R-Ab, then AT_1R activation is more sustained and prolonged in time. Thus, AT_1R-Ab - AT_1R binding may exert significant deleterious effects, such as malignant hypertension development, enhanced fibrosis and immune cell recruitment (256). In fact, the presence of AT_1R-Ab has been associated to

preeclampsia development (259), hypertension (260) and for the first time in 2005, to severe steroid-refractory vascular rejection in KT without HLA-DSA (130). More recently, pre- or post-transplant AT_1R-Ab detection have also been linked to both rejection and increased risk of KT allograft failure (138, 261, 262), although to this day, their impact in graft survival remains unclear, with controversial results in the literature (146, 263-266). We did not find a relationship between AT_1R-Ab detection and graft loss. Of note, our study and some others might not be powered enough to assess the relationship between AT_1R-Ab and graft loss, and thus the relationship between those antibodies and graft outcome remains to be further elucidated in its totality.

Of note, some reports have suggested that non-HLA and HLA-DSA antibodies may function in synergy (138, 262), and that AT_1R-Ab detection may be a risk factor for dnHLA-DSA development (146, 267). Here we report an association of pre-transplant AT_1R-Ab with post-transplant HLA-DSA and importantly, this was observed either with *de novo* and persistent preformed HLA-DSA, but not with preformed cleared HLA-DSA. This could be relevant for pre-transplant immunological risk stratification and also for allograft outcomes, considering the strong association among persistent preformed HLA-DSA and lower ABMR-free survival compared to preformed cleared HLA-DSA (91). The exact mechanisms whereby HLA-DSA and AT_1R-Ab may function in synergy have not been totally unravelled yet, but it has been proposed that AT_1R-Ab may promote proinflammatory changes in vascular cells, triggering the activation of graft EC. After graft EC activation, an increase in the expression of HLA molecules could follow, promoting antigen recognition by immune cells and subsequently, the development of HLA-DSA (267, 268). Given the presence of AT_1R on immune cells, AT_1R-Ab could also interfere and magnify immune responses (146). Finally, pre-transplant HLA-DSA and AT_1R-Ab were independent $ABMR_h$ predictors. Given the strong and already stated association between ABMR and increased risk of kidney allograft loss (14, 15, 17, 91), our data support that pre-transplant AT_1R-Ab assessment should be carefully considered in KT candidates.

The basis of the HLA-EM analysis came from the evidence that HLA antigens share HLA epitopes (149). This was firstly suspected when a significant cross-reactivity between HLA antigens was observed: the sensitization against one HLA antigen led to the generation of antibodies towards apparently unrelated HLA antigens (148). In the last years, the analysis of HLA-EM has been proposed as a better strategy than HLA-AM for: 1) preventing dnHLA-DSA development (154), 2) predicting ABMR development (269), 3) optimizing immunosuppression treatment (157) and 4) improving kidney allocation in highly sensitized

patients (153, 270). Here, we show that class II and DRB dnHLA-DSA development can be successfully predicted in KT recipients with HLA-EM, as previously reported (157), but not with HLA-AM. The main reason for this could be that each HLA-AM is associated with a wide range of HLA-EM. Thus, as an example, a pair of donor and recipient with one DR HLA-AM may share up to 35 HLA-EM, whereas another pair of donor and recipient with two DR HLA-AM may receive only two or three HLA-EM (157). HLA-EM analysis did not reach statistical significance to predict DQB dnHLA-DSA, and that is probably due to the restricted number of cases included in our cohort. Both class II and DRB HLA-EM were associated with $ABMR_hDSA_{pos}$ development, although the existence of preformed HLA-DSA or AT_1R-Ab were more potent predictors of $ABMR_hDSA_{pos}$ in our experience.

A matter of interest in the field of HLA-EM has been the characterization of harmful HLA-EM. All individual HLA-EM show differences in immunogenicity, thus hindering or promoting antibody formation (160, 271). Several reasons have been proposed, including the type of amino acid substitutions, which may trigger different physiochemical properties, or the absence or presence of accompanying T-helper cell epitopes (271). In order to identify clinically relevant HLA-EM, some groups have tried to verify HLA-EM with antibodies (272), although only a subset of them have been already registered as antibody-verified. Of note, Sapir-Pichhadze *et al.* reported that $HLA\text{-}EM^{ver}$ were found to be independent predictors of death-censored graft failure, but no effect of non-verified HLA-EM could be found on graft loss, except for HLA-DRB1 loci (158). Given the available evidence, we followed the same approach, confirming in our cohort that the use of $HLA\text{-}EM^{ver}$ is more useful than the use of total HLA-EM for dnHLA-DSA development prediction (data not shown). Interestingly, HLA-EM may also be predictive of primary humoral and cellular alloimmunity, as it has been shown by Meneghini *et al.* in a very recent article (273). The field of HLA-EM analysis has advanced rapidly in the last few years, and it is feasible to consider that HLA-EM analysis will become soon the most reliable tool for donor-recipient compatibility analysis.

To conclude, the results presented herein confirm that immune monitoring in KT recipients in a combine effort of HLA and non-HLA antibodies, HLA epitope mismatch and immune cell phenotyping is useful for better immunological risk assessment. All these tools are of utmost importance for moving towards a better understanding of immune responses in KT, and for helping tailoring treatments to achieve the best outcome for each individual KT patient.

7. CONCLUSIONS

7. Conclusions

1. Both steroid withdrawal and conversion from tacrolimus to mTOR inhibitors in KT recipients are safe in terms of renal function and development of *de novo* HLA-DSA after two years of follow-up.

2. Steroid withdrawal and conversion to mTOR inhibitors triggered a significant redistribution of peripheral blood immune cell subsets: steroid withdrawal exerted its principal effects in the B-cell compartment, whereas conversion to mTORi modulated the NK-cell repertoire. These changes were mostly maintained after two years of follow-up. These findings may have a relevant impact on the proper understanding of immunological changes in studies including KT recipients.

3. The detection of pre-transplant HLA-DSA is frequent in KT recipients with histological ABMR. The presence of pre-transplant AT_1R antibodies associates with ABMR in the presence of HLA-DSA. These pre-transplant AT_1R antibodies may act synergistically with HLA-DSA to produce ABMR or could facilitate the *de novo* HLA-DSA development.

4. Kidney transplant recipients with ABMR histology without detectable HLA-DSA presented less class II HLA epitope mismatches compared to patients with ABMR in the presence of HLA-DSA, suggesting that non-detected HLA-DSA may not be responsible for this type of damage. HLA epitope mismatch but not antigen mismatch analysis was able to discriminate KT recipients developing *de novo* class II and DRB1 HLA-DSA.

5. Immune monitoring of KT recipients, including HLA and non-HLA antibodies, HLA-EM and peripheral blood immune cell subpopulations, is useful for better tailoring the immunological risk of KT recipients, and represents an opportunity for better guided clinical decisions.

8. LIMITATIONS AND FUTURE PERSPECTIVES

8. Limitations and future perspectives

8.1 Limitations

Regarding the first two studies presented in this doctoral thesis, some limitations can be stated:

- The restricted number of patients included in both studies and the high number of statistical tests performed, which imply a weakness in terms of taking conclusions of individual results and may increase the chance of type 1 error.
- The absence of protocol graft biopsies, which precluded the analysis of potential correlations between peripheral blood lymphocytes and intra-graft infiltrations, and also if the observed changes in peripheral blood cells could associate with the detection or not of subclinical damage.
- The absence of additional markers in the immunophenotypic analyses (e.g., CD16, FoxP3, CD24, CD45RA and CCR7), which would have allowed us to more precisely characterize the lymphocyte subsets redistribution reported herein.

Concerning the third study included in the present doctoral thesis, some restrictions should be also considered:

- The limited number of $ABMR_hDSA_{neg}$ cases in the studied cohort, which may have hampered the detection of some harmful non-HLA antibodies.
- The inclusion of both indication and surveillance biopsies, which introduces heterogeneity both in the timing post-transplantation and in the clinical picture of KT patients.
- The crossmatches with EC were performed with aortic cells, which may not express the same proteins as the microvascular renal EC do.
- The use of the inferred four-digit HLA typing for HLA-EM analysis may allow that some rare HLA genotypes are not correctly assigned, and therefore influence the data presented herein (274).

8.2 Future perspectives

In this doctoral thesis, the influence of immunosuppression treatment on the distribution of peripheral blood T, B and NK cell subsets has been unravelled. It has been also reported a strong association between pre-transplant AT_1R-Ab detection and $ABMR_h$ development in the presence of HLA-DSA, but no clear role could be attributed to non-HLA antibodies in $ABMR_hDSA_{neg}$ cases. Furthermore, the analysis of HLA epitope mismatches has shown better performance in predicting *de novo* HLA-DSA development than classical HLA antigen matching. Taking into account all the data presented herein, further studies may be performed:

- Validation of the previous immunophenotype redistribution after immunosuppressive treatment changes in another KT recipient cohort. This study should include a more exhaustive set of immune markers and protocol biopsies, to decipher the putative impact of immune cell subsets changes in the development or not of subclinical damage.

- Studies aimed to decipher the role of NK cells in ABMR and HLA-DSA development. To better characterize the NK cell phenotype in KT patients with ABMR in the presence or not of HLA-DSA and the one induced after mTORi conversion by *in vitro* assays.

- Analysis and comparison of several putative non-HLA antibodies in a larger cohort composed by KT patients with normal biopsies, $ABMR_hDSA_{pos}$ and $ABMR_hDSA_{neg}$.

- A prospective assessment of HLA-EM in a large cohort of KT recipients with surveillance biopsies and sequential HLA-DSA detection, to better characterize its impact in allograft outcomes and HLA-DSA development.

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10. ANNEX

10. Annex

10.1 Participation in congresses

10.1.1 International congresses

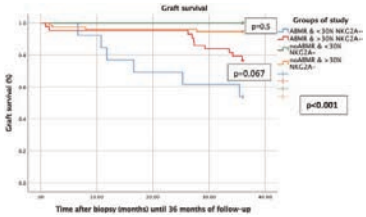
1. Llinàs-Mallol, L; Redondo-Pachón, D; Raïch-Regué, D; Pérez-Sáez, MJ; Sanz, S; Arias-Cabrales, C; Buxeda, A; Burballa, C; López-Botet, M; Pascual, J; Crespo, M. Changes in peripheral NK cells in kidney transplant recipients with and without HLA DSA. Nephrology Dialysis Transplantation 2021; 36(S1): i88. Oral presentation, 58th ERA-EDTA Congress, June 5-8, 2021, virtual. *Abstract awarded with an ERA-EDTA Travel Grant.*

FC129 **CHANGES IN PERIPHERAL NK CELLS IN KIDNEY TRANSPLANT RECIPIENTS WITH AND WITHOUT HLA DSA**

Laura Llinas[1], Dolores Redondo Pachon[1], Dàlia Raïch Regué[1], Maria Jose Perez-Saez[1], Sara Sanz[1], Carlos Arias-Cabrales[1], Anna Buxeda[1], Carla Burballa[1], José Miguel López-Botet[2,3], Julio Pascual Santos[1], Marta Crespo[1]

[1]Hospital del Mar and Hospital del Mar Medical Research Institute, Department of Nephrology, Barcelona, Spain, [2]Hospital del Mar and Hospital del Mar Medical Research Institute, Department of Immunology, Barcelona, Spain and [3]University Pompeu Fabra, Barcelona, Spain

BACKGROUND AND AIMS: Antibody-mediated rejection (ABMR) is a frequent cause of renal allograft loss. There is increasing evidence of the role of Natural Killer (NK) cells in the establishment of ABMR damage. Our group described that patients with donor-specific antibodies (DSA) and ABMR present higher proportions of NKG2A+ NK cell subset in peripheral blood than those without HLA DSA or HLA antibodies.
METHOD: We selected 177 kidney transplant recipients (KT) with renal biopsies 2011-2017: 77 with ABMR (DSA+: 53, DSA-: 24) and 100 without ABMR (DSA+: 15, DSA-: 85). We assessed graft survival with a median time of follow-up since the renal biopsy of 53 months. In 138 KT we evaluated the peripheral blood NK cell immunophenotyping and its value as a prognostic biomarker.
RESULTS: Graft survival was worse in ABMR-KT at the end of follow-up ($p<0.001$) independently of DSA detection ($p=0.63$). Regarding NK cell immunophenotyping, we observed a lower proportion and absolute NK cell count in ABMR+DSA+-KT and ABMR+DSA–KT compared with ABMR-DSA–KT ($p=0.027$, $p=0.017$). ABMR+DSA+-KT showed higher proportion of NKG2A+ NK cells compared with ABMR-DSA–KT ($p=0.007$). All ABMR+ patients, independently of DSA detection, presented lower absolute NKG2A- NK cell count in comparison with ABMR-DSA–KT ($p=0.001$, $p=0.017$). Finally, a proportion of NKG2A- <30% was associated with lower graft survival 36 months after graft biopsy with ABMR ($p=0.067$) (Figure).



FC129 Figure: Graft survival in patients with and without ABMR grouped according to NKG2A$^-$ > or <30% at biopsy.

CONCLUSION: Graft survival is worse in ABMR+ compared with ABMR- KT independently of DSA detection. Kidney transplant recipients with ABMR show reduced peripheral absolute numbers of NK cells and NKG2A- NK cells regardless of undetectable DSA. This NK cell phenotype associated with a worse medium-term graft survival in cases with ABMR.

2. Crespo, M**; Llinàs-Mallol, L**; Redondo-Pachón, D; Butler, C; Gimeno, J; Pérez-Sáez, MJ; Buxeda, A; Arias, C; Folgueiras, M; Sanz, S; Valenzuela, N; Reed, E; Pascual, J. Non-HLA antibodies and eplet mismatches in kidney transplant recipients with a histological picture of antibody-mediated rejection with and without HLA donor-specific antibodies. Nephrology Dialysis Transplantation 2021;36(S1): i88. Oral presentation, 58th ERA-EDTA Congress, June 5 – 8 2021, virtual.

FC128 **NON-HLA ANTIBODIES AND EPLET MISMATCHES IN KIDNEY TRANSPLANT RECIPIENTS WITH A HISTOLOGICAL PICTURE OF ANTIBODY-MEDIATED REJECTION WITH AND WITHOUT HLA DONOR-SPECIFIC ANTIBODIES**

Marta Crespo[1], Laura Llinas[1], Dolores Redondo Pachon[1], Carrie L. Butler[2,3], Javier Gimeno[4], Maria Jose Perez-Saez[1], Anna Buxeda[1], Carlos Arias-Cabrales[1], Montserrat Folgueiras[1], Sara Sanz[1], Nicole M. Valenzuela[2,3], Elaine F. Reed[2,3], Julio Pascual Santos[1]

[1]Hospital del Mar and Hospital del Mar Medical Research Institute, Department of Nephrology, Barcelona, Spain, [2]UCLA Immunogenetics Center, University of California, Los Angeles, CA, United States of America, [3]David Geffen School of Medicine, University of California, Department of Pathology and Laboratory Medicine, Los Angeles, CA, United States of America and [4]Hospital del Mar and Hospital del Mar Medical Research Institute, Department of Pathology, Barcelona, Spain

BACKGROUND AND AIMS: Correlation between antibody-mediated rejection (ABMR) and HLA donor-specific antibodies (DSA) is strong but imperfect in kidney transplant (KT) recipients, raising the possibility of other detrimental antibodies contributing to ABMR. The role of non-HLA antibodies on outcomes is not well known.
METHOD: We retrospectively assessed KT biopsies scored according to Banff'15 classification. Pre- and post-KT serum samples were checked for HLA and non-HLA antibodies (MICA-Ab, angiotensin II type 1 receptor (AT1R)-Ab, endothelin-1 type A receptor (ETAR)-Ab and crossmatches with primary aortic endothelial cells (EC-XM)). We also analyzed HLA epitope mismatches between donors and recipients.
RESULTS: One-hundred eighteen patients with normal (n=19), ABMR histology (n=52) or IFTA (n=47) in their biopsy were studied. Graft survival was worse in ABMR patients (p=0.003). Pre-KT HLA-DSA were more frequent in ABMR cases (p=0.006). At biopsy, 73% ABMR patients had HLA-DSA (p<0.001). Pre-KT AT1R-Ab were more frequent in ABMR compared with IFTA and normal cases (p=0.003), without differences in other non-HLA antibodies. Fourteen patients with histological ABMR (27%) had no detectable HLA-DSA post-KT and only 3 had non-HLA Ab. However, these ABMR-DSA- cases showed similar biopsy changes and graft survival compared with ABMR-DSA+. Pre- or post-KT non-HLA antibodies other than AT1R-Ab were detected similarly in ABMR and in normal or IFTA cases. Both total class II and DRB1 epitope mismatches were associated with postransplant DSA and ABMR-DSA+. Multivariate analysis showed that both pre-KT HLA-DSA and AT1R-Ab (DSA: OR: 3.39 [1.20-9.59], p=0.021; AT1R-Ab: OR: 5.31 [1.75-16.10], p=0.003) were strong independent predictors of postransplant ABMR-DSA+ (Table 1).

CONCLUSION: Despite highly prevalent HLA-DSA before and after transplantation in KT with histological ABMR, 27% of cases did not show circulating HLA-DSA. Pre-KT AT1R-Ab associated with ABMR-DSA+, but not MICA-Ab, ETAR-Ab or EC-XM+. Any of them associated significantly with ABMR-DSA-. Epitope mismatch predicted both postransplant DRB-DSA and ABMR-DSA+. Detection of pre-KT HLA-DSA and/or AT1R-Ab, together with HLA epitope mismatch assessment, are valuable tools for better DSA and ABMR prediction in KT patients.

FC128 Table 1. Logistic regression analysis of ABMR-DSA$^+$ risk factors.

Risk factor	Univariate OR (95% CI)	Univariate p-value	Multivariate OR (95% CI)	Multivariate p-value
HLA-DSA pre-KT	5.26 (2.03-13.62)	0.001	3.39 (1.20-9.59)	0.021
AT1R-Ab pre-KT	7.86 (2.75-22.41)	<0.001	5.31 (1.75-16.10)	0.003
ETAR-Ab pre-KT	1.56 (0.60-4.05)	0.36		
MICA-Ab pre-KT	0.97 (0.23-4.14)	0.96		
Positive EC-XM pre-KT	0.81 (0.08-8.23)	0.86		
Class I antibody-verified epitope mismatches	0.97 (0.88-1.07)	0.53		
Class II antibody-verified epitope mismatches	1.12 (1.01-1.25)	0.029	1.07 (0.95-1.21)	0.26

3. Crespo, M**; Llinàs-Mallol, L**; Redondo, D; Butler, C; Gimeno, J; Pérez, MJ; Buxeda, A; Arias, C; Folgueiras, M; Sanz, S; Valenzuela, NM; Reed, EF; Pascual; J. Non-HLA antibodies and eplet mismatches in cases with histological picture of antibody-mediated rejection with and without HLA donor-specific antibodies. Am J Transplant. 2021; 21 (suppl. 3). Poster presentation, American Transplant Congress, June 4 – 9 2021, virtual.

Non-HLA Antibodies and Eplet Mismatches in Cases with Histological Picture of Antibody-Mediated Rejection with and without HLA Donor-Specific Antibodies

M. Crespo[1], L. Llinás[2], D. Redondo[2], C. Butler[3], J. Gimeno[4], M. Pérez-Sáez[2], A. Buxeda[2], C. Arias[2], M. Folgueiras[2], S. Sanz[2], N. Valenzuela[3], E. E. Reed[3], J. Pascual[2]

[1]Nephrology and Kidney Transplantation, Hospital del Mar, Barcelona, Spain, [2]Nephrology, Hospital del Mar, Barcelona, Spain, [3]UCLA Immunogenetics Center, UCLA, Los Angeles, CA, [4]Pathology, Hospital del Mar, Barcelona, Spain

Meeting: 2021 American Transplant Congress

Abstract number: 1044

Keywords: Antibodies, Epitopes, HLA antibodies, Rejection

Topic: Clinical Science » Kidney » Kidney Chronic Antibody Mediated Rejection

Session Information

Session Name: Kidney Chronic Antibody Mediated Rejection

Location: Virtual

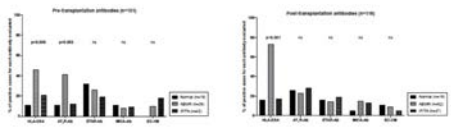
Session Date & Time: None. Available on demand.

***Purpose:** Correlation between antibody-mediated rejection (ABMR) and HLA donor-specific antibodies (DSA) is strong but imperfect in kidney transplant (KT) recipients, raising the possibility of other detrimental antibodies contributing to ABMR. The role of non-HLA antibodies on outcomes is not well known.

***Methods:** We retrospectively assessed KT biopsies scored according to Banff'15 classification. Pre- and post-KT serum samples were checked for HLA and non-HLA antibodies (MICA-Ab, angiotensin II type 1 receptor (AT_1R)-Ab, endothelin-1 type A receptor (ETAR)-Ab and crossmatches with primary aortic endothelial cells (EC-XM)). We also analyzed HLA epitope mismatches between donors and recipients.

***Results:** One-hundred eighteen patients with normal (n=19), ABMR histology (n=52) or IFTA (n=47) in their biopsy were studied. Graft survival was worse in ABMR patients (p=0.003). Pre-KT HLA-DSA were more frequent in ABMR cases (p=0.006). At biopsy, 73% ABMR patients had HLA-DSA (p<0.001). Pre-KT AT_1R-Ab were more frequent in ABMR compared with IFTA and normal cases (p=0.003), without differences in other non-HLA antibodies. Both total class II and DRB1 epitope mismatches were associated with ABMR-DSA$^+$. Fourteen patients with histological ABMR (27%) had no detectable HLA-DSA post-KT and only 3 had non-HLA Ab. However, they showed similar biopsy changes and graft survival compared with ABMR-DSA$^+$. Pre- or post-KT non-HLA antibodies other than AT_1R-Ab were detected only in a small subset of ABMR ($graphic_). Multivariate analysis showed that both pre-KT HLA-DSA and AT_1R-Ab (DSA: OR: 3.39 [1.20-9.59], p=0.021; AT_1R-Ab: OR: 5.31 [1.75-16.10], p=0.003) were strong independent predictors of ABMR-DSA$^+$.

***Conclusions:** Despite highly prevalent HLA-DSA before and after transplantation in KT with histological ABMR, 27% of cases did not show circulating HLA-DSA. Pre-KT AT_1R-Ab associated with ABMR-DSA$^+$, but not MICA-Ab, ETAR-Ab or EC-XM$^+$. Epitope mismatch predicted both de novo appearance of DRB-DSA and ABMR-DSA$^+$. Detection of pre-KT HLA-DSA and/or AT_1R-Ab, together with HLA epitope mismatch assessment, are valuable tools for better DSA and ABMR prediction in KT patients.



Graphic. Pre and post-KT HLA and non-HLA antibodies in KT recipients with normal, ABMR or IFTA biopsies.

To cite this abstract in AMA style:

Crespo M, Llinás L, Redondo D, Butler C, Gimeno J, Pérez-Sáez M, Buxeda A, Arias C, Folgueiras M, Sanz S, Valenzuela N, Reed EE, Pascual J. Non-HLA Antibodies and Eplet Mismatches in Cases with Histological Picture of Antibody-Mediated Rejection with and without HLA Donor-Specific Antibodies [abstract]. *Am J Transplant.* 2021; 21 (suppl 3). https://atcmeetingabstracts.com/abstract/non-hla-antibodies-and-eplet-mismatches-in-cases-with-histological-picture-of-antibody-mediated-rejection-with-and-without-hla-donor-specific-antibodies/. Accessed July 4, 2021.

4. Burballa, C; **Llinàs-Mallol, L**; Buxeda, A; Arias-Cabrales, C; Pérez-Sáez, MJ; Redondo-Pachón, D; Mir, M; Faura, A; Crespo, M; Pascual, J. Antibody mediated rejection: clinical phenotype matters. Nephrology Dialysis Transplantation 2020;35(S3): 2018. Poster presentation, 57th ERA-EDTA Congress, June 6 - 9 2020, virtual.

P1701 **ANTIBODY MEDIATED REJECTION: CLINICAL PHENOTYPE MATTERS**

Carla Burballa Tarrega[1,2], Laura Llinás[2], Anna Buxeda[1,2], Carlos Arias Cabrales[1,2], María José Pérez Sáez[1,2], M. Dolores Redondo Pachón[1,2], Marisa Mir Fontana[1,2], Anna Faura[1], Marta Crespo Barrio[1,2], Julio Pascual Santos[1,2]

[1]Hospital del Mar. Barcelona, Nephrology Department, Barcelona, Spain and [2]IMIM – Institute Hospital del Mar for Medical Research. Barcelona, Nephrology Department, Barcelona, Spain

Background and Aims: Protocol biopsies following kidney transplantation (KT) allow the histological diagnosis of antibody-mediated rejection (ABMR) with stable renal function (RF). Controversy arises when considering isolated proteinuria as a clinical biomarker. Currently, there is no effective treatment for ABMR and transplant units may decide on treatment independently of the clinical expression.

Method: KT recipients (1987-2017) with post-KT biopsies (2008-2018) showing *ABMR* graft *lesions* (category 2-Banff'2015) >1year post-KT were included. Cases were grouped into phenotypes of ABMR according to the clinical picture at biopsy : 1) acute RF impairment (↑creatinine >15% three weeks before biopsy) with/without proteinuria and with/without DSA detection. 2) sub-acute RF impairment (↑creatinine >15% six months before biopsy) with/without proteinuria and with/without DSA detection, 3) performed for DSA detection with stable RF and no proteinuria or 4) protocol biopsy, with stable RF, no proteinuria or DSA detection. We considered an additional category: 5) isolated proteinuria (↑>500 mg or x2 six months before biopsy). Categories 1), 2) and 5) were considered clinical ABMR. Categories 3) and 4) were considered subclinical ABMR. We aimed to evaluate graft outcomes in the different ABMR phenotypes.

Results: In a cohort of 105 KT recipients with histologic lesions of ABMR, biopsies corresponded to phenotypes 1) in 35 (33%), 2) 10 (9,5%), 3) 21 (20,3%), 4) 14 (13,4%) and 5) in 25 23,8%). No differences between clinical and subclinical ABMR were found in baseline characteristics except for donors 'age, who were older within the clinical group (51.8±18.8 vs. 43.88 ±16.1; p=0.04). At time of biopsy, subclinical had better RF than clinical ABMR (creatinine 1.3±0.4 mg/dl vs. 2.2±1.1 mg/dl; p=0.02) and less proteinuria (161 mg/g [IQR 93-269] vs 939 mg/g [IQR 412-2000]; p=0.001)

Graft survival was worse in those patients with acute and sub-acute RF impairment, followed by those with isolated proteinuria (Figure 1). In comparison to subclinical ABMR, those with RF impairment and isolated proteinuria had an increased risk of graft lost; HR 9.4 (95% IC 2.2-40.7, p=0.002) and 4.8 (95% IC 1.01-23.2, P=0.05) respectively. DSA detection in these groups did not impact graft survival. Specific treatment was not different among groups, except for steroid pulses, which were more frequently applied in cases of ABMR with clinical manifestation.

Conclusion: The clinical phenotypes of ABMR influence long-term graft survival independently from treatment. Understanding graft evolution according to clinical phenotype at the time of histologic diagnosis should guide the therapeutic strategy, to balance risk-benefit ratio.

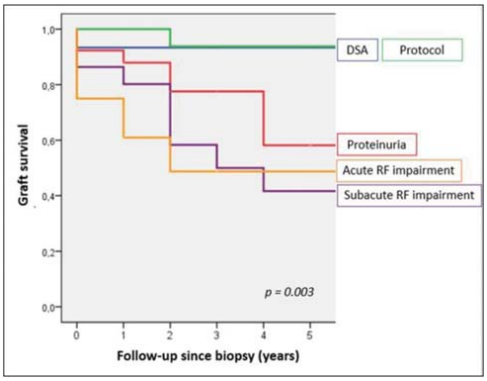


Figure:

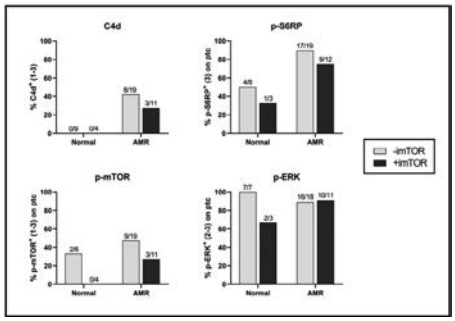
5. Raïch-Regué, D; Gimeno, J; Menéndez, S; Benito, D; Redondo-Pachón, D; Pérez-Sáez, MJ; **Llinàs-Mallol, L**; Arias-Cabrales, C; Riera, M; Pascual, J; Crespo, M. Phosphorylation of S6RP in peritubular capillaries is associated with antibody-mediated rejection in kidney allografts. Transpl. Int. 2019;32(S2): 143. Oral presentation, 19th Congress of the European Society for Organ Transplantation, September 15 – 18 2019, Copenhagen, Denmark.

evaluated whether paracrine factors found in the perfusion fluid of ECD kidneys could induce endothelial senescence in vitro.
Results: The angiogenic function and quantitative distribution of the stromal and endothelial cell compartments within the perirenal stromal vascular fraction exhibited high inter-individual variability among donors. SVF from ECD donors displayed a differential signature characterized by over-expression of CD144 and inflammatory transcripts. In vitro exposure of endothelial cells derived from the PR-SVF of young donors to machine perfusion fluid of ECD donors was shown to induce endothelial senescence.
Conclusions: Our study shows that PR-SVF allows an individualized assessment of donor-related parameters that associate to the dysfunction of kidney allografts. Such appraisal of biomarkers that reflect the quality of transplants may open perspectives for targeted approaches aimed at preserving the regenerative function of aging kidneys.

FG061 PHOSPHORYLATION OF S6RP IN PERITUBULAR CAPILLARIES IS ASSOCIATED WITH ANTIBODY-MEDIATED REJECTION IN KIDNEY ALLOGRAFTS

Dalia Raïch-Regué, Javier Gimeno, Silvia Menendez, David Benito, Dolores Redondo, M. Jose Pérez-Sáez, Laura Llinàs, Carlos Arias, Marta Riera, Julio Pascual, Marta Crespo
IMIM/Hospital del Mar

Background: Antibody mediated rejection (AMR) in the presence of donor-specific HLA antibodies (DSA) is recognized as key factor in late renal allograft loss. DSA have been reported to activate microvascular endothelial cells through the mTOR pathway. The phosphorylation of the mTOR pathway proteins S6RP and 70S6K have been proposed as new markers of AMR on transplanted hearts. Our aims were (1) to evaluate the mTOR pathway activation because of HLA DSA in kidney grafts with AMR compared to normal biopsies, and (2) to evaluate the potential modulation of the mTOR pathway by immunosuppression with mTOR inhibitors (mTORi).
Methods: We included 45 kidney transplant patients with graft-biopsies (RB) performed in 2011–15: 34 with AMR diagnosis (Banff 2015), all with HLA-DSA, and 11 age-matched recipients with normal biopsies. 17 had received mTORi (13 AMR) and 28 had not (21 AMR). RB were stained for C4d and phosphorylation of the mTOR pathway proteins S6RP (Ser235/236), ERK (Thr202/204) and mTOR (Ser2448) in peritubular capillaries (PTC) by immunohistochemistry in paraffin sections. PTC labelling was graded according to the scale: 0, no staining; 1, rare staining of single cells; 2, focal staining; 3, multifocal to diffuse staining.
Results: Staining of RB with AMR showed a significant increase in C4d staining ($p = 0.006$) and expression of pS6RP in PTC compared to controls ($p = 0.012$). No association was found between AMR and pERK or pmTOR staining. The presence of circulating HLA-DSA associated with pS6RP ($p = 0.034$) in PTC. There was no correlation between C4d and staining of the studied mTOR phosphoproteins. Treatment with mTORi had no significant impact neither on C4d, pERK, pmTOR, pS6RP staining in PTC, or chronicity.
Conclusion: Our findings support that S6RP phosphorylation in PTC is associated with AMR in RB. Consequently, pS6RP staining may be useful for AMR diagnosis. Treatment with mTORi does not seem to modify pS6RP, pERK or mTOR in kidney allografts.



FG062 A URINARY METABOLITE CONSTELLATION TO DETECT ACUTE REJECTION IN KIDNEY ALLOGRAFTS

Miriam Banas[1], Sindy Neumann[2], Philipp Pagel[2], Franz Josef Putz[1], Bernhard K. Krämer[3], Petra Rümmele[4], Johannes Eiglsperger[2], Eric Schiffer[2], Bernhard Banas[1]
[1]Department of Nephrology, University Hospital Regensburg; [2]Numares AG; [3]Fifth Department of Medicine, University Medical Center Mannheim; [4]Department of Pathology, University Hospital Erlangen

Background: Post-transplant surveillance for acute rejection is mainly based on regular monitoring of serum creatinine levels and transplant biopsies upon functional renal impairment. Recently, we developed a novel method to detect kidney allograft rejection via a characteristic constellation of the urine metabolites alanine, citrate, lactate, and urea investigated by nuclear magnetic resonance (NMR) spectroscopy (Banas M et al. Metabolomics 2018).
Methods: Within the prospective, observational UMBRELLA study 986 urine specimens were collected from 109 consecutively enrolled renal transplant recipients and metabolite constellations were analyzed by NMR spectroscopy. A metabolite rejection score was calculated and compared to histopathological results of corresponding allograft biopsies ($n = 206$).
Results: The metabolite constellation was found to be a useful biomarker to non-invasively detect acute allograft rejection (AUC = 0.75; 95% confidence interval (CI) 0.68–0.83; based on 46 cases with biopsy-proven rejection and 520 controls). A combination of the metabolite rejection score and the estimated glomerular filtration rate (eGFR) at the time of urine sampling further improved the overall test performance significantly (AUC = 0.84; 95% CI 0.76–0.91; based on 42 cases and 468 controls). In a subgroup of patients without rejection episodes the test results remained well below a diagnostic threshold associated with high risk of acute rejection. In other cases a marked increase above this threshold indicated an acute allograft rejection already 6–10 days before diagnostic renal biopsies were performed.
Conclusions: In conclusion, a combination of a NMR-based urine metabolite analysis and glomerular filtration rate is promising as a non-invasive test for post-transplant surveillance and to support decision making whether renal allografts need histopathological evaluation.

FG063 EXPRESSION PROFILING OF EXOSOMAL MIRNAS DERIVED FROM THE PERIPHERAL BLOOD OF KIDNEY RECIPIENTS WITH DGF USING HIGH-THROUGHPUT SEQUENCING

Junpeng Wang[1], Xin Li[2], Xiaoqiang Wu[1], Zhiwei Wang[1], Chan Zhang[1], Guanghui Cao[1], Tianzhong Yan[1]
[1]Henan Provincial People's Hospital; [2]Zhengzhou University

Background: Delayed graft function (DGF) is one of the major obstacles for graft survival for kidney recipients. It is profound to reduce the incidence of DGF for maintaining long-term graft survival. However, the molecular regulation of DGF is still not adequately explained and the biomarkers for DGF are limited. Exosome-derived proteomic and RNA signature profiles are often used to account for the molecular regulation of diseases or reflect the conditional state of their tissue as biomarkers. Few researches have been done to demonstrate the function of exosomes associated with DGF.
Methods: In this study, high-throughput sequencing was used to explore the miRNA expression profiling of exosomes in the peripheral blood of kidney recipients with or without DGF. Two algorithms miRanda, and Targetscan were used to predict the target genes of exosomal miRNAs which were differentially expressed between DGF and control groups. Subsequently, the gene ontology terms (http://www.geneontology.org/) and KEGG pathway terms (http://www.genome.jp/kegg) enriched in predicted target genes were determined to explore the function and related pathway of the targets.
Results: We identified 52 known and 5 conserved exosomal miRNAs specifically expressed in recipients with DGF. Three co-expressed miRNAs, hsa-miR-33a-5p_R-1, hsa-miR-98-5p and hsa-miR-151a-5p, were observed significantly up-regulated in kidney recipients with DGF. Moreover, hsa-miR-151a-5p was positively correlated with the first-week serum CR, BUN and UA levels of the kidney recipients after transplantation. Furthermore, we also analyzed functions and signaling pathways of the three up-regulated miRNAs target genes to uncover putative mechanism that how these exosomal miRNAs functioned in DGF.
Conclusions: Overall, these findings identified biomarker candidates for DGF and provided new insights into the important role of the exosomal miRNAs regulation in DGF.

6. Llinàs-Mallol, L; Redondo-Pachón, D; Pérez-Sáez, MJ; Raïch-Regué, D; Mir, M; Yélamos, J; López-Botet, M; Pascual, J; Crespo, M. Different effects on transitional B lymphocytes after conversion to mTOR inhibitors or steroid withdrawal in renal transplant recipients. Nephrology Dialysis Transplantation 2019;34(S1): i619-i620. Poster presentation, 56th ERA-EDTA Congress, June 13-16, 2019, Budapest, Hungary.

SP779 **DIFFERENT EFFECTS ON TRANSITIONAL B LYMPHOCYTES AFTER CONVERSION TO MTOR INHIBITORS OR STEROID WITHDRAWAL IN RENAL TRANSPLANT RECIPIENTS**

Laura Llinàs-Mallol[1], Dolores Redondo[1], Maria José Pérez-Sáez[1], Dàlia Raïch-Regué[1], Marisa Mir[1], José Yélamos[1], Miguel López-Botet[1], Julio Pascual[1], Marta Crespo[1]

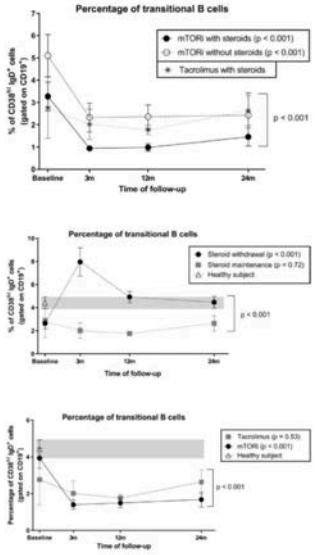
[1]Hospital del Mar, Barcelona, Spain

INTRODUCTION: Despite high graft-survival rates during the first year after kidney transplantation, long-term graft survival is still limited. Antibody-mediated rejection is the main cause of graft loss and current immunosuppressive therapies are still not able to control it. In the other side, immunosuppression is associated with side effects, which produce morbidity and may limit long-term graft survival. Conversion from CNI to mTOR inhibitors (mTORi) or steroid withdrawal pursues the challenge to reduce these drawbacks. Since transitional B cells have been linked with increased possibilities of acquiring operational tolerance, we aimed to assess whether changes in immunosuppression therapies may affect this particular subset of B lymphocytes.

METHODS: We analyzed the immunophenotype of peripheral blood lymphocyte subsets before, 3, 12 and 24 months after: a) conversion from tacrolimus to mTORi in 29 patients; b) steroid withdrawal in 19 patients; c) no changes with maintenance of tacrolimus and steroids in 16 patients, as a control group.

RESULTS: We found opposed effects of these immunosuppression changes in transitional B lymphocytes. Transitional B cells decreased in patients who started mTORi treatment, whereas increased in patients who withdraw steroids (both $p<0.001$). These results were maintained during the 24 months of follow-up. We further assessed the additive effect of introducing mTORi in patients who did not receive steroids. Patients with and without steroid treatment showed significant decrease of transitional B cells after mTORi conversion (both $p<0.001$). Patients who maintained tacrolimus and steroids showed no changes in transitional B cells ($p=0.53$), and consequently confirm the influence of these two immunosuppressive agents on this particular subset of B lymphocytes.

doi:10.1093/ndt/gfz103 | i619



CONCLUSIONS: These results indicate that mTORi and steroids reduce the frequency of transitional B cells in peripheral blood. It is of interest to know the functionality of this subpopulation and to clarify whether mTORi and steroids are hindering cell-related operational tolerance.

7. Llinàs-Mallol, L; Redondo-Pachón, D; Pérez-Sáez, MJ; Raïch-Regué, D; Mir, M; Yélamos, J; López-Botet, M; Pascual, J; Crespo, M. Peripheral lymphocyte changes in renal allograft recipients after conversion to mTOR inhibitors. Nephrology Dialysis Transplantation 2019;34(S1): i618. Poster presentation, 56th ERA-EDTA Congress, June 13-16, 2019, Budapest, Hungary. *Abstract awarded with an ERA-EDTA Travel Grant.*

SP775 **PERIPHERAL LYMPHOCYTE CHANGES IN RENAL ALLOGRAFT RECIPIENTS AFTER CONVERSION TO MTOR INHIBITORS**

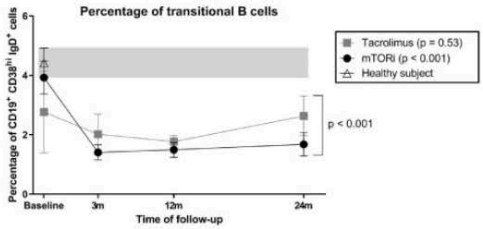
Laura Llinàs-Mallol[1], Dolores Redondo[1], Maria José Pérez-Sáez[1], Dàlia Raïch-Regué[1], Marisa Mir[1], José Yélamos[1], Miguel López-Botet[1], Julio Pascual[1], Marta Crespo[1]

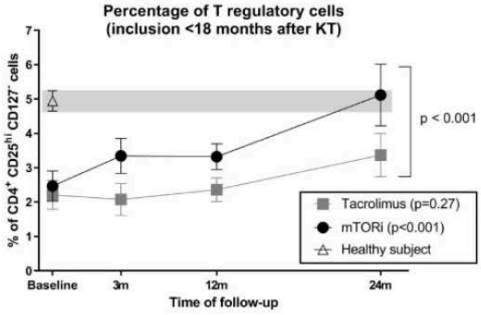
[1]*Hospital del Mar, Barcelona, Spain*

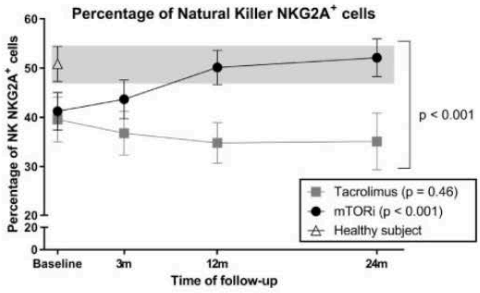
INTRODUCTION: Despite high early graft-survival rates after kidney transplantation (KT), long-term graft survival is still limited in time under immunosuppression based on steroids, calcineurin inhibitors (CNI) and antiproliferative agents. Therefore, conversion to mTOR inhibitors (mTORi) has been proposed to reduce CNI-associated nephrotoxicity and to decrease cardiovascular risk on KT recipients. Changes in immunosuppression treatment may also have an impact on lymphocyte subpopulations

METHODS: We designed a prospective study to evaluate the long-term immunological impact of tacrolimus to mTORi conversion in 29 KT recipients compared with 16 KT recipients maintained on tacrolimus per clinical practice. We evaluated renal function, HLA antibodies and peripheral blood lymphocyte subsets at inclusion and 3, 12 and 24 months later. The immunophenotype of 20 healthy subjects was also analyzed for reference.

RESULTS: At follow up, renal function remained stable in both groups and proteinuria increased slightly in the mTORi group (308 g/g vs. 156 g/g in tacrolimus group, p=0.007). Two patients in the mTORi group developed *de novo* HLA donor-specific antibodies (DSA) and none in the control group (7 vs 0%, p=0.53). Overall both groups of patients showed a progressive increase in T regulatory cells, but the restricted analysis of patients recruited within the first 18 months post-KT showed increase in regulatory T cells only in those converted to mTORi (p<0.001). Patients treated with mTORi showed a decrease of total B cells and naïve B cells, except for those converted to mTORi without steroids. mTORi patients showed a significant decrease in transitional B cells (p<0.001) compared to those on tacrolimus, even patients who did not receive steroids. Natural Killer NKG2A+ cells increased in mTORi patients compared to tacrolimus (p<0.001), reaching healthy subjects´percentages.







CONCLUSIONS: In summary, patients who switched to an mTORi displayed a significant redistribution of peripheral blood lymphocyte subpopulations that might influence graft outcomes. The co-administration of steroids modified some of these changes.

8. Burballa, C; Outón, S; Vázquez, S; **Llinàs-Mallol, L**; Redondo-Pachón, D.; Pérez-Sáez, MJ; García, C; Mir, M; Arias-Cabrales, C; Pascual, J; Crespo, M. HLA antibody immunization: pregnancy as a natural source. Nephrology Dialysis Transplantation 2019;34(S1): i16-i17. Oral presentation, 56th ERA-EDTA Congress, June 13-16, 2019, Budapest, Hungary.

Nephrology Dialysis Transplantation

FO037 **HLA ANTIBODY IMMUNIZATION: PREGNANCY AS A NATURAL SOURCE**

Carla Burballa[1], Sara Outon[1], Susana Vazquez[2], Laura Llinàs-Mallol[1], Dolores Redondo[1], Maria José Pérez-Sáez[1], Carme Garcia[2], Marisa Mir[1], Carlos Arias-Cabrales[1], Julio Pascual[3], Marta Crespo[1]

[1]*Hospital del Mar, Barcelona, Spain, Spain,* [2]*Hospital del Mar, Barcelona, Spain,* [3]*Laboratori de Referencia de Catalunya, Barcelona, Spain and* [1]*Institut Hospital del Mar d'Investigacions Mèdiques IMIM, Barcelona, Spain*

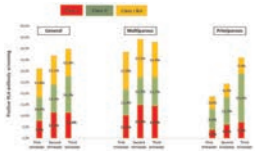
INTRODUCTION: Donor specific antibodies (DSA) complicate the access to transplantation in alloimmunized potential recipients. Pregnancy may induce the production of anti *human* leukocyte antigen (HLA) antibodies. Most studies have evaluated pregnancy-induced sensitization mainly by *Complement-Dependent Cytotoxicity* (CDC) assays *or after birth*. The dynamics of anti-HLA antibodies appearance during pregnancy by new techniques based on *Solid Phase Immunoassays* (SPI) has not been well described.

METHODS: From 2012 to 2017, serum samples from pregnant women registered at Hospital del Mar, Barcelona, were prospectively collected during the first (1T), second (2T) and third (3T) trimesters of pregnancy. The presence of HLA antibodies was assessed by Luminex screening kits for HLA class I and class II antibodies. The aim of the study was to determine the incidence and dynamics of HLA antibody appearance during pregnancy.

RESULTS: In a cohort of 141 pregnant women (mean age 34.2 years 36.8% primiparous women) 45.4% presented HLA antibodies at some point during pregnancy. Around 40% of multiparous women had a positive screening in each of the three trimesters with similar ratio of class I, class II and class I & II antibodies. As for primiparous pregnant women, there was a gradual increase in antibody detection (19% 1T, 24% 2T and 36% 3T) with a more significant rise in HLA class II antibodies, especially during the third trimester (figure 1). The number of previous live births (0.8±0.85 vs. 1.02±0.9;p=0.2) or abortions (0.69±0.8vs. 0.88±1.03; p=0.2) was not associated with sensitization.

Nephrology Dialysis Transplantation

Abstracts



CONCLUSIONS: Pregnancy is a relevant source of HLA antibody sensitization, so that more multi than primiparous women showed antibodies during pregnancy. Class II antibodies showed a more important increase than class I antibodies in primiparous women, especially during the third trimester. This is a relevant piece of information to consider when assessing the immunization status for kidney transplantation in women

doi:10.1093/ndt/gfz096 | i17

9. Llinàs-Mallol, L; Redondo-Pachón, D; Raïch-Regué, D; Yélamos, J; Pérez-Sáez, MJ; Mir, M; López-Botet, M; Pascual, J; Crespo, M. Steroid withdrawal promotes B lymphocyte phenotypes of operational tolerance predisposition in renal allograft recipients. Transplantation 2018;102(7S): S403. Oral presentation, 27th International Congress of The Transplantation Society (TTS), June 30 – July 5, 2018, Madrid, Spain.

616.3

Steroid withdrawal Promotes B Lymphocyte Phenotypes of Operational Tolerance Predisposition in Renal Allograft Recipients

Laura Llinàs Mallol[1], Dolores Redondo Pachón[1,2], Dàlia Raïch Regué[1], Jose Yélamos[3,4], María José Pérez Sáez[1,2], Marisa Mir[1], Miguel López Botet[3,4,5], Julio Pascual Santos[1,2], Marta Crespo Barrio[1,2]
[1]Nephropathies Research Group, Hospital del Mar Medical Research Institute, Barcelona, Spain; [2]Nephrology Department, Hospital del Mar, Barcelona, Spain; [3]Immunology Department, Hospital del Mar, Barcelona, Spain; [4]Immunity and Infection Research Group, Hospital del Mar Medical Research Institute, Barcelona, Spain; [5]Experimental and Health Sciences Department, University Pompeu Fabra, Barcelona, Spain.

Despite high graft-survival rates one year after kidney transplantation, long-term graft survival is still limited. Chronic immunological rejection associated with donor-specific antibodies (DSA) is recognized as a cause of graft loss and current immunosuppressive therapies are still not able to control it. Steroids are effective in reducing the incidence of acute rejection but also are an important cause of morbidity, which may limit long-term graft survival. Several studies have assessed clinical outcomes after steroid withdrawal, but not the effects on peripheral blood lymphocyte subsets. We aimed to assess whether steroid withdrawal modifies the distribution of peripheral blood lymphocyte subsets and circulating anti-HLA antibodies.

We analyzed the immunophenotype of peripheral blood lymphocyte subsets and determined circulating anti-HLA antibodies of 21 patients before, 3, 12 and 24 months after steroid withdrawal and 18 patients who maintained steroids. All patients showed stable renal function during the study. Our results depict that long-term steroid withdrawal promotes an increase in percentage of B lymphocytes compared to basal condition ($p = 0.006$) and control group ($p = 0.04$).This is accompanied with the expansion of naïve B lymphocytes and significant decrease of memory B lymphocytes compared to basal condition (both $p < 0.001$) and control group (both $p < 0.001$).We observed an expansion of transitional B lymphocytes compared to basal condition ($p = 0.002$) and control group ($p = 0.013$). No significant differences were found on T lymphocyte subpopulations or Natural Killer (NK) cells. No development of de novo DSA was identified and only 2 patients developed non DSA HLA antibodies.

Operational tolerant kidney transplant receptors, characterized by long-term acceptance of a mismatched kidney allograft after immunosuppressive drug withdrawal, have been described to display increased numbers and frequency of B lymphocytes and naïve B lymphocytes and increased frequency of transitional B lymphocytes in comparison with stable patients. Taken together, these data suggest that steroid withdrawal may be beneficial and patients could have increased possibilities to acquire operational tolerance.

PI13/00598 (Institute of Health Carlos III, ISCIII).

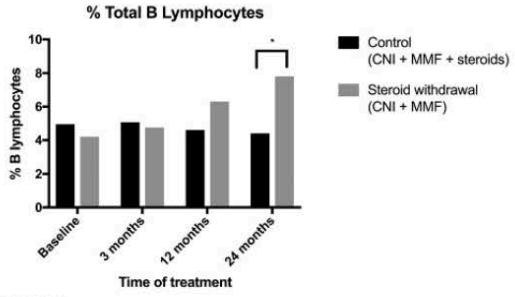


FIGURE 1.

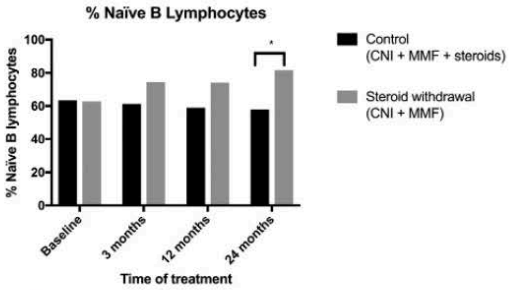


FIGURE 2.

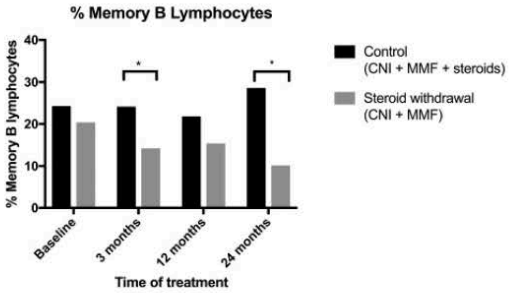


FIGURE 3.

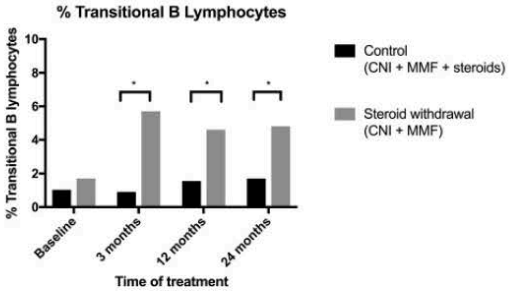


FIGURE 4.

10. Llinàs-Mallol, L; Redondo-Pachón, D; Raïch-Regué, D; Yélamos, J; Pérez-Sáez, MJ; Mir, M; López-Botet, M; Pascual, J; Crespo, M. Different effects on transitional B lymphocytes after conversion to mTOR inhibitors or steroid withdrawal in renal transplant recipients. Transplantation 2018; 10(7S): S280. Oral presentation, 27th International Congress of The Transplantation Society (TTS), June 30 – July 5, 2018, Madrid, Spain.

517.2

Different Effects on Transitional B Lymphocytes after Conversion to mTOR Inhibitors or Steroid Withdrawal in Renal Transplant Recipients

Laura Llinàs Mallol[1], Dolores Redondo Pachón[1,2], Dàlia Raïch Regué[1], Jose Yélamos[3,4], María José Pérez Sáez[1,2], Marisa Mir[1], Miguel López Botet[3,4,5], Julio Pascual Santos[1], Marta Crespo Barrio[1]
[1]Nephropathies Research Group, Hospital del Mar Medical Research Institute, Barcelona, Spain; [2]Nephrology Department, Hospital del Mar, Barcelona, Spain; [3]Immunology Department, Hospital del Mar, Barcelona, Spain; [4]Immunity and Infection Research Group, Hospital del Mar Medical Research Institute, Barcelona, Spain; [5]Experimental and Health Sciences Department, University Pompeu Fabra, Barcelona, Spain.

Despite high graft-survival rates during the first year after kidney transplantation, long-term graft survival is still limited. Chronic immunological rejection is the main cause of graft loss and current immunosuppressive therapies are still not able to control it. Calcineurin inhibitors (CNI) and steroids are associated with the development of side effects, which produce morbidity and may limit long-term graft survival. Conversion from CNI to mTOR inhibitors (mTORi) or steroid withdrawal pursue the challenge to reduce these drawbacks. Since transitional B cells have been linked with increased possibilities of acquiring operational tolerance, we aimed to assess whether changes in immunosuppression therapies may affect this particular subset of B lymphocytes.

We analyzed the immunophenotype of peripheral blood lymphocyte subsets before and 3, 12 and 24 months after: a) conversion from CNI to mTORi in 39 patients; b) steroid withdrawal in 21 patients; c) no changes with maintenance of CNI and steroids in 18 patients, as a control group. We found paradoxical effects of immunosuppression treatments in transitional B lymphocytes. Patients who started with mTORi therapy showed a significant decrease of transitional B lymphocytes after 3 months compared to basal condition ($p < 0.001$), whereas patients who withdraw steroids showed a significant increase at 3 months ($p = 0.001$). These results did not change with time. Results in patients who maintained CNI therapy and steroids revealed no changes in transitional B cells compared to basal condition ($p = 0.64$), and consequently confirm influence of immunosuppression therapy on this particular subset of B lymphocytes.

These results indicate that both steroids and mTORi reduce the frequency of transitional B cells. Therefore, it is of great interest to clarify whether mTORi and steroids are hindering cell-related operational tolerance.

PI13/00598 (Institute of Health Carlos III, ISCIII).

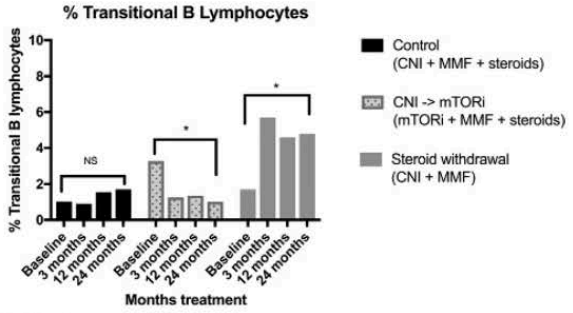


FIGURE 1.

10.1.2 National congresses

1. Llinàs-Mallol, L; Redondo-Pachón, D; Raïch-Regué, D; Pérez-Sáez, MJ; Micó, M; Arias-Cabrales, C; Buxeda, A; Burballa, C; Pascual, J; Crespo, M. Fenotipo específico de células NK periféricas con impacto pronóstico independiente en receptores de trasplante renal con rechazo mediado por anticuerpos. Nefrología 2020;40(S1): 120. Oral presentation, L Congress Spanish Society of Nephrology (SEN), November 6 – 9 2020, virtual. *Abstract awarded with the Young Nephrologists - Spanish Society of Nephrology prize to the Best Young Oral Communication presented in the L SEN Congress.*

Trasplante Renal - Resultados y estudios epidemiológicos

432 ••• **DISEÑO Y OPTIMIZACIÓN CON ALTA EFICIENCIA DE ALGORITMO PREDICTOR Y RED ARTIFICIAL NEURONAL MEDIANTE EL USO DE HYBRID DATA ENVELOPMENT ANALYSIS-ARTIFICIAL NEURAL NETWORK COMO MÉTODO PREDICTOR DE LA EVOLUCIÓN DEL TRASPLANTE RENAL**

I. REVUELTA[1], FJ. SANTOS-ARTEAGA[2], D. DI CAPRIO[3], D. CUCCHIARI[2], P. VENTURA-AGUIAR[1], JM. CAMPISTOL[1], F. OPPENHEIMER[1], F. DIEKMANN[1]

[1]SERVICIO DE NEFROLOGIA Y TRASPLANTE RENAL. HOSPITAL CLINIC (BARCELONA/ESPAÑA), [2]FACULTY OF ECONOMICS AND MANAGEMENT. FREE UNIVERSITY OF BOLZANO (BOLZANO/ITALIA),[3]DEPARTMENT OF ECONOMICS AND MANAGEMENT. UNIVERSITY OF TRENTO (TRENTO/ITALIA)

Justificación: El trasplante renal no ha experimentado una gran mejora en las tasas de supervivencia. La enorme cantidad de variables que pueden afectar a los resultados hace complicado el manejo holístico del paciente. El presente estudio presenta el diseño y validación de una técnica híbrida de Data Envelopment Analysis (DEA)- Artificial Neural Network (ANN) para predecir la evolución de los pacientes trasplantados en función de las variables que definen sus perfiles en el momento del trasplante.

Método: A través de una cohorte retrospectiva no-Big Data de 485 receptores (2006-2015), identificamos por métodos estadísticos doce variables para generar los perfiles de los pacientes en el pretrasplante. Los outcomes del trasplante (rechazo, pérdida injerto, muerte, neoplasia) se evalúan individualmente y a través de una sola variable de desenlace que englobe todos los outcomes. La categorización de los pacientes según su rendimiento relativo se define a través de dos etapas: la primera identifica las variables en las que cada paciente tiene un rendimiento inferior, y genera un índice de evaluación, utilizado de entrenamiento de referencia para la ANN (implementada en la segunda etapa). El Comité Ética Local aprobó el estudio.

Resultados: Cuando usamos el algoritmo predictor con la categorización en dos capas, con DEA como optimización de datos previamente, dados los perfiles de los pacientes pretrasplante, el DEA optimiza e identifica cada variable pretrasplante donde ocurre una ineficiencia por paciente. También DEA identifica la magnitud de estas variables en relación con el punto de referencia (definido por los pacientes con mejores resultados). Con ello podemos dividir, para cada variable pretrasplante, los pacientes en categorías de cuartil y quintil según a cómo están respecto al paciente con mejores resultados. Con todo entrenamos la ANN correspondiente. Cuando usamos el algoritmo predictor en inteligencia artificial- ANN sin la categorización en dos capas con DEA obtenemos una eficiencia del 11%, que con el método DEA-ANN asciende al 86.5% para cada variable de desenlace o a este común set de variables de outcome del trasplante.

Conclusiones: DEA-ANN se basa en la matriz de eficiencia derivada de la etapa DEA, que permite capacitar a la ANN para extrapolar el comportamiento de los pacientes en función de sus perfiles pre-trasplante aún sin tener BigData. Con ello el análisis por el modelo DEA se puede utilizar para incorporar variables difusas e inciertas al análisis, al igual que usar cohortes no-BigData que de otra manera no podrían evaluarse a través de una ANN.

433 ••• **ALTA PREDICCIÓN DE LA EVOLUCIÓN DE PACIENTES TRASPLANTADOS INGRESADOS POR COVID-19 A TRAVÉS DE UN MODELO HIBRIDO PREDICTIVO DE MACHINE-LEARNING**

I. REVUELTA[1], FJ. SANTOS-ARTEAGA[2], E. MONTAGUD-MARRAHI[1], P. VENTURA-AGUIAR[1], F. COFAN[1], JV. TORREGROSA[1], D. CUCCHIARI[2], N. ESFORZADO[1], F. OPPENHEIMER[1], F. DIEKMANN[1]

[1]SERVICIO DE NEFROLOGIA Y TRASPLANTE RENAL. HOSPITAL CLINIC (BARCELONA), [2]FACULTY OF ECONOMICS AND MANAGEMENT. FREE UNIVERSITY OF BOLZANO (BOLZANO / ITALY)

Justificación: En un escenario de demanda abrumadora, como ha sido la pandemia del SARS-CoV-2, la presión sobre los sistemas de salud puede superar su capacidad prevista para enfrentar una situación tan extrema, por lo que se necesita evidencia científica y modelos validados predictors, especialmente para población de alto riesgo, como serían los receptores de trasplantes.

Método: Estudio retrospectivo de pacientes trasplantados hospitalizados por COVID-19. Terapia de combinación con lopinavir/ritonavir, hidroxicloroquina y azitromicina. Retirada temporal de micofenolato e inhibidor mTOR en todos los pacientes y tambien de anticalcienurinico (sobre todo si lopinavir/ritonavir y graves). En todos casos prednisona 20 mg/día hasta resolución de COVID-19 y posterior reducción e incorporación progresiva de los inmunosupresore. Para desarrollar el modelo de predicción para evaluar un peor comportamiento (definido como admisión en UCI o necesidad intensificación terapeutica con agentes antiinflamatorios) se integraron datos de los pacientes en el momento de la admisión al hospital. Las predicciones se realizaron utilizando el híbrido: Data Envelopment Analysis (DEA)-Artificial Neural Network (ANN), cuya precisión en relación con varias configuraciones alternativas se ha validado mediante una batería de múltiples técnicas de machine-learning. El Comité de Ética aprobó el studio.

Resultados: De 1006 receptores seguidos presencial o telemáticamente durante el período de observación, treinta y ocho ingresaron por COVID-19, con una edad media de 59 años (rango,33-87). La mayoría consultaron por fiebre (94.7%), tos (60.5%), disnea (39.5%) y diarrea (28.9%), y fueron ingresados después de una media de 7.3±5.9 días desde el comienzo de los síntomas. Veinticuatro pacientes (63.2%) mostraron una peor evolución (tasa de mortalidad:13.2%), con una media de 12 días de ingreso. La tos como síntoma de presentación (P=0.000), la neumonía (P=0.011) y los niveles de LDH (P=0.031) fueron factores de admisión asociados con peores resultados. El modelo híbrido de predicción que trabaja con un conjunto de 17 variables inputs al ingreso mostró una precisión del 96,3%, superando a cualquier otro modelo, como la regresión logística(65,5%) o random forest(44,8%). Además, el modelo de predicción nos permite clasificar la evolución de los pacientes a través de los valores al ingreso hospitalario e identificar qué variables.

Conclusiones: El modelo de predicción basado en Data Envelopment Analysis(DEA)-Artificial Neural Network(ANN) pronostica el comportamiento del paciente trasplantado por COVID-19 con una precisión del 96,3%. El modelo predictivo propuesto puede ayudar a guiar el manejo del COVID-19 mediante la identificación de predictores claves que permitan una distribución sostenible de recursos ante una pandemia.

434 ••• **FENOTIPO ESPECÍFICO DE CÉLULAS NK PERIFÉRICAS CON IMPACTO PRONÓSTICO INDEPENDIENTE EN RECEPTORES DE TRASPLANTE RENAL CON RECHAZO MEDIADO POR ANTICUERPOS**

L. LLINÀS MALLOL[1], D. REDONDO PACHÓN[1], D. RAÏCH REGUÉ[1], MJ. PÉREZ SÁEZ[1], M. MICÓ[1], C. ARIAS CABRALES[1], A. BUXEDA[1], C. BURBALLA[1], J. PASCUAL[1], M. CRESPO[1]

[1]NEFROLOGÍA. HOSPITAL DEL MAR (BARCELONA)

Introducción: El rechazo mediado por anticuerpos (AMR) se asocia con peor supervivencia del injerto renal. Se ha sugerido un papel de las células NK circulantes en la fisiopatología del daño en AMR. Nuestro grupo describió la asociación entre la proporción de células NKG2A+ periféricas y la detección de anticuerpos anti-HLA donante-específicos (DSA).

Materiales y métodos: Seleccionamos 177 receptores de trasplante renal (TR) con biopsia 2011-2017: 77 con AMR (DSA+: 53, DSA-: 24) y 100 sin AMR (DSA+: 15, DSA-: 85). Evaluamos la supervivencia del injerto (tiempo medio de seguimiento desde la biopsia: 53 meses) en los 4 grupos; en 138 analizamos el inmunofenotipado de células NK en sangre periférica contemporáneo a la biopsia y su valor como biomarcador de supervivencia.

Resultados: Al final del seguimiento, se observó peor supervivencia del injerto en TR-AMR+ (p<0.001) independientemente de la detección de DSA (p=0.63), y una supervivencia similar entre los TR-AMR-DSA+ y los TR-AMR-DSA- (p=0.42) (Figura). Entre los 138 TR con inmunofenotipado, los TR-AMR+DSA+ y TR-AMR+DSA- mostraron menor proporción (p=0.06) y menor número absoluto de células NK respecto TR-AMR-DSA- (p=0.027, p=0.017). Observamos mayor porcentaje de células NKG2A+ y menor de NKG2A- en TR-AMR+DSA+ respecto TR-AMR-DSA- (p=0.007), con menor número absoluto de células NKG2A- en TR-AMR+DSA+ y TR-AMR+DSA- respecto TR-AMR-DSA- (p=0.001, p=0.017). Un porcentaje de células NKG2A-<30% en TR-AMR+ se asoció a peor supervivencia del injerto en los 36 meses posteriores a la biopsia renal (p=0.067) (Figura).

Figura A. Supervivencia desde la biopsia hasta fin de seguimiento en los TR incluidos.

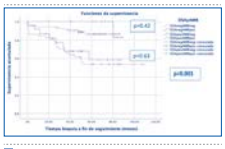
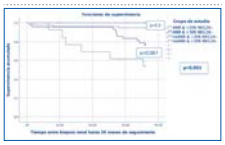


Figura B. Supervivencia desde la biopsia hasta 36 meses de seguimiento estratificada según grupo de estudio (AMR+ vs. AMR-) y la detección de un porcentaje de células NKG2A- mayor o menor del 30%.



Conclusiones: La supervivencia a largo plazo de los injerto con AMR es significativamente menor respecto a los injertos sin AMR independientemente de la presencia de DSA. Existe un menor número de células NK en TR-AMR+, con menor número de células NKG2A- circulantes indistintamente de la detección de DSA. Esta presencia disminuida de NK se asocia a una peor supervivencia del injerto a medio plazo.

435 ••• **AKI EN PACIENTES TRASPLANTADOS RENALES CON ENFERMEDAD POR SARS-COV-2. EXPERIENCIA DE UN CENTRO**

M. SANCHEZ-BAYA[1], C. ARANA[1], L. FAYOS[1], F. BERNA[1], MJ. LLORET[2], R. GELPI[2], A. VILA[2], C. FACUNDO[2], L. GUIRADO[2], N. SERRA[2]

[1]NEFROLOGÍA. FUNDACIÓ PUIGVERT (BARCELONA), [2]NEFROLOGÍA, UNIDAD DE DIÁLISIS. FUNDACIÓ PUIGVERT (BARCELONA), [2]NEFROLOGÍA, UNIDAD DE TRASPLANTE RENAL. FUNDACIÓ PUIGVERT (BARCELONA)

Introducción: La enfermedad por SARS-CoV-2(COVID-19) tiene una presentación clínica muy variable y la mortalidad en la población general varía del 1,4 al 8%, siendo mayor(24-27%) en pacientes en diálisis y trasplantados renales(TR). La incidencia de daño renal agudo (AKI por sus siglas en inglés) en la población general es variable 3-15%, dependiendo de la severidad de la enfermedad del grupo analizado y es más frecuente en pacientes que desarrollan inflamación, hipoxia y sepsis. Los datos de AKI en pacientes TR son escasos.

Objetivo: Describir la frecuencia y las características del AKI en pacientes TR con COVID-19 y analizar su impacto en la mortalidad.

Material y Método: Estudio descriptivo, unicéntrico. Se incluyeron pacientes TR que desarrollaron COVID-19 de marzo a mayo/2020. Se obtuvieron datos demográficos, de laboratorio y de tratamiento.

Resultados: Se incluyeron 25 pacientes TR diagnosticados de COVID-19, edad media 58±11 años, 64% varones, 56% TR de donante cadáver, con un tiempo medio de TR 93±72 meses. Entre las comorbilidades destacaba HTA(100%), diabetes(28%), EPOC/SAHS/asma(16%) y bajo riesgo según escala de Charlson del 96%. El 36% estaban tratados con IECA y 12% con ARA II. 96% con inmunosupresión de mantenimiento basada en tacrolimus.

92% requirieron ingreso hospitalario con una media de días de hospitalización de 15±11, 68% clínica compatible de COVID-19 y al 72% se les administró tratamiento específico. 12% requirió ingreso en UCI.

El 47% (9/19 pacientes) desarrollaron AKI durante el ingreso, siendo el 44% AKI2 y el 55% AKI3. El 25% requirió técnicas de reemplazo renal (4 técnica intermitente y 1 técnica continua) recuperando la función del injerto únicamente el 20%. El AKI tuvo una mayor incidencia en aquellos pacientes que tenían una peor función renal basal.

En ninguno de los pacientes se realizó biopsia renal. No obstante, en ningún caso se sospechó que la causa fuera un rechazo agudo. Los pacientes que presentaron AKI (cualquier grado) presentaron de manera significativa una mayor mortalidad.

Conclusiones: La incidencia de AKI en el contexto de COVID-19 en el paciente TR es elevada y mayor que la población no trasplantada. Un porcentaje significativo requiere técnicas de reemplazo renal. Se relaciona significativamente con mayor mortalidad por lo que es necesario estudiar la etiopatogenia en este paciente y la detección precoz para optimizar el manejo. Es importante analizar el impacto que puede tener a medio y largo plazo tanto en la supervivencia del injerto como del paciente.

2. Burballa, C; **Llinàs-Mallol, L**; Buxeda, A; Arias-Cabrales, C; Mir, M; Faura, A; Pascual, J; Crespo, M. Rechazo mediado por anticuerpos: el fenotipo clínico importa. Nefrología 2019;39(S1): 144. Oral presentation, XLIX SEN Congress, October 5 – 8 2019, A Coruña, Spain.

Resúmenes

Trasplante Renal - Aspectos clínicos y complicaciones

532 LA FRAGILIDAD DURANTE EL TIEMPO DE ESPERA PARA TRASPLANTE RENAL ES UN FACTOR DETERMINANTE DE LA SUPERVIVENCIA PRECOZ DESPUÉS DEL TRASPLANTE

C. ARIAS-CABRALES[1], MJ. PERÉZ-SÁÉZ[1], A. BACH[1], M. VERA[1], A. FAURA[1], E. JUNYENT[1], C. BURBALLA[1], M. MIR[1], M. CRESPO[1], J. PASCUAL[1]
[1]NEFROLOGÍA. HOSPITAL DEL MAR (BARCELONA)

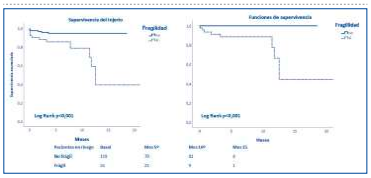
Introducción: La fragilidad se define como la falta de reserva fisiológica del individuo ante eventos estresantes y se ha relacionado con mayor morbimortalidad en TR. Quisimos evaluar el impacto pronóstico de la fragilidad en pacientes en lista de espera (LE) de TR, así como la evolución de este estado en el post-TR.

Métodos: Estudio prospectivo observacional de pacientes incluidos en LE de TR julio-2015/diciembre-2018. Evaluamos la fragilidad (fenotipo Fried), así como otros parámetros (escalas dependencia/depresión, analíticos, bioimpendancia) al acceso a LE, y a los 3 y 12 meses post- TR.

Resultados: De los 330 pacientes incluidos en LE, 170 se trasplantaron, mediana de seguimiento to 6.8 meses [RIQ 3.2-10.7]. El 30% (n=51) eran frágiles pre-TR, sin encontrar diferencias en la mayoría de características basales incluido la edad o comorbilidades. Los pacientes frágiles presentaron más función retardada del injerto (36.7 vs 16.1%; p=0.007), incluso tras ajustar por otras variables como edad del donante y hemodiálisis pre-TR (HR: 2.3, 95%IC 1.05-5.36; p=0.038). No encontramos diferencias significativas en complicaciones quirúrgicas, días de ingreso o tasa de reingresos precoces. Durante el seguimiento, registramos 13 pérdidas del injerto censurando por muerte y 8 muertes, estas últimas todas en pacientes frágiles, el 50% de causa infecciosa y 37% cardiovascular. Los frágiles tuvieron menor supervivencia del injerto y del paciente; no falleció ningún paciente no frágil pre-TR (Figura). A los 3 meses posTR evaluamos fragilidad en 116 pacientes. De ellos, 26 eran frágiles pre-TR, 17 (65.4%) de los cuales mejoraron post-TR; de los 90 no frágiles, 16 (17%) empeoraron a frágiles tras el TR.

Conclusiones: El estado de fragilidad es independiente de la edad y la comorbilidad. Los pacientes frágiles presentaron más función retrasada del injerto y tienen peor supervivencia del paciente y del injerto. Dos tercios de los pacientes frágiles mejoran ese estatus tras el TR.

Figura 1.



533 ENDARTERITIS TEMPRANA: FACTOR DE MAL PRONÓSTICO EN EL INJERTO RENAL

AB. ANNA BUXEDA[1], MS. MARÍA LUISA SERRANO SALAZAR[2], JG. JAVIER GIMENO[3], CA. CARLOS ARIAS-CABRALES[1], LL. LAURA LLINÀS-MALLOL[4], DR. DOLORES REDONDO- PACHÓN[1], CB. CARLA BURBALLA[1], MC. MARTA CRESPO[1], JP. JULIO PASCUAL[1], MP. MARÍA JOSÉ PÉREZ-SÁEZ[1]
[1]NEFROLOGÍA. HOSPITAL DEL MAR (BARCELONA), [2]NEFROLOGÍA. HOSPITAL UNIVERSITARIO PUERTA DE HIERRO MAJADAHONDA (MADRID),[3]ANATOMÍA PATOLÓGICA. HOSPITAL DEL MAR (BARCELONA), [4]NEFROLOGÍA. INSTITUTO HOSPITAL DEL MAR DE INVESTIGACIONES MÉDICAS (BARCELONA)

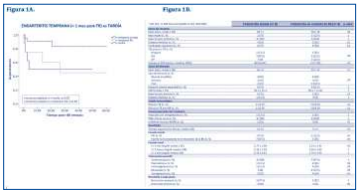
Introducción: Las lesiones de endarteritis (v>0) en biopsias (BR) de trasplante renal (TR) pueden aparecer en contexto de rechazo (celular y/o mediado por anticuerpos- v-RA), o de forma aislada (v-aislada). El significado y pronóstico de estas lesiones según el tiempo post-TR no está bien definido. Analizamos el impacto de la endarteritis temprana y su fenotipo histológico (primer mes post-TR) vs. tardía en una cohorte de TR (mediana 26.6 meses post-TR [RIQ 15.8-53]).

Métodos: Estudio retrospectivo, 38 TR (2011-2018) con lesiones de endarteritis en BR por indicación o seguimiento entre I/2011-I/2019. Los diagnósticos histológicos corresponden a la clasificación Banff-2017. La mediana de seguimiento desde la BR fue 19.9 meses [RIQ 7.4-44.4].

Resultados: De los pacientes, 42.1% (n=16) presentaron v-temprana y tuvieron peor supervivencia del TR a 3 años comparados con v-tardía (84 vs 44%, p=0.02). Los TR con v-temprana tenían más anticuerpos donante-específicos (DSA) pre-TR (37.5 vs 9.1%, p=0.05), especialmente hemaglutininas en TR ABO-incompatibles, y más injertos eran no-funcionantes en el momento de la BR (56.2 vs 0%, p<0.001). Dentro de los TR con v-temprana, el 50% presentaba v-aislada y 50% v-RA; el fenotipo v-aislada se asoció a más isquemia fría (IF) (12±6 vs 5±5h, p=0.03), más función retrasada del injerto (FRI) (75 vs 12.5%, p=0.04) y más injertos no-funcionantes en el momento de la biopsia (INF-BR) (87.5 vs 25%, p=0.04). El grupo v-RA mostraba más DSA pre- y post-TR (62.5 vs 12.6%, p=0.12). La supervivencia a 3 años en ambas formas de presentación fue similar (45 vs 50%, p=0.89).

Conclusiones: La endarteritis temprana post-TR condiciona mala supervivencia del injerto. Observamos dos fenotipos diferenciados de v- temprana: 1) v-aislada asociada a factores sugestivos de isquemia (FRI, INF-BR e IF) y 2) v-RA asociada DSA (particularmente incompatibilidad- ABO); ambos con pronóstico similar.

Figura 1.



534 EVOLUCION DE LAS CAUSAS DE MUERTE CON INJERTO FUNCIONANTE EN EL TRASPLANTADO: ANÁLISIS DE 35 AÑOS DE TRASPLANTE RENAL

A. MAZUECOS BLANCA[1], F. VILLANEGO FERNÁNDEZ[2], V. LÓPEZ JIMÉNEZ[2], G. BERNAL BLANCO[3], MC. GRACIA GUINDO[4], A. RODRIGUEZ BENOT[5], P. CASTRO DE LA NUEZ[6]
[1]NEFROLOGÍA. HOSPITAL UNIVERSITARIO PUERTA DEL MAR (CÁDIZ),[2]NEFROLOGÍA. HOSPITAL REGIONAL UNIVERSITARIO DE MÁLAGA (MÁLAGA), [3]NEFROLOGÍA. HOSPITAL UNIVERSITARIO VIRGEN DEL ROCÍO (SEVILLA), [4]NEFROLOGÍA. HOSPITAL UNIVERSITARIO VIRGEN DE LAS NIEVES (GRANADA),[5]NEFROLOGÍA. HOSPITAL UNIVERSITARIO REINA SOFÍA (CÓRDOBA),[6]NEFROLOGÍA. COORDINACIÓN AUTONÓMICA DE TRASPLANTES DE ANDALUCÍA (SEVILLA)

Introducción: La muerte con injerto funcionante (MCIF) es la causa más frecuente de pérdida del trasplante renal (TR). La información sobre las causas de MCIF en los últimos años es limitada, a pesar de los cambios en las características demográficas y en los tratamientos inmunosupresores.

Material y métodos: Estudio observacional de cohortes retrospectivo de los pacientes TR en Andalucía desde 1984-2018. Analizamos causas de MCIF, evolución en el tiempo y según periodo post-TR [muerte precoz (MP): primer año post-TR; muerte tardía (MT): tras primer año post-TR]. Variables analizadas: demográficas, etiología ERC, diabetes, tiempo y tipo de tratamiento renal sustitutivo (TRS), tipo de donante, re- TR, tipo de neoplasia.

Resultados: Se realizaron 10902 TR. Excluidos receptores pediátricos y trasplantes combinados, en los restantes 9905 TR se produjeron 1861 MCIF. La causa más frecuente fue cardiovascular (25.1%) seguida de infecciones (21.5%) y neoplasias (19.9%). Las muertes cardiovasculares fueron más frecuentes en diabéticos (p=0.026), y el tiempo en TRS era superior (p=0.002). La muerte por infecciones fueron más frecuente en mujeres y por neoplasias en hombres (p=0.001).

Comparamos MCIF por etapas excluyendo las ocurridas más allá de los 10 años (n=1290; Etapa-1984/1995, 287; Etapa-1996/2007, 555; Etapa-2008/2018, 448). El porcentaje de reTR (p=0.027), la diabetes (p=0.000) y la edad del receptor (p=0.000) fueron incrementándose en las sucesivas etapas. En las MP, no observamos cambios siendo siempre las infecciones la causa más frecuente (1984/1995, 36.6%; 1996/2007, 41.9%; 2008/2018, 43.7%; p=0.768). En las MT se produce un descenso en muerte cardiovascular (1984/1995, 35.2%; 1996/2007, 22.6%; 2008/2018, 23.9%) e incremento significativo del cáncer (1984/1995, 21.8%; 1996/2007, 29%; 2008/2018, 26.8%) (p=0.000) siendo este la causa más frecuente de MT en las etapas recientes. En el análisis multivariante para MT, la muerte cardiovascular se relacionó con edad del receptor, re-TR, diabetes y etapa; sexo varón y etapas recientes, pero no edad del receptor, se asociaron significativamente con MCIF por neoplasia.

Analizamos los tipos de neoplasias causantes de MCIF. En el primer año posTR la más frecuente fue la enfermedad linfoproliferativa posTR, y tras el primer año el cáncer de pulmón (25.4%), sin diferencias significativas al analizar por etapas.

Conclusiones: A pesar de la mayor comorbilidad del receptor las muertes cardiovasculares han descendido significativamente. Las neoplasias son la principal causa de muerte tardía en los últimos años, sin que observemos asociación con la edad del receptor. El cáncer de pulmón es la neoplasia más frecuente causante de MCIF en nuestros trasplantados.

535 RECHAZO MEDIADO POR ANTICUERPOS: EL FENOTIPO CLÍNICO IMPORTA

C. BURBALLA[1], L. LLINÁS[1], A. BUXEDA[1], C. ARIAS-CABRALES[1], M. MIR[1], A. FAURA[1], J. PASCUAL[1], M. CRESPO[1]
[1]NEFROLOGÍA. HOSPITAL DEL MAR (BARCELONA/ESPAÑA)

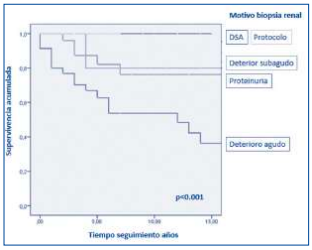
Introducción: En trasplante renal (TR), las biopsias (BR) de protocolo permiten detectar rechazo mediado por anticuerpos (ABMR) sin deterioro de función renal (FR) +/- anticuerpos donante específicos (DSA), pudiendo identificar ABMR subclínico. Existe controversia sobre considerar la proteinuria aislada como marcador clínico. No existe tratamiento eficaz del ABMR y algunos centros tratan todos, sean/no clínicos.

Material y métodos: Estudio retrospectivo de TR (1987-2018) con BR (2008-2018) compatibles con ABMR (categoría 2-Banff´2015) >1 año post-TR. Diferenciamos ABMR clínico: 1) deterioro agudo FR (aumento creatinina>15% tres semanas previas a BR) con/sin proteinuria; 2) deterioro subagudo FR (aumento creatinina>15% seis meses previos) con/sin proteinuria; y ABMR subclínico: 3) DSA o 4) BR protocolo. Consideramos otra categoría:
5) proteinuria aislada (aumento>500 mg o x2 6 meses previos a BR). Nuestro objetivo fue describir diferentes fenotipos de ABMR y evaluar su pronóstico a largo plazo.

Resultados: De 105 TR con ABMR, realizamos BR por 1) en 35 (33%), 2) 10 (9,5%), 3) 21 (20,3%), 4) 14 (13,4%) y 5) en 25 (23,8%). No hubo diferencias basales entre grupos aunque sí en el tratamiento recibido para ABMR (inmunoglobulinas+plasmaféresis+rituximab): 40% en 1, 0% en 2, 9,5% en 3, 0% en 4 y 16% en 5 (p=0.002). La supervivencia del injerto muerte-censurada fue peor en el grupo con deterioro agudo de FR seguido por el de deterioro subagudo y el grupo con proteinuria aislada. La detección de DSA en estos grupos no condicionó la supervivencia. No hubo ninguna pérdida entre los injertos biopsiados por protocolo o por detección aislada de DSA durante el seguimiento.

Conclusión: Existen diferencias pronósticas entre distintos fenotipos de ABMR, independientemente del tratamiento. La proteinuria aislada muestra peor pronóstico que el ABMR subclínico. Conocer la evolución de diferentes fenotipos de ABMR según su forma de presentación permite adecuar la estrategia terapéutica considerando el riesgo-beneficio.

Figura 1.



••• Presentación oral •• E-póster • Póster

3. Llinàs-Mallol, L; Ferrés Llach, J; Redondo-Pachón, D; Raïch-Regué, D; Pérez-Sáez, MJ; Yélamos López, J; Gimeno Beltrán, J; López-Botet, M; Pascual Santos, J; Crespo Barrio, M. La distribución de subpoblaciones linfocitarias en sangre periférica en receptores de trasplante renal se asocia con el diagnóstico histológico. Nefrología 2018;38(S1): 160. Oral presentation, XLVIII Congress SEN and IX Iberoamerican Nephrology Congress, November 16 – 19 2018, Madrid, Spain.

Trasplante Renal - Resultados y estudios epidemiológicos

594 ••• 10 AÑOS ROMPIENDO BARRERAS INMUNOLÓGICAS CON EL TRASPLANTE RENAL DE DONANTE VIVO

I. REVUELTA[1], E. DE SOUSA[1], E. PALOU[2], M. LOZANO[3], M. VALENTIN[4], J. CID[5], J. MARTORELL[2], JM. CAMPISTOL[6], F. OPPENHEIMER[1], F. DIEKMANN[1]

[1]UNIDAD DE TRASPLANTE RENAL. SERVICIO DE NEFROLOGIA Y TRASPLANTE RENAL. HOSPITAL CLÍNIC (BARCELONA/ESPAÑA),[2]SERVICIO DE INMUNOLOGIA. HOSPITAL CLÍNIC (BARCELONA/ESPAÑA), [3]SERVICIO DE AFÉRESIS. HOSPITAL CLÍNIC (BARCELONA/ESPAÑA),[4]ONT. ONT (MADRID/ESPAÑA), [5]SERVICIO DE AFERESIS. HOSPITAL CLÍNIC (BARCELONA/ESPAÑA), [6]DIRECCIÓN GENERAL. HOSPITAL CLÍNIC (BARCELONA/ESPAÑA)

Introducción: Diferentes aproximaciones de alocación y esquemas terapéuticos se han establecido ante las incompatibilidades del trasplante con controversias acerca de la mejor estrategia. Nuestro objetivo es analizar los resultados de las diferencias estrategias en nuestro centro.

Material y Método: Trasplante renal de donante vivo(TRDV, 2006- 2015) con incompatibilidad ABO(ABOi)/crossmatch positivo(CMP) tratados con DS(rituximab, recambios plasmáticos/inmunoadsorción, inmunoglobulinas) o en programa cruzado(KPD) comparados con TRDV clásico y entre ellos. Los pacientes firmaron el consentimiento informado y el estudio se aprobó por el Comité de Ética de Investigación.

Resultados: De 486 TRDV, 121 incompatibles (ABOi:70;CMP: 29;KPD:22) tuvieron más rechazo agudo(p=0.002), trasplantes previos (p=0.000) e igual pérdida de injerto(p=0.260) y paciente(p=0.336) en menor tiempo de seguimiento(47.95vs34.66m; p=0.000). Entre ABOi(70) y ABOc(416) en mismo tiempo de seguimiento no hubo diferencias de rechazo, supervivencia de paciente e injerto, aunque sí más terapia de inducción(p=0.004). Del grupo CMP, hubo más rechazo agudo mediado por anticuerpos en DS e HS(58) al año y 5 años(p<0.01) comparados con no sensibilizados (390); peor función(p=0.002) y supervivencia del injerto renal a los 5 años en DS(p=0.002) sin diferencias en supervivencia del paciente(p=0.14). 7 KPD tuvieron rechazo(31.8%) sin pérdidas de injerto ni muertes de paciente. Al comparar ABOi, CMP y KPD, más varones receptores y mujeres donantes en ABOi y CMP(p=0.003) y menos trasplantes previos en CMP y KPD(p=0.001). KPD tuvieron menor pérdida de injerto y sin diferencias en supervivencia del paciente que ABOi y CMP(0% vs17.1% vs 24,1%, p=0.056; 0%vs 5.7% vs 3.4%, p= 0.490, respectivamente) y necesitaron menos inducción(p=0.000). Más rechazo(p=0.056) y episodios de rechazo(p=0.017) en CMP que ABOi y KPD, con menor tiempo de seguimiento(p=0.015) en KPD(19.4±14.8m) que en ABOi(38.9±28.3m) y CMP(37.4±29.6m).

Conclusiones: El manejo de la incompatibilidad inmunológica con el trasplante renal de donante vivo ofrece buenos y comparables resultados, aunque se tienen que seleccionar individualmente las estrategias por paciente.

595 ••• LA DISTRIBUCIÓN DE SUBPOBLACIONES LINFOCITARIAS EN SANGRE PERIFÉRICA EN RECEPTORES DE TRASPLANTE RENAL (TR) SE ASOCIA CON EL DIAGNÓSTICO HISTOLÓGICO

L. LLINÀS-MALLOL[1], J. FERRÉS LLACH[1], D. REDONDO PACHÓN[2], D. RAÏCH-REGUÉ[1], MJ. PÉREZ-SÁEZ[2], J. YÉLAMOS LÓPEZ[3], J. GIMENO BELTRÁN[4], M. LÓPEZ-BOTET[3], J. PASCUAL SANTOS[2], M. CRESPO BARRIO[2]

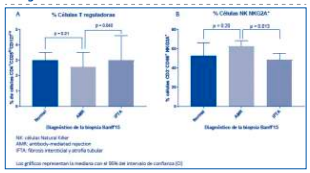
[1]NEFROLOGÍA. INSTITUTO HOSPITAL DEL MAR DE INVESTIGACIONES MÉDICAS (BARCELONA), [2]NEFROLOGÍA. HOSPITAL DEL MAR (BARCELONA), [3]INMUNOLOGÍA. HOSPITAL DEL MAR (BARCELONA), [4]ANATOMÍA PATOLÓGICA. HOSPITAL DEL MAR (BARCELONA)

Introducción: El rechazo mediado por anticuerpos (AMR) es causa frecuente de pérdida del injerto renal. Los anticuerpos anti-HLA donante- específicos (DSA) juegan un papel relevante en su aparición. La participación de las diferentes subpoblaciones linfocitarias en el desarrollo del AMR no se conoce bien. Tampoco la relación del predominio de unas subpoblaciones sobre otras y los hallazgos clínicos e histopatológicos. Diseñamos este estudio para analizar la relación entre poblaciones linfocitarias y hallazgos histológicos en biopsias post- trasplante.

Métodos: Se incluyeron pacientes TR con biopsias renales con diagnóstico definitivo de AMR (Banff´15) y se compararon con un grupo control sin hallazgos de AMR, pero con fibrosis intersticial-atrofia tubular (IFTA) y otro grupo control de biopsias normales. Determinación de anticuerpos anti-HLA (Luminex) y estudio contemporáneo de poblaciones linfocitarias en sangre periférica con citometría de flujo: subpoblaciones linfocitarias T y B (CD3, CD4, CD8, CD25, CD127, CD19, CD27, CD38, IgD) y células NK con receptores inhibidores o activadores (CD56, NKG2A, NKG2C, ILT2, KIR, CD161).

Resultados: Se evaluaron 91 pacientes: AMR (n=37), IFTA (n=41) y normales (n=13). Los grupos fueron semejantes en edad y sexo, sin embargo, entre los pacientes con AMR había mayor tasa de retrasplante (35% vs. 9.8% en IFTA y 15.4% en normales, p=0.02). La función renal en el momento de la biopsia fue similar en los tres grupos. Los pacientes con AMR mostraron menor porcentaje de linfocitos T reguladores y mayor proporción de células NK NKG2A+ que los pacientes con IFTA (2.5% vs. 3%, p=0.04; 62.7% vs. 48.3%, p=0.013, respectivamente) (Figura). No se encontraron diferencias significativas en otras poblaciones linfocitarias.

Figura 1.



Conclusión: El diagnóstico de AMR se asocia a una menor proporción de células T reguladoras y una mayor proporción de células NK NKG2A+ circulantes. Estas células pueden ser biomarcadores adecuados para una monitorización completa post-trasplante.

596 ••• LOS MIRNAS URINARIOS COMO POTENCIALES BIOMARCADORES EN EL TRASPLANTE RENAL

I. BANCU[1], I. LOZANO[2], L. CAÑAS[1], J. JUEGA[1], M. PEREZ[1], R. LAUZURICA[1], F. BORRAS[1]

[1]NEFROLOGIA. HOSPITAL UNIVERSITARI GERMANS TRIAS I PUJOL (BADALONA, ESPAÑA),[2]REMAR IVECAT GROUP. INSTITUTO DE INVESTIGACION GERMANS TRIAS I PUJOL, CAN RUTI CAMPUS (BADALONA, ESPAÑA), [1]REMAR IVECAT GROUP. INSTITUTO DE INVESTIGACION GERMANS TRIAS I PUJOL, CAN RUTI CAMPUS (BADALONA, ESPAÑA)

La orina es ideal para el estudio de posibles biomarcadores, su colección es fácil, no invasiva y repetitible.

Nuestro objetivo fue analizar el contenido de miRNA de las vesiculas extracelulares urinarias de los pacientes trasplantados y definir patrones diferenciales de expresión de miRNA específicos para las diferentes patologías del riñón trasplantado.

Material y metódos: Se seleccionaron pacientes trasplantados con una biopsia renal realizada por indicación clínica. Las biopsias se evaluaron según la clasificación de Banff. Se seleccionaron las que presentaban rechazo agudo celular (RAC), fibrosis intersticial-atrofia tubular (FIAT), toxicidad por anticalcineurínicos (TAC). Los pacientes trasplantados con función renal normal (CKD-EPI>90 ml/min/1,72m2), sin proteinuria se definieron como grupo control. Se colectó la primera orina de la mañana. Después de centrifugación a baja velocidad para eliminar restos celulares se obtuvieron las microvesiculas de orina mediante la cromatografía de exclusión por tamaño. EL RNA total se extrajo utilizando el kit mirCURY y se realizó secuenciación. Taqman qPCR se realizaron utilizando Taqman Advance miRNA para los hsa-miR-1-10. Los valores de Ct de los triplicados se usaron para los cálculos de delta Ct. El análisis de componentes principales, el análisis de la característica operativa del receptor y la prueba de Mann-Whitney se realizaron con GraphPrim v5 y SPSS v15; p<0,05 se establecieron como significativos.

Resultados: Se procesaron un total de 14 muestras patológicas (3 FIAT, 6 RAC, 5 TAC) y 8 muestras control. Se indentificaron un total de 1500 miRNAs. Tras la normalización y el análisis de componentes principales 12 miRNAs se seleccionaron para validarse en una nueva cohorte. Se seleccionaron un nuevo grupo de 24 muestras de pacientes (9 FIAT, 9 RAC, 6 TAC) y 8 muetsras control, se aislaron las microvesiculas y se extrajo el ARN.

Encontramos que 8 miRNAs tenían diferentes patrones de expresión en la orina de pacientes con cualquier tipo de patología del injerto renal en comparación con el grupo control.

Conclusiones: El perfil de los miRNAs de la orina tiene potencial como nuevo metódo para detectar patología en el riñón trasplantado.

597 ••• TRASPLANTE RENAL (TR) CON INJERTOS PROCEDENTES DE DONANTES EN ASISTOLIA CONTROLADA (DAC) DE EDAD AVANZADA: COMPARACIÓN CON LA DONACIÓN EN MUERTE ENCEFÁLICA (DME)

A. BUXEDA[1], C. ARIAS-CABRALES[1], MJ. PÉREZ-SÁEZ[1], G. VELIS[1], D. REDONDO-PACHÓN[1], A. ZAPATERO[2], ML. MIR[1], M. CRESPO[1], J. PASCUAL[1]

[1]NEFROLOGIA. HOSPITAL DEL MAR (BARCELONA), [2]MEDICINA INTENSIVA. HOSPITAL DEL MAR (BARCELONA)

Introducción: La DAC ha crecido de manera exponencial en España en los últimos años. No se dispone de evidencia suficiente en resultados de TR con DAC>65años. Analizamos los resultados de TR de nuestra cohorte DAC>65años y los comparamos con los obtenidos de DAC<65años y DME)>65años.

Métodos: Estudio retrospectivo observacional de 85 TR-DAC y 101 TR-ME>65años desde enero-2013 hasta diciembre-2017, con una mediana de seguimiento de 9 meses [RIQ 4.6-29.6meses] para DAC y 20meses [7.7-38.28] para DME>65años.

Resultados: Los DAC>65años representan el 53% de toda nuestra cohorte TR-DAC y en un 64.4% de los casos los injertos se trasplantaron en receptores >65años. Los receptores de DAC>65años presentaron más función retrasada del injerto (FRI) comparado con DAC<65años (43.2% vs 17.9%,p=0.013), pero no observamos diferencias respecto a DME>65años (38.3%,p=0.585). La función renal al mes fue peor respecto a ambos grupos (creatinina 2.71, 1.89 y 2.11mg/dl, respectivamente, p=0.013 y 0.05); sin embargo, la función renal a los 6 meses fue similar (creatinina 1.92, 1.53 y 1.93mg/dl, p=0.055 y 0.934). No encontramos otras diferencias entre las cohortes en cuanto a no función renal primaria o pérdidas de origen vascular. Tabla 1A. En el análisis multivariante, los determinantes de FRI en DAC fueron la edad del donante (OR 1.07-por año-, IC95% [1.02-1.13]) y el uso de timoglobulina (OR 6.12, IC95% [1.46-25.65]). Tabla 1B.

Conclusiones: Los receptores de DAC>65años tienen más FRI comparado con los receptores de DAC<65años, pero similar a los de DME>65años. La función renal a los 6m es similar entre los 3 grupos. Los determinantes de FRI entre DAC fueron la edad del donante y el uso de timoglobulina.

4. Llinàs-Mallol, L; Redondo-Pachón, D; Raïch-Regué, D; Pérez-Sáez, MJ; Yélamos López, J; Mir Fontana, M; Faura Vendrell, A; López-Botet, M; Pascual Santos, J; Crespo Barrio, M. La distribución de linfocitos B en sangre periférica tras la retirada de esteroides en receptores de trasplante renal (TR) es similar a la de individuos sanos. Nefrología 2018;38(S1): 156. Poster presentation, XLVIII Congress SEN and IX Iberoamerican Nephrology Congress, November 16 – 19 2018, Madrid, Spain.

Resúmenes

Trasplante Renal - Inmunosupresión y ensayos clínicos

580 LA INFLAMACIÓN SUBCLÍNICA EN BIOPSIAS DE PROTOCOLO SE ASOCIA CON LAS CARACTERÍSTICAS CLÍNICAS E INMUNOLÓGICAS Y LA PAUTA DE TRATAMIENTO INMUNOSUPRESOR

I. TORRES RODRIGUEZ[1], B. CHAMOUN HUACÓN[1], J. SELLARÉS ROIG[1], M. PERELLÓ CARRASCOSA[1], M. SALCEDO ALLENDE[2], D. SERÓN MICAS[1], F. MORESO MATEOS[1]

[1]NEFROLOGÍA. HOSPITAL UNIVERSITARIO VALL D' HEBRÓN (ESPAÑA),[2]ANATOMÍA PATOLÓGICA. HOSPITAL UNIVERSITARIO VALL D' HEBRÓN (ESPAÑA)

Objetivos / Introducción: Introducción. La presencia de inflamación subclínica en biopsias de protocolo del aloinjerto renal con función estable se ha relacioando con progresión de la fibrosis intersticial / atrofia tubular y con la aparición de anticuerpos anti-HLA donante-específicos (DSA). Objetivo. Analizar la relación entre inflamación subclínica en aloinjertos renales estables y las variables clínicas.

Material y métodos: Se analizan biopsias de protocolo realizadas en injertos con función estable (creatinina sérica < 2 mg/dL y proteinuria < 0.8 g/g) a los 3-6 (precoces) y a los 12-18 meses (tardías) post-trasplante. Se consideran injertos de bajo riesgo inmunológico (ausencia de DSA y PRAc < 25%) tratados con tacrolimus-micofenolato durnate todo el seguimiento. Las biopsias se han valorado utilizando los criterios de Banff 2015. Se define inflamación subclínica como i-score = ó >1. La exposición a tacrolimus se evalúa como el área bajo la curva de los niveles valle y el tiempo de exposición (AUC_TAC).

Resultados: Se ha observado inflamación subclínica en el 26% de las biopsias precoces (34 de 131) y en el 20% de las tardías (15 de 76). Las variables significativas en el análisis univariante para las biopsias precoces y tardías se muestra en la tabla 1. En el análisis multivariante la inflamación subclínica se asocia con el AUC_TAC_3 meses-biopsia (OR: 0.64, p=0.005), la incompatilidad HLA-DR (OR=2.09, p=0.043) y la retirada de corticoides (OR:2.1, p=0.004) en las biopsias precoes y con la edad del donante (OR=1.07, p=0.012) y el AUC_TAC_4 meses-biopsia (OR=0.57, p=0.040) en las biopsias tardías.

Conclusiones: En biopsias de protocolo realizadas en aloinjertos renales de bajo riesgo inmumológico las características clínicas (edad de donante y receptor) e inmunológicas (incompatibilidad DR) así como la exposición a tacrolimus se asocian con la presencia de inflamción subclínica

Tabla 1.

	i-score=0	i-score ≥1	p-valor
Biopsias precoces	n=97	n=34	
Edad receptor	54±12	59±14	0.023
Incompatib. HLA-DR	1.04±0.63	1.26±0.65	0.083
Tiempo biopsia	4.6±1.5	4.1±1.1	0.091
Retirada corticoides	2	4	0.039
AUC_TAC_2-3 m	10.4±2.2	9.7±1.9	0.077
AUC_TAC_3 m-Bx	10.0±2.1	8.6±1.8	0.001
CV_TAC (%)	31.1±12.6	37.6±14.9	0.016
Biopsias tardías	n=61	n=15	
Edad donante	51±16	63±10	0.005
Edad receptor	51±13	59±12	0.041
Incompatib. HLA-DR	1.03±0.67	1.20±0.56	0.083
AUC_TAC_4m-Bx	9.0±1.8	7.8±2.0	0.027

581 SUPERANDO LA BARRERA ANTI HLA: RESULTADOS DE DOS PROTOCOLOS DE TRATAMIENTO

S. ELÍAS TRIVIÑO[1], I. NIETO GAÑAN[2], S. JIMÉNEZ ÁLVARO[1], C. GALEANO ÁLVAREZ[1], A. FERNÁNDEZ RODRÍGUEZ[1], A. COLLADO ALSINA[3], JL. CATAÑER[2], M. FERNÁNDEZ LUCAS[1]

[1]NEFROLOGÍA. HOSPITAL RAMÓN Y CAJAL (MADRID/ESPAÑA), [2]INMUNOLOGÍA. HOSPITAL RAMÓN Y CAJAL (MADRID/ESPAÑA), [3]INVESTIGACIÓN. INSTITUTO RAMÓN Y CAJAL DE INVESTIGACIÓN SANITARIA (MADRID/ESPAÑA)

Introducción: Uno de los desafíos que presenta la lista de espera es el aumento de pacientes con alto número de anticuerpos anti HLA. Buscamos soluciones para poder trasplantar a estos receptores.

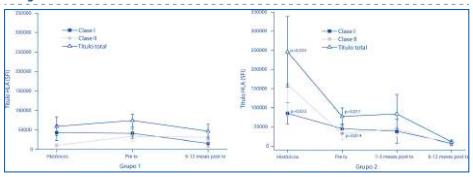
Material y método: Desde Octubre de 2014 trasplantamos pacientes con prueba cruzada virtual positiva (PCV), PRA<98% y suma de anticuerpos anti HLA menor de 80000 SFI con inducción con timoglobulina (grupo 1). Desde Julio 2016 pacientes con PRA>98% y/o ADEs mayor de 80000 SFI se trasplantan con timoglobulina, plasmaféresis e inmunoglobulina (grupo2). Analizamos resultados clínicos, analíticos y de anticuerpos en cada uno de los grupos.

Resultados: Estudiamos 44 pacientes, 32 del grupo 1 y 12 del grupo 2. Las poblaciones son equiparables salvo en el PRA máximo (p<0,001) y al momento del trasplante (p=0,013) e incompatibilidad de eplets (p=0,002), que son mayores en el grupo 2. Se observa una correlación significativa entre el número de eplets incompatibles y el título de anticuerpos (R2=0,37).

En el grupo 1 el descenso de anticuerpos anti HLA desde los históricos y pretrasplante, hasta los 6-12 meses del trasplante no presenta diferencias, y en el grupo 2 tanto la clase I como la suma total de anticuerpos presentan un descenso significativo (Fig1). Hubo 5 casos de rechazo humoral, sólo 1 de ellos en el grupo 2 que respondió al tratamiento. De los 4 del grupo 1, 3 respondieron al tratamiento y 1 no se trató, perdiendo finalmente el injerto. Hubo 8 pérdidas de injerto: 6 en el grupo 1 (1 rechazo humoral crónico); 2 en el grupo 2, ninguna por rechazo humoral.

Conclusiones: Nuestros resultados preliminares sugieren que el trasplante con anticuerpos donante específicos, junto con la desensibilización post trasplante puede ser una alternativa para los pacientes hiperinmunizados.

Figura 1.



582 LA DISTRIBUCIÓN DE LINFOCITOS B EN SANGRE PERIFÉRICA TRAS LA RETIRADA DE ESTEROIDES EN RECEPTORES DE TRASPLANTE RENAL (TR) ES SIMILAR A LA DE INDIVIDUOS SANOS

L. LLINÀS-MALLOL[1], D. REDONDO-PACHÓN[2], D. RAÏCH-REGUÉ[1], MJ. PÉREZ-SÁEZ[2], J. YÉLAMOS LÓPEZ[3], M. MIR FONTANA[2], A. FAURA VENDRELL[2], M. LÓPEZ-BOTET[3], J. PASCUAL SANTOS[2], M. CRESPO BARRIO[2]

[1]NEFROLOGÍA. INSTITUTO HOSPITAL DEL MAR DE INVESTIGACIONES MÉDICAS (BARCELONA), [2]NEFROLOGÍA. HOSPITAL DEL MAR (BARCELONA), [3]INMUNOLOGÍA. HOSPITAL DEL MAR (BARCELONA)

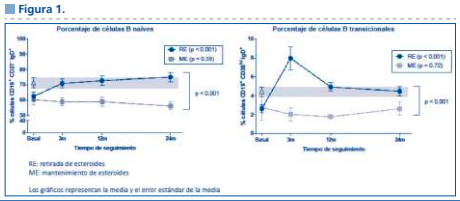
Introducción: El tratamiento inmunosupresor en TR se basa en combinaciones de inhibidores de calcineurina, fármacos antiproliferativos y esteroides, a pesar de los efectos adversos asociados y la ineficacia para prolongar la vida del injerto a muy largo plazo. Los esteroides son causa de morbilidad con potencial impacto en la supervivencia del injerto. Diversos estudios han evaluado los resultados clínicos de la retirada de esteroides (RE), pero existe poca evidencia de su influencia en biomarcadores inmunológicos.

Métodos: Hemos diseñado un estudio prospectivo para analizar el efecto de RE en TR comparado con pacientes sin cambios de inmunosupresión (mantenimiento de esteroides, ME). Evaluamos su evolución clínica, el desarrollo de anticuerpos anti-HLA con Luminex y la distribución de subpoblaciones linfocitarias mediante citometría de flujo en el momento de la inclusión y a los 3, 12 y 24 meses. Además, incluimos 20 sujetos sanos de referencia.

Resultados: La función renal y la proteinuria permanecieron estables en los 21 TR tras RE y en los 18 TR con ME durante los 24 meses de estudio. No se detectaron anticuerpos donante-específicos tras RE. Los TR-RE mostraron reducción de linfocitos T (p<0.001), incremento de linfocitos B (p=0.017) y ningún cambio en células NK respecto a los TR-ME. Además, los TR-RE mostraron una disminución de células T helper (p=0.046) y un incremento de células B naïve y transicionales (p<0.001) en comparación con TR-ME (Figura). La distribución de células B fue similar a los sujetos sanos al final del seguimiento. Los cambios se detectaron 3 meses después de RE y persistieron durante los 24 meses.

Conclusiones: La RE alteró de manera significativa la distribución de subpoblaciones linfocitarias T y B. Los pacientes después de RE mostraron un fenotipo B similar al de los sujetos sanos. Actualmente existe debate sobre si este fenotipo B podría ser marcador de tolerancia.

Figura 1.



583 TACROLIMUS DE LIBERACIÓN PROLONGADA (LCP-TAC) INHIBE LA ACTIVIDAD DE CALCINEURINA DE FORMA MÁS SOSTENIDA COMPARADO CON LA FORMULACIÓN ADMINISTRADA CADA 12 HORAS

P. FONTOVA[1], A. VIDAL[1], N. MONTERO[1], E. MELILLI[1], A. MANONELLES[1], J. TORRAS[1], O. BESTARD[1], JM. CRUZADO[1], JM. GRINYÓ[1], N. LLOBERAS[1]

[1]NEFROLOGIA. HOSPITAL UNIVERSITARIO BELLVITGE (HOSPITALET LLOBREGAT)

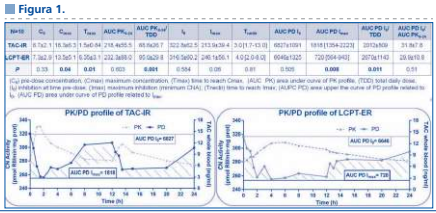
El tacrolimus (TAC) es el inhibidor de la calcineurina más usado en la prevención del rechazo en trasplante renal. La nueva formulación de liberación prolongada (LCPT-ER) (Envarsus®) presenta una exposición al TAC más estable y menos fluctuante que podría traducirse con un perfil farmacodinámico (PD) más optimizado con una inhibición más sostenida de la actividad de calcineurina (CNA) en comparación con la formulación de liberación inmediata (TAC-IR) (Prograf® o Adoport®). El objetivo de este estudio es comparar el área bajo la curva (AUC) de CNA de ambas formulaciones en pacientes trasplantados renales y su correlación con sus correspondientes exposiciones de TAC.

Metodología: Estudio prospectivo, no aleatorizado e impulsado por investigador. Diez pacientes recibieron TAC-IR se convirtieron posteriormente a LCPT-ER. Se realizaron análisis intensivos farmacocinéticos (PK) y PD (15 puntos muestreo), 24 horas antes y 3 semanas después de la conversión. Los niveles de TAC y CNA se determinaron por UPLC-MS. La CNA se analizó mediante la defosforilación de péptidos en células mononucleares de sangre periférica. Se calculó en relación al punto pre-dosis (I0) y el de máxima inhibición (Imax).

Los resultados PK mostraron que LCPT-ER presentaba una Cmax menor y AUC PK0-24 normalizada por dosis significativamente más alta que TAC-IR. La AUC PD de LCPT-ER mostró una inhibición de la CNA significativamente mayor y más mantenida que TAC-IR. Los parámetros de PK y PD se muestran en la tabla y figura adjunta.

Conclusiones: Con dosis más bajas de LCPT-ER se obtuvo una exposición similar de TAC e inhibición de CNA. Además, LCPT-ER mostró una curva de tiempo de inhibición de CNA fuertemente mantenida a lo largo de intervalos de dosis de fármaco en comparación con la formulación de TAC-IR. Solo con la formulación de LCPT-ER, los niveles de CNA no alcanzaron la dosis previa en ningún punto del estudio de PD.

Figura 1.



5. Burballa, C; Vázquez, S; Outón, S; **Llinàs-Mallol, L**; Redondo, D.; Pérez, MJ; Arias, C; Faura, A; Pascual, J; Crespo, M. El embarazo como fuente natural de sensibilización de anticuerpos anti-HLA. Nefrología 2018;38(S1): 161. Oral presentation, XLVIII Congress SEN and IX Iberoamerican Nephrology Congress, November 16 – 19 2018, Madrid, Spain.

598 ••• DISCORDANCIA EN LA DETECCIÓN DE ANTICUERPOS ANTI-HLA DONANTE-ESPECÍFICOS (ADS) POSTRASPLANTE RENAL (TR) CON TESTS DE ANTÍGENO AISLADO DE DOS CASAS COMERCIALES

C. BURBALLA TÁRREGA[1], S. OUTON GONZÁLEZ[1], D. REDONDO PACHÓN[1], MJ. PÉREZ SÁEZ[1], C. GARCIA[2], M. MIR FONTANA[1], G. VELIS ESPINOZA[1], A. FAURA VENDRELL[1], J. PACUAL SANTOS[1], M. CRESPO BARRIO[1]

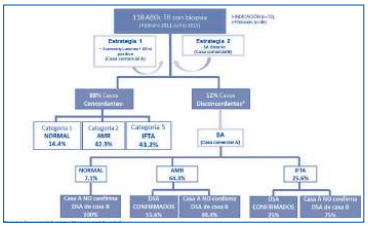
[1]NEFROLOGÍA. HOSPITAL DEL MAR (BARCELONA), [2]LABORATORI REFERENCIA CATALUNYA. LABORATORI REFERENCIA CATALUNYA (BARCELONA)

Introducción: La detección de ADS es criterio diagnóstico de rechazo mediado por anticuerpos(AMR) en receptores de TR. Se emplean dos estrategias para estudiar los ADS: kits de especificidad de antígeno aislado(SAB) siempre o sólo en casos con "screening" positivo. Dos casas comerciales ofrecen estos productos. La combinación de estrategias y casas comerciales puede influir en la capacidad de identificarlos.

Métodos: Evaluamos la sensibilidad y especificidad de la detección de ADS peri-biopsia en 118 TR (normales [n=16], AMR [n=53], fibrosis intersticial-atrofia tubular(IFTA) [n=49]), comparando 2 estrategias, cada una con una casa comercial: 1) screening y SAB si era positivo; casa A, y 2) SAB directo; casa B. En 14 casos (12%) se obtiene "screening" negativo - con la estrategia 1 y ADS positivos con la estrategia 2, casa B. Realizamos SAB en estos casos discordantes con la casa A y comparamos el resultado de SAB con ambas casas.

Resultados: En los casos discordantes, encontramos que el estudio SAB con la casa A confirmaba la presencia de DSA en 43% de los casos (36% AMR y 7% IFTA) y no lo confirmaba en 57% (7% normales, 28,4% AMR y 21,4% IFTA) (figura). En los casos con AMR, el estudio de SAB directo con la casa A confirmó la presencia de ADS en el 55.5% de los casos y no la confirmó en 44.5%. En el 50% casos confirmados, el ADS era el mismo, en el 33.4% era de la misma clase pero distinta especificidad y en 16.6% de distinta clase. Los casos confirmados y no confirmados eran semejantes en características basales, función renal y supervivencia del injerto.

Figura.



Conclusiones: Descartar presencia de ADS con tests de screening postrasplante es insuficiente. El estudio de anticuerpos anti-HLA con estudio directo de antígeno aislado con dos casas comerciales también muestra discordancia hasta en la mitad de los casos.

599 ••• EL EMBARAZO COMO FUENTE NATURAL DE SENSIBILIZACIÓN DE ANTICUERPOS ANTI-HLA

C. BURBALLA TÁRREGA[1], S. VAZQUEZ GONZÁLEZ[1], S. OUTÓN GONZÁLEZ[1], L. LLINÀS MALLOL[2], D. REDONDO PACHÓN[1], MJ. PÉREZ SÁEZ[1], C. ARIAS CABRALES[1], A. FAURA VENDRELL[1], J. PACUAL SANTOS[1], M. CRESPO BARRIO[1]

[1]NEFROLOGÍA. HOSPITAL DEL MAR (BARCELONA), [2]NEFROLOGÍA. IMIM.HOSPITAL DEL MAR (BARCELONA)

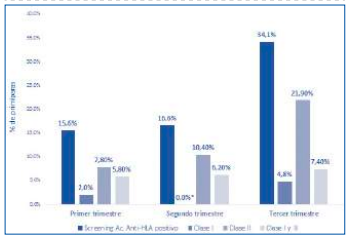
Introducción: El acceso al trasplante renal y de otros órganos sólidos se ve condicionado por la presencia de anticuerpos donante específicos. El embarazo puede inducir la producción de anticuerpos contra los antígenos leucocitarios humanos (ac.anti-HLA). Varios estudios han evaluado la aparición de anticuerpos durante el embarazo, la mayoría con las técnicas tradicionales de citotoxicidad mediada por complemento (CDC) y pocos mediante ensayos de fase sólida. La dinámica de aparición de ac.anti-HLA durante la gestación mediante las nuevas técnicas no está bien descrita.

Población y métodos: Entre 2012-2017, en el Hospital del Mar, Barcelona, se recogieron prospectivamente muestras sanguíneas de embarazadas durante el primer (1T), segundo (2T) y tercer trimestre (3T) de gestación. Se evaluó la presencia de ac.anti-HLA mediante tests de cribaje por Luminex. Quisimos evaluar la incidencia de sensibilización y en qué momento del embarazo se produce.

Resultados: En una cohorte de 142 embarazadas (edad media 34.2años, 38.7% primíparas) con muestras analizadas de más de 1 trimestre, 40.7% presentaron ac.anti-HLA durante el embarazo, suponiendo el 34% de las primíparas y el 44% de las multíparas. El porcentaje de multíparas con ac.anti-HLA se mantuvo alrededor de 40% durante los tres trimestres. En primíparas, con el porcentaje se dobló entre 2T y el 3T (alrededor 16% en 1T y 2T, 34% en 3T) con un incremento mayor de ac.anti-HLA clase II mayor, especialmente en el 3T (Figura 1).

Conclusiones: El embarazo supone una fuente importante de sensibilización frente a antígenos HLA, siendo mayor en mujeres con embarazos previos que en primeras gestaciones. Se produce un incremento más marcado de anticuerpos HLA clase II en el tercer trimestre del embarazo. Esta información es relevante en la evaluación de mujeres para trasplante renal.

Figura.



600 •• SUPERVIVENCIA DE INJERTO Y PACIENTE A LARGO PLAZO EN EL TRASPLANTE RENAL EN CATALUNYA

C.. CANAL[1], J.. COMAS[2], C.. FACUNDO[1], N.. SERRA[1], A.. VILA[1], N.. AYASREH[1], B.. BARDAJÍ[1], J.. BALLARÍN[1], L.. GUIRADO[1]

[1]NEFROLOGÍA. FUNDACIÓ PUIGVERT (BARCELONA), [2]REGISTRE DE MALALTS RENALS DE CATALUNYA. ORGANITZACIÓ CATALANA DE TRASPLANTAMENTS (BARCELONA)

Introducción: El trasplante renal es el tratamiento de elección de la enfermedad renal crónica terminal. Los trabajos publicados en los últimos años objetivan, en la mayoría de casos, que la supervivencia de paciente e injerto ha mejorado claramente a corto plazo, mientras que a largo plazo los resultados no son tan concluyentes.

Con la premisa de un constante aumento de edad y comorbilidades de nuestros receptores y el uso creciente de donantes con criterios expandidos, se plantea como objetivo principal comprobar que la supervivencia del paciente trasplantado renal y del injerto renal ha mejorado tanto a corto como a largo plazo, cuando se desglosan los donantes y receptores en diferentes grupos de edad. Y como objetivos secundarios: estudiar los diferentes factores que pueden influir en estas supervivencias y las causas de pérdida del injerto por períodos y por grupos de edad.

Material y Métodos: Se han considerado todos los primeros trasplantes renales de donante cadáver aislado realizados en Catalunya a pacientes mayores de 18 años entre los años 1992-2010 (n=5.010) y se ha dividido la muestra en dos períodos: 1992-2001; 2002-2010.

Resultados: Los resultados demuestran que, a pesar de tratarse de una población más añosa, el paciente trasplantado en el periodo 2002-2010 tiene mayor supervivencia que el del periodo 1992-2001.

La supervivencia del injerto aumenta en el periodo 2002-2010 y lo hace en todos los grupos de edad en el análisis univariante, pero en el modelo multivariante el periodo no influye de forma significativa pues se interpreta que es la nueva terapia inmunosupresora de inducción la que influye en la mejora de supervivencia y no el periodo en sí.

La supervivencia del injerto censurando la muerte aumenta en el periodo 2002-2010 en el análisis univariante, mejorando en todos los grupos de edad y, aunque en el grupo de edad más avanzada no es estadísticamente significativo, en éste se observa una tendencia al aumento que es especialmente importante en la supervivencia a 10 años. No obstante, en el análisis multivariante el periodo no es un factor significativo que influya en esta supervivencia.

Al analizar las causas de pérdida del injerto destaca que la principal causa en el grupo de receptores de edad avanzada es la muerte con injerto funcionante.

Conclusiones: En conclusión podemos decir que la supervivencia de injerto y paciente ha aumentado en los últimos años a pesar del aumento de edad y comorbilidades de nuestros receptores.

601 •• INMUNOADSORCIÓN COMO TRATAMIENTO DEL RECHAZO HUMORAL Y EL ACONDICIONAMIENTO DE GRUPO SANGUÍNEO INCOMPATIBLE EN EL TRASPLANTE RENAL

E. MONTAGUD[1], E. GUILLEN[1], A. MOLINA[1], M. XIPELL[1], LF. QUINTANA[1], L. RODAS[1], F. DIEKMANN[1], F. OPPENHEIMER[1], E. POCH[1], M. BLASCO[1]

[1]NEFROLOGÍA Y TRASPLANTE RENAL. HOSPITAL CLÍNIC (BARCELONA / ESPAÑA)

Introducción: El trasplante renal (TR) es la mejor opción terapéutica para los pacientes con insuficiencia renal crónica terminal. La introducción de diferentes técnicas de aféresis ha permitido realizar trasplantes con incompatibilidad de grupo sanguíneo (ABOi) de forma segura y con una supervivencia del injerto comparable a los TR del mismo grupo. También forma parte del tratamiento del rechazo mediado por anticuerpos (RMA) que puede limitar en gran medida la supervivencia del injerto.

Presentamos nuestra experiencia en la introducción de una nueva técnica extracorpórea, la inmunoadsorición no selectiva (IAD), con el objetivo de permitir la desensibilización previa a un TR ABOi y el tratamiento del RMA.

Material y métodos: Estudio retrospectivo, descriptivo, de los primeros 18 pacientes tratados en nuestro centro con IAD (2012 - 2017). En los casos de ABOi se realizaron un número variable de sesiones con el objetivo de conseguir un título de isoaglutininas pre-trasplante < 4/4 (IgG/IgM). Mientras en los casos de RMA se realizaron 5 sesiones de IAD. En ambos casos se completó el tratamiento con el uso de anticuerpo monoclonal anti-CD20 (Rituximab) y reposición con inmunoglobulina policlonal inespecífica (IGIV). El protocolo inmunosupresor se adaptó de forma individual en cada receptor.

Resultados: Durante un periodo de 4 años se han analizado un total de 126 sesiones de IAD en 18 pacientes. En el 44,4% de los casos para el tratamiento del RMA y en el 55,54% restante para TR ABOi.

La edad media de los pacientes acondicionados para TR ABOi fue de 45,3±12,7 años (85,7% hombres). Se realizaron una media de 8,3±0,49 sesiones de IAD previas al TR. Un 42,9% requirieron la realización de recambios plasmáticos adicionales. Actualmente, el 100% de los pacientes mantienen el injerto funcionante con un nivel medio de creatinina plasmática de 1,5±0,59 mg/dL y una proteinuria de 304,7±179,3 mg/24h.

Los pacientes tratados frente a la presencia de RMA presentaban una edad media de 51,2±14,9 años (72,7% hombres). En la actualidad el 81,8% de los pacientes mantienen un injerto funcionante (seguimiento medio de 11 meses). La creatinina media es de 2,2±1,3 mg/dL, con claro peor pronóstico de los pacientes con signos histológicos de cronicidad al diagnóstico.

Conclusiones: La introducción de la IAD en nuestro servicio ha permitido de forma eficaz y segura realizar TR ABOi, con un 100% de supervivencia del injerto. Además, supone una herramienta imprescindible en el manejo terapéutico concomitante del rechazo mediado por anticuerpos, especialmente en el rechazo agudo.

10.2 Funding

This thesis was supported by funding from the following grants:

1. Fondo de Investigación Sanitaria-Instituto Carlos III-FEDER (ISCIII-FEDER PI10/01370, PI13/00598, PI16/00617 and PI20/00090).

Principal Investigators: Dr. Julio Pascual Santos and Dr. Marta Crespo Barrio.

2. Red de Investigación Renal. Fondo de Investigación Sanitaria-Instituto Carlos III. Subprograma RETICS (ISCIII-REDinREN RD12/0021/0024 and RD16/0009/0013).

Principal Investigator: Dr. Julio Pascual Santos.

3. 2018 Grant for Scientific Research in Solid Organ Transplantation. Catalan Transplant Foundation, year 2018.

4. Fundació la Marató de TV3 (201822-10).

Principal Investigator: Dr. Miguel López-Botet Arbona.

10.3 Grants and awards

1. European Dialysis and Transplant Association (ERA-EDTA) Travel Grant for the 58th ERA-EDTA Congress, year 2021.

2. JovSEN (Young Nephrologists - Spanish Society of Nephrology (SEN)) prize to the Best Young Oral Communication presented in the 50th SEN Congress, year 2020.

3. Spanish Society of Transplantation (SET) Grant 2019 for Research Stays in Excellence Centres, year 2019.

Awarded for a stay in the Transplant Immunology Research Group, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands.

4. European Dialysis and Transplant Association (ERA-EDTA) Travel Grant for the 56th ERA-EDTA Congress, year 2019.

5. 2018 Grant for Scientific Research in Solid Organ Transplantation. Catalan Transplant Foundation, year 2018.