UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN
SCIENZE MEDICHE E CHIRURGICHE TRASLAZIONALI

CICLO XXXIV

NON-INVASIVE MONITORING OF CELL-MEDIATED REJECTION AND BKV-ASSOCIATED UROTHELIAL CANCER IN KIDNEY TRANSPLANT RECIPIENTS

Coordinatore:
Chiar.mo Prof. NICOLA SVERZELLATI

Tutore:
Chiar.mo Prof. UMBERTO MAGGIORE

Dottoranda: ILARIA GANDOLFINI

Anni Accademici 2018/2019 – 2020/2021

ABSTRACT

Background

Kidney transplantation is the treatment of choice for end-stage kidney disease, however in order to avoid rejection kidney transplant recipients (KTRs) are undergoing a lifelong immunosuppression increasing the risk of infection and cancer. While humoral immunity can be easily monitored through anti-HLA alloantibodies, non-invasive biomarkers to study the cellular immunity are lacking before and after transplant.

Donor specific (d-sp) ELISPot and Panel-reactive T (PRT) cell before transplantation have been developed to detect alloreactive memory T cells, however no studies ever investigated the combination of these two techniques for estimating the risk of acute T-cell mediated rejection (TCMR).

After transplantation, urinary CXCL9 can help diagnosing TCMR, however the current ELISA technique requires 24 hour for processing therefore is not feasible for clinical decision-making. Moreover, the response of CXCL9 to rejection treatment has not been investigated yet.

Finally, recent studies suggested a potential role of BKV in the oncogenesis of urothelial cancer and in the context of BKV infection, conventional urinary cytology specificity for detecting neoplastic cells is jeopardized by the presence of atypical infected urothelium.

Methods

We performed a retrospective multicentric study on 168 KTRs to assess the role of pre-transplant d-sp ELISPot and PRT assays in predicting the risk of biopsy-proven acute rejection (BPAR), de novo donor-specific antibodies (DSA), and eGFR decline over a 48-month follow-up period.

We further validated a new rapid Bio-Layer Interferometry (BLI)-based assay to measure urinary CXCL9 in <1 hour. The performance of this assay was retrospectively compared to the standard ELISA technique in 86 KTRs with various diagnoses. To assess utility in detecting adequacy of therapy we serially measured serum creatinine and urinary CXCL9 in 6 subjects after treatment for TCMR and correlated the results with histological and clinical response to rejection treatment.

Finally, we conducted a retrospective single-center study on 147 KTRs to explore the association between BKV infection and urothelial cancer (UC) and the role of specific immunohistological stains on urinary cytology for the diagnosis of UC in the context of BKV infection.

Results

d-sp ELISPOT was independently associated with TCMR (adjusted OR: 4.20 [95%CI: 1.06 to 21.73; P=0.041]). Median PRT and PRT+IL15 were independently associated with higher eGFR decline at 48month post-transplantation.

BLI accurately and reproducibly detected urinary CXCL9 in <1h for a non-invasive diagnosis of TCMR in KTRs. In samples obtained after treatment for TCMR, BLI CXCL9 measurements detected biopsy-proven intragraft infiltrates despite treatment-induced reduction in serum creatinine.

Finally, patients with diagnosis of UC were more likely to have a BKV persistent infection with increased viral replication (higher plasmatic detection)

Conclusions

Pre-transplant T-cell immune-monitoring using d-sp ELISPOT and PRT assays identifies kidney transplant candidates at high risk of TCMR and worse kidney allograft progression.

BLI-based urinary CXCL9 detection has potential as a point-of-care, noninvasive biomarker to diagnose and guide therapy for TCMR in kidney transplant recipients.

BKV infection is associated with increased risk of UC. In the context of BKV infection, special immunohistochemical stains for p16 can increase the sensitivity and specificity of urinary cytology for the diagnosis of UC.

Publications during the PhD Program

Regolisti G, Maggiore U, Sabatino A, **Gandolfini I**, Pioli S, Torino C, Aucella F, Cupisti A, Pistolesi V, Capitanini A, Caloro G, Gregorini M, Battaglia Y, Mandreoli M, Dani L, Mosconi G, Bellizzi V, Di Iorio BR, Conti P, Fiaccadori E; Gruppo di Studio "Esercizio fisico nel paziente con insufficienza renale cronica" of the Società Italiana di Nefrologia. Interaction of healthcare staff's attitude with barriers to physical activity in hemodialysis patients: A quantitative assessment. PLoS One. 2018 Apr 27;13(4):e0196313.

Piotti G, **Gandolfini I**, Palmisano A, Maggiore U. Metabolic risk profile in kidney transplant candidates and recipients. Nephrol Dial Transplant. 2019 Mar 1;34(3):388-400.

Pistolesi V, Regolisti G, Morabito S, **Gandolfini I**, Corrado S, Piotti G, Fiaccadori E. Contrast medium induced acute kidney injury: a narrative review.J Nephrol. 2018 Dec; 31(6):797-812

Sabatino A, Piotti G, Cosola C, **Gandolfini I**, Kooman JP, Fiaccadori E. Dietary protein and nutritional supplements in conventional hemodialysis. Semin Dial. 2018 Nov;31(6):583-591.

Gandolfini I, Crespo E, Baweja M, Jarque M, Donadei C, Luque S, Montero N, Allesina A, Perin L, Maggiore U, Cravedi P, Bestard O. Impact of preformed T-cell alloreactivity by means of donor-specific and panel of reactive T cells (PRT) ELISPOT in kidney transplantation. PLoS One. 2018 Jul 30;13(7):e0200696. doi: 10.1371/journal.pone.0200696. eCollection 2018.

Greco P, Regolisti G, Maggiore U, Ferioli E, Fani F, Locatelli C, Parenti E, Maccari C, **Gandolfini I**, Fiaccadori E. Sustained low-efficiency dialysis for metformin-associated lactic acidosis in patients with acute kidney injury.J Nephrol. 2019 Apr;32(2):297-306

Montero N, Farouk S, **Gandolfini I**, Crespo E, Jarque M, Meneghini M, Torija A, Maggiore U, Cravedi P, Bestard O. Pretransplant Donor-specific IFNγ ELISPOT as a Predictor of Graft Rejection: A Diagnostic Test Accuracy Meta-analysis. Transplant Direct. 2019 Apr 25;5(5):e451.

Gandolfini I, Regolisti G, Bazzocchi A, Maggiore U, Palmisano A, Piotti G, Fiaccadori E, Sabatino A. Frailty and Sarcopenia in Older Patients Receiving Kidney Transplantation. Front Nutr. 2019 Nov 12;6:169.

Gandolfini I, Delsante M, Fiaccadori E, Zaza G, Manenti L, Degli Antoni A, Peruzzi L, Riella LV, Cravedi P, Maggiore U. COVID-19 in kidney transplant recipients. Am J Transplant. 2020 Jul;20(7):1941-1943.

Delsante M, Rossi GM, **Gandolfini I**, Bagnasco SM, Rosenberg AZ. Kidney Involvement in COVID-19: Need for Better Definitions. J Am Soc Nephrol. 2020 Sep;31(9):2224-2225.

Cravedi P, Mothi SS, Azzi Y, Haverly M, Farouk SS, Pérez-Sáez MJ, Redondo-Pachón MD, Murphy B, Florman S, Cyrino LG, Grafals M, Venkataraman S, Cheng XS, Wang AX, Zaza G, Ranghino A, Furian L, Manrique J, Maggiore U, **Gandolfini I**, Agrawal N, Patel H, Akalin E, Riella LV. COVID-19 and kidney transplantation: Results from the TANGO International Transplant Consortium. Am J Transplant. 2020 Nov;20(11):3140-3148.

Gandolfini I, Zanelli P, Palmisano A, Salvetti D, Parmigiani A, Maltzman JS, Labate C, Fiaccadori E, Cravedi P, Maggiore U. Anti-HLA and anti-SARS-CoV2 Antibodies in Kidney Transplant Recipient with COVID-19. Transpl Int. 2021 Jan 22.

Palmisano A, **Gandolfini I**, Delsante M, Cantarelli C, Fiaccadori E, Cravedi P, Maggiore U. Acute Kidney Injury (AKI) before and after Kidney Transplantation: Causes, Medical Approach, and Implications for the Long-Term Outcomes. J Clin Med. 2021 Apr 2;10(7):1484

Mitra S, Jayanti A, Vart P, Coca A, Gallieni M, Øvrehus MA, Midtvedt K, Abd ElHafeez S, **Gandolfini I**, Büttner S, Franssen CFM, Hemmelder MH; ERACODA Collaborators. Clinical triage of patients on kidney replacement therapy presenting with COVID-19: an ERACODA registry analysis. Nephrol Dial Transplant. 2021 Dec 2;36(12):2308-2320.

Maggiore U, Palmisano A, Buti S, Claire Giudice G, Cattaneo D, Giuliani N, Fiaccadori E, **Gandolfini I**, Cravedi P. Chemotherapy, targeted therapy and immunotherapy: Which drugs can be safely used in the solid organ transplant recipients? Transpl Int. 2021 Dec;34(12):2442-2458.

Oniscu GC, Abramowicz D, Bolignano D, **Gandolfini I**, Hellemans R, Maggiore U, Nistor I, O'Neill S, Sever MS, Koobasi M, Nagler EV. Management of obesity in kidney transplant candidates and recipients: A clinical practice guideline by the DESCARTES Working Group of ERA. Nephrol Dial Transplant. 2021 Dec 24;37(Supplement_1):i1-i15.

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Kidney transplantation is the treatment of choice for end-stage renal disease, indeed it is associated with a lower mortality and a better quality of life compared to dialysis [1,2].

In the absence of immunosuppression the immune system of the recipient is attacking the graft and rejecting it with subsequent loss of the kidney [3].

The immunosuppression can however increase the risk of infections [4] and some neoplasms [5] with higher morbidity and mortality compared to the general population.

It is therefore crucial to find biomarkers that are able to stratify the patients according to their risk of developing rejection in order to minimize the therapy as much as possible in the low-risk group and tailored the immunosuppression

in the high-risk population [6,7].

The ideal biomarker should be cheap, quick and available at the time of the transplant to assess the appropriate therapy for every patient but also it should be easily repeatable after transplant to follow up the immunological status and avoid graft biopsies and other invasive tests [8-10].

A lot of biomarkers have been recently proposed to assess the humoral and cellular [11,12] immunological reactivity.

The assessment of circulating donor-specific anti-HLA antibodies (DSA) has led to a dramatic reduction in the incidence of acute antibody-mediated rejection (ABMR) [13,14]. Conversely, no immune assay is currently performed on a standard basis to predict the risk of T-cell mediated rejection (TCMR) that still occurs in up to 10-15% of kidney transplant patients [6].

In the second chapter of my thesis, I will focus on pre-transplant evaluation of alloreactive memory T cells by measuring the release of interferon-gamma after stimulation with donor-derived B cells or with a pool of B cells representative of the cadaveric donor population, and I will correlate these results with major clinical outcomes. These pre-transplant evaluation of cellular alloresponse could be helpful to stratify the risk for rejection and choose the best induction therapy to be administered at the time of transplantation.

The gold standard for TCMR diagnosis after kidney transplantation is the pathological evaluation of kidney biopsy. This procedure is, however, invasive, and poorly repeatable. Moreover, most of the transplant centers are performing renal biopsy only *for-cause*, that means for a rising of serum creatinine, or detection of proteinuria or DSA, when the kidney damage is already involving most of the renal parenchyma, limiting the chances of an effective

treatment[15,16]. A few experienced centers perform follow-up biopsies to assess efficacy of anti-rejection therapy, but this practice is impractical, risky and therefore not done routinely in most transplant centers[17].

Noninvasive monitoring tools capable of rapidly assessing intragraft inflammation could help early diagnosing and treating rejection, thereby potentially improve graft function and patient health.

Urinary chemokines are among candidate biomarkers for detecting kidney allograft inflammation.[18,19] CXCL9 is an interferon-gamma (IFN□□-induced, T cell chemoattractant chemokine produced by monocyte/macrophages, endothelial cells and renal parenchymal cells[20].

Results of previous studies[21-27] showed that measurements of urinary CXCL9 (uCXCL9) can differentiate TCMR from most other causes of acute post-transplant kidney dysfunction in the absence of infection with a negative predictive value of 92% [25].

In a follow-up tacrolimus withdrawal trial (CTOT-09, NCT01517984), serial ELISA uCXCL9 measurements detected TCMR up to 30 days prior to clinical presentation [27], emphasizing the potential role of this non-invasive biomarker in the early diagnosis of rejection.

One of the major obstacle for “real-time” implementation of therapeutic changes based on uCXCL9 is the 12-24 hour requisite turn-around time for ELISA tests. Herein, in the third chapter, I report an alternative, automated, “point-of-care” uCXCL9 assay that can be performed in <1 hour.

In addition to confirming that this innovative technology can diagnose TCMR noninvasively, we provide proof-of-concept that serial uCXCL9 measurements

following therapy of TCMR could be employed to guide subsequent clinical decision-making.

The fourth chapter of my thesis is dealing with another clinical dilemma in kidney transplantation: the non-invasive diagnosis of urothelial cancer in the context of BK virus (BKV) infection in kidney transplant recipients.

BKV is a polyomavirus that can often reactivate in immunosuppressed individuals and can be responsible for cytopathic alteration of the urinary tract and renal tubular epithelium in up to one third of the kidney transplant recipients[28]. This condition strongly limits the sensitivity and specificity of urinary cytology for non-invasive diagnosis of urothelial cancer in the context of a high-risk population. Moreover, recent studies suggested a role of BKV in altering cell cycle, through p16 and p53 oncogene dysregulation, and potentially inducing urothelial cancer[29-33] in human and animal models. For this reason, many transplant associations are suggesting to periodically monitor through invasive cystoscopy these patients.

In this last project I investigated the potential role of immunohistochemistry for BKV p16 oncogene on urinary cytology in the non-invasive diagnosis of urothelial cancer in the context of BKV infection.

REFERENCES

1. Wolfe RA, Ashby VB, Milford EL, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. N Engl J Med 1999;341:1725-30.

2. Port FK, Wolfe RA, Mauger EA, Berling DP, Jiang K. Comparison of survival probabilities for dialysis patients vs cadaveric renal transplant recipients. JAMA 1993;270:1339-43.

3. Lakkis FG, Lechler RI. Origin and biology of the allogeneic response. Cold Spring Harb Perspect Med 2013;3.

4. Lopez-Medrano F, Fernandez-Ruiz M, Silva JT, et al. Multinational retrospective case-control study of risk factors for the development of late invasive pulmonary aspergillosis following kidney transplantation. Clin Microbiol Infect 2017.

5. Baccarani U, Adani GL, Montanaro D, et al. De novo malignancies after kidney and liver transplantations: experience on 582 consecutive cases. Transplant Proc 2006;38:1135-7.

6. Bestard O, Cravedi P. Monitoring alloimmune response in kidney transplantation. J Nephrol 2017;30:187-200.

7. Augustine JJ, Poggio ED, Heeger PS, Hricik DE. Preferential benefit of antibody induction therapy in kidney recipients with high pretransplant frequencies of donor-reactive interferon-gamma enzyme-linked immunosorbent spots. Transplantation 2008;86:529-34.

8. Massart A, Ghisdal L, Abramowicz M, Abramowicz D. Operational tolerance in kidney transplantation and associated biomarkers. Clin Exp Immunol 2017.

9. Christians U, Klawitter J, Klawitter J. Biomarkers in Transplantation--Proteomics and Metabolomics. Ther Drug Monit 2016;38 Suppl 1:S70-4.

10. Thomas FT, Lee HM, Lower RR, Thomas JM. Immunological monitoring as a guide to the management of transplant recipients. Surg Clin North Am 1979;59:253-81.

11. Cherkassky L, Lanning M, Lalli PN, et al. Evaluation of alloreactivity in kidney transplant recipients treated with antithymocyte globulin versus IL-2 receptor blocker. Am J Transplant 2011;11:1388-96.

12. Ghobrial, II, Morris AG, Booth LJ. Clinical significance of in vitro donor-specific hyporesponsiveness in renal allograft recipients as demonstrated by the MLR. Transpl Int 1994;7:420-7.

13. Lee KW, Kim SJ, Lee DS, et al. Effect of panel-reactive antibody positivity on graft rejection before or after kidney transplantation. Transplant Proc 2004;36:2009-10.

14. Zhang R. Donor-Specific Antibodies in Kidney Transplant Recipients. Clin J Am Soc Nephrol 2017.

15. McDonald S, Russ G, Campbell S, Chadban S. Kidney transplant rejection in Australia and New Zealand: relationships between rejection and graft outcome. Am J Transplant 2007;7:1201-8.

16. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. N Engl J Med 2010;363:1451-62.

17. Nankivell BJ, Chapman JR. The significance of subclinical rejection and the value of protocol biopsies. Am J Transplant 2006;6:2006-12.

18. Anglicheau D, Naesens M, Essig M, Gwinner W, Marquet P. Establishing Biomarkers in Transplant Medicine: A Critical Review of Current Approaches. Transplantation 2016;100:2024-38.

19. Menon MC, Murphy B, Heeger PS. Moving Biomarkers toward Clinical Implementation in Kidney Transplantation. J Am Soc Nephrol 2017.

20. Lazzeri E, Romagnani P. CXCR3-binding chemokines: novel multifunctional therapeutic targets. Curr Drug Targets Immune Endocr Metabol Disord 2005;5:109-18.

21. Hu H, Aizenstein BD, Puchalski A, Burmania JA, Hamawy MM, Knechtle SJ. Elevation of CXCR3-binding chemokines in urine indicates acute renal-allograft dysfunction. Am J Transplant 2004;4:432-7.

22. Hauser IA, Spiegler S, Kiss E, et al. Prediction of acute renal allograft rejection by urinary monokine induced by IFN-gamma (MIG). J Am Soc Nephrol 2005;16:1849-58.

23. Schaub S, Nickerson P, Rush D, et al. Urinary CXCL9 and CXCL10 levels correlate with the extent of subclinical tubulitis. Am J Transplant 2009;9:1347-53.

24. Jackson JA, Kim EJ, Begley B, et al. Urinary chemokines CXCL9 and CXCL10 are noninvasive markers of renal allograft rejection and BK viral infection. Am J Transplant 2011;11:2228-34.

25. Hricik DE, Nickerson P, Formica RN, et al. Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury. Am J Transplant 2013;13:2634-44.

26. Kim SC, Page EK, Knechtle SJ. Urine proteomics in kidney transplantation. Transplant Rev (Orlando) 2014;28:15-20.

27. Hricik DE, Formica RN, Nickerson P, et al. Adverse Outcomes of Tacrolimus Withdrawal in Immune-Quiescent Kidney Transplant Recipients. J Am Soc Nephrol 2015;26:3114-22.

28. Manzano Sanchez D, Jimeno Garcia L, Manzano Sanchez D, et al. Renal Function Impairment in Kidney Transplantation: Importance of Early BK Virus Detection. Transplant Proc 2019;51:350-2.

29. Alexiev BA, Drachenberg CB, Papadimitriou JC. Polyomavirus-cystitis associated with in situ and invasive urothelial carcinoma in a heart transplant recipient: evidence suggesting sequential progression/evolution from infection to carcinoma. Transplantation 2015;99:e3-4.

30. Gaur L, Gupta A, Meena P, Shingada A, Gupta P, Rana DS. Bladder Carcinoma Associated with BK Virus in a Renal Allograft Recipient. Indian J Nephrol 2019;29:135-9.

31. Muller DC, Ramo M, Naegele K, et al. Donor-derived, metastatic urothelial cancer after kidney transplantation associated with a potentially oncogenic BK polyomavirus. J Pathol 2018;244:265-70.

32. Prom A, Jorgenson M, Alagusundaramoorthy S, Parajuli S. Persistent BK polyomavirus-DNAemia may warrant cystoscopy to rule out urologic carcinoma: A case report and review of the literature. Transpl Infect Dis 2020;22:e13316.

33. Yan L, Salama ME, Lanciault C, Matsumura L, Troxell ML. Polyomavirus large T antigen is prevalent in urothelial carcinoma post-kidney transplant. Hum Pathol 2016;48:122-31.

CHAPTER 2

Pre-transplant risk assessment of cellular immunity:

Impact of Preformed T-cell Alloreactivity by means of Donor-specific and Panel of Reactive T cells (PRT) ELISPOT in Kidney Transplantation

Ilaria Gandolfini, MD [1,2*], Elena Crespo, PhD[3*], Mukta Baweja, MD[1*], Marta Jarque, BS [3*], Chiara Donadei, PhD[1], Sergi Luque, BS[3], Núria Montero, MD[4], Anna Allesina, MD[5], Laura Perin, PhD [6,7], Umberto Maggiore, MD[2**], Paolo Cravedi, MD, PhD[1**], Oriol Bestard, MD, PhD[3,4**]

[1] Department of Medicine, Translational Transplant Research Center, Icahn School of Medicine at Mount Sinai, New York, NY.

[2] Kidney and Kidney-Pancreas Transplant Unit (Department of Nephrology), Parma University Hospital, Parma, Italy.

[3] Experimental Nephrology Laboratory, IDIBELL. Barcelona University. Barcelona, Spain.

[4]*Kidney Transplant Unit, Bellvitge University Hospital, IDIBELL, Barcelona University. Barcelona, Spain.*

[5] *Department of Internal Medicine and Therapeutics, University of Pavia, Pavia, Italy.*

[6] *GOFARR Laboratory, Saban Research Institute, Division of Urology, Children's Hospital Los Angeles, Los Angeles, California, USA.*

[7] *Department of Urology, Keck School of Medicine, University of Southern California, Los Angeles, California, USA.*

* First authors contributed equally to the article

**Senior authors contributed equally to the article

INTRODUCTION

Currently, immunosuppressive therapy in kidney transplant patients is largely chosen on the basis of center-specific protocols and is empirically guided by nonspecific clinical parameters including serum creatinine, circulating drug levels, and kidney biopsies[1-4] As a result, there are some patients receiving too little immunosuppression and others unnecessarily exposed to the toxicities of inadequately high doses of immunosuppressive drugs[5,6]. Therefore, tools to monitor alloimmune response in a non-invasive and specific manner are urgently needed to tailor immunosuppression according to the individual-patient immunological risk[7-11].

The notion that alloreactive memory T cells (Tmem) are crucial mediators of allograft rejection led to the development of the cytokine enzyme-linked immunospot (ELISPOT) assay which is able to quantify circulating alloreactive Tmem at the single cell level[12,13]. Initial studies have shown that pre-transplantation d-sp ELISPOT correlates with biopsy-proven acute rejection (BPAR) after kidney transplantation[14-19] and could be used to tailor immunosuppression[20-23]. In 2015, a large prospective-cohort study of 176 kidney transplant patients surprisingly failed to observe a relationship between positive pre-transplant d-sp ELISPOT and BPAR, but detected a relationship between and lower one-year graft function in patients not receiving thymoglobulin induction. This relationship was absent in patients induced with thymoglobulin, suggesting that thymoglobulin diminishes the risk of graft injury in patients with a positive d-sp ELISPOT before transplant[24].

A major drawback of the d-sp ELISPOT assay is that it requires donor cells and over 24 hours to be performed, making it impractical in cadaveric kidney

recipients[25]. To address this issue, a T-cell reactivity index, or panel of reactive T-cells (PRT) has been proposed utilizing the ELISPOT responses to common HLA antigens from a pool of donors reflective of general organ donor pool. Similar to the panel-reactive antibody test for identifying individuals with elevated levels of anti-HLA antibodies, the PRT may identify patients at risk for post-transplantation cellular-mediated graft injury[25]. In a small study of 30 kidney transplant patients, six of the seven (86%) patients with acute rejection were PRT-positive whereas only one had low PRT before transplantation[26]. Other small, retrospective studies reported an association between positive pre-transplant PRT and increased risk of acute rejection[27]. However, the utility of PRT in predicting graft outcomes has not yet been investigated in larger cohorts of kidney transplant patients. Additionally, a comparison of the performance of the d-sp ELISPOT assay and the PRT assay has not been evaluated yet.

Emerging data identified circulating $CD28^-$ T cells as crucial population for both allograft tolerance and rejection[28-30]. These cells do not proliferate nor produce IFN-γ in regular mixed lymphocyte reaction assays, but can be unraveled by adding IL-15 to the assay[31,32]. In vivo, IL-15 is produced by renal epithelial cells and promotes the recruitment and activation of alloreactive $CD28^-$ T cells. Therefore, quantifying these cells pre-transplant by adding IL15 to standard PRT may be important in stratifying the risk of BPAR[31].

In this large retrospective-cohort study, we performed pre-transplant d-sp ELISPOT and PRT (±IL15) in 168 consecutive kidney transplant recipients and evaluated their independent relationship with the development of BPAR and

other transplant outcomes including *de novo* DSA, graft function and graft failure.

METHODS

Patients and interventions

This retrospective-cohort study included all consecutive adult patients (≥18 years) who received a kidney transplant from living or deceased donors at Bellvitge University Hospital, Barcelona, Spain, from 2011 to 2013 who had donor and recipient blood or spleen samples available. Exclusion criteria included multiple organ transplant recipients, ABO incompatible transplants, and positive complement-dependent cytotoxicity cross-match. The study was approved by Institutional Review Board (IRB) (HUB PR228/13) of the Bellvitge University Hospital and all eligible patients provided written informed consent for study participation.

Estimated GFR (CKD-EPI)[33] and 24h-proteinuria were assessed at 3, 6, 12, 24, 36 and 48 months post-transplantation. All patients underwent graft biopsy in the case of clinical dysfunction. All biopsy samples were scored following the international Banff 2013 classification criteria[34].

Delayed graft function (DGF) was defined as the need for dialysis during the first week after transplant. Patients were followed-up until October 31st 2016 or graft failure (dialysis or death with a functioning graft).

Immunosuppressive therapy consisted of induction with thymoglobulin (total dose: 4.5 mg/kg over 5 days) or basiliximab (20 mg on days 0 and 4). Maintenance immunosuppressive treatment consisted of tacrolimus or cyclosporine, mycophenolate mofetil (MMF) and steroids. Steroid withdrawal was undertaken at 3 months after transplant based on clinical criteria in those patients with stable renal function and no previous BPAR episodes.

Donor-specific ELISPOT and PRT results were unavailable to the clinicians in charge of the patients and therefore had no influence on the choice of immunosuppression and clinical management of the transplant patients.

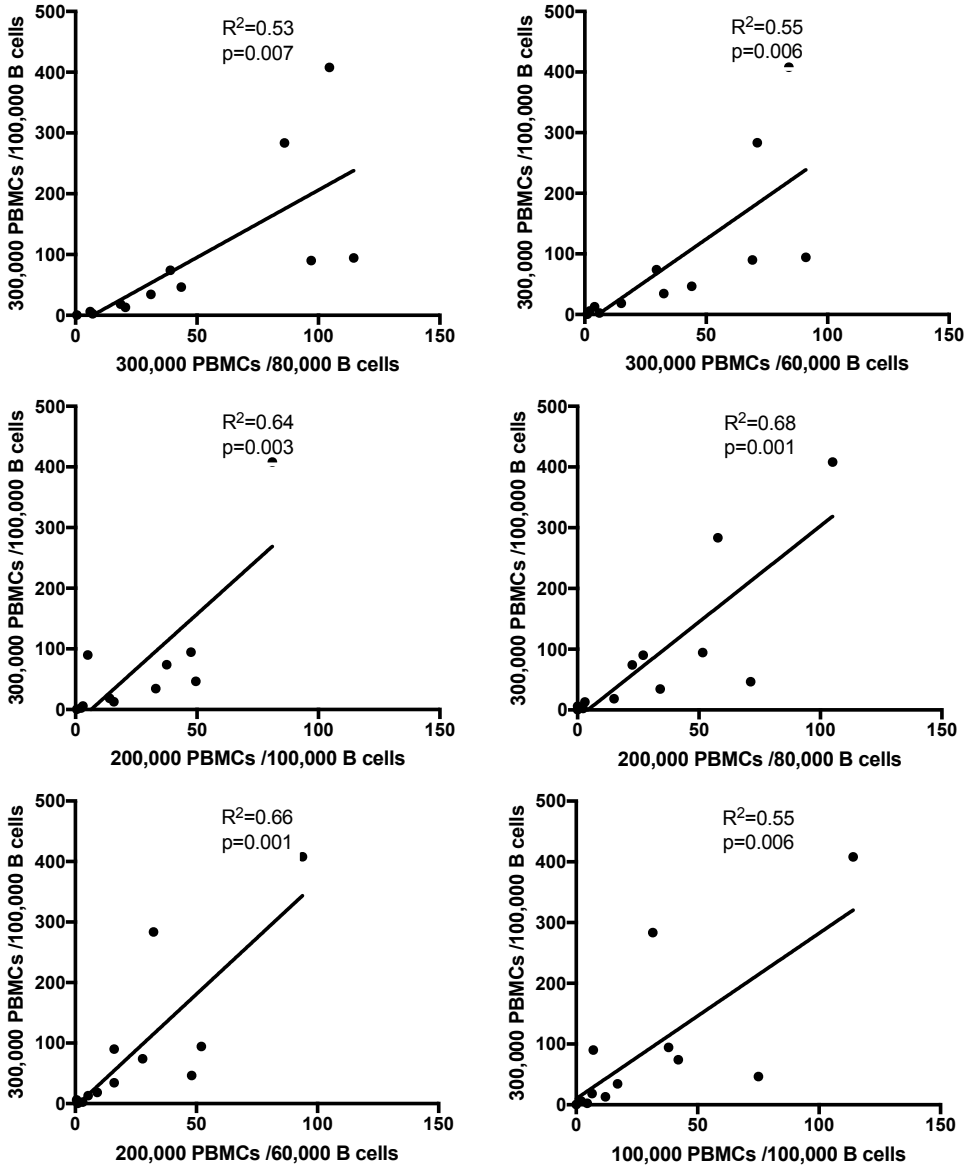
Donor-specific ELISPOT assay

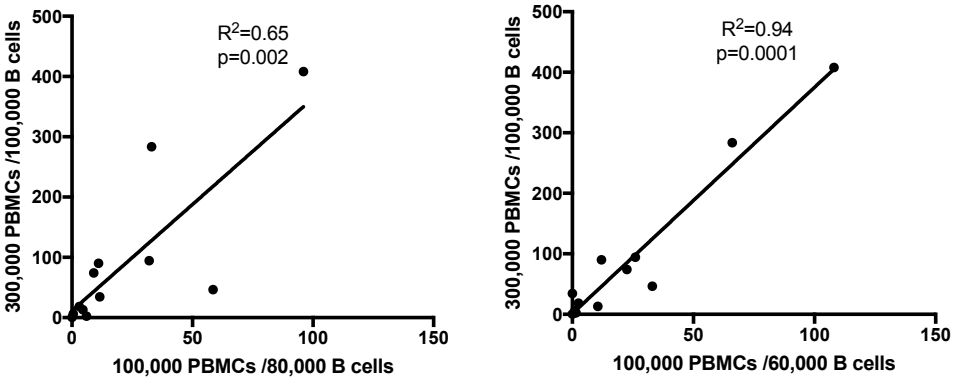
Donor-specific ELISPOT assay was performed as previously described[20,35]. In brief, recipient peripheral blood mononuclear cells (PBMCs) (harvested on the day of transplant before the administration of any immunosuppressive drug) were isolated by Ficoll gradient centrifugation. Donor cells were obtained from donor spleens or PBMCs in deceased and living donors, respectively. Donor and recipient cells were frozen in liquid nitrogen and defrosted on the day of the ELISPOT or PRT assays. Deceased-donor splenocytes were CD2-depleted and living-donor PBMCs were CD3-depleted. Recipient PBMCs ($3x10^5$ cells/well) were tested in triplicate wells with respective donor cells ($3x10^5$ cells/well) in 96-well plates. Anti-third party cells (full mismatch A, B and DR splenocytes) were also used as stimulators and evaluated in triplicate wells. PBMCs plus medium alone served as a negative control and phytohemagglutinin (PHA) stimulation was used as a positive control.

PRT assay

The lowest number of stimulator B cells and PBMCs able to provide results highly correlated (**Supplemental Figure S1**; R2: 0.94; P<0.0001) with the ones obtained with standard technique[26,27] was 100.000 responder PBMCs against 60.000 *in vitro* expanded (not EBV transformed) allogeneic B-cell stimulators. Therefore, we used these numbers of cells for all our experiments in a 384-well plate.

Figure s1. Analysis of the best choice among different combinations of PBMCs and B cells in order to reproduce the results from the standard PRT assay, which is traditionally based on a 300,000 PBMCs to 100,00 B cells ratio per well. Each plot represents the linear correlation between a PBMCs to B cells ratio combination (x-axis) selected in order to minimize the number of cell used, and the standard 300,000 PBMCs/100,000 B cells combination (*y* axis). The 100,000 PBMCs to 60,000 B cells ratio (left-lower-most panel) showed an excellent correlation with the standard 300,000 PBMCs to 100,00 B cells ratio (R2=0.94) and was therefore used for the PRT assays in the current study. Each dot represents the average of two wells.

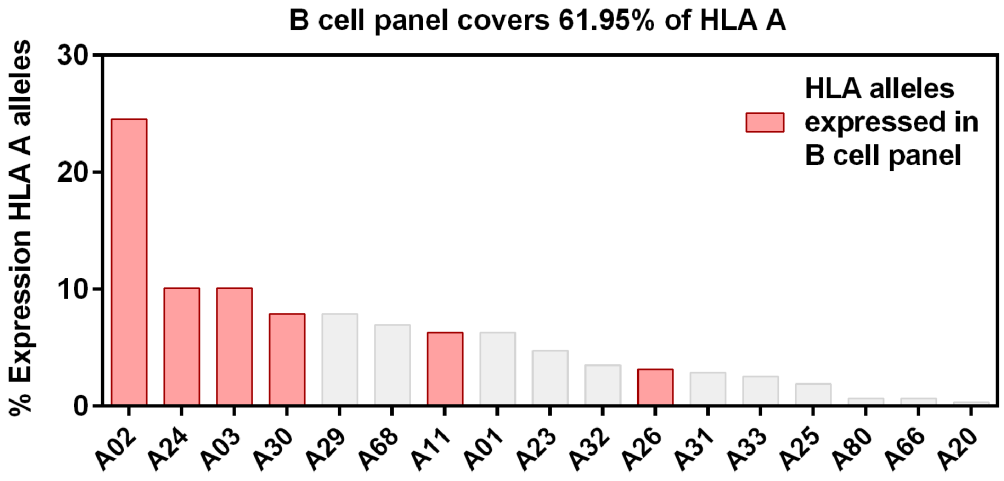


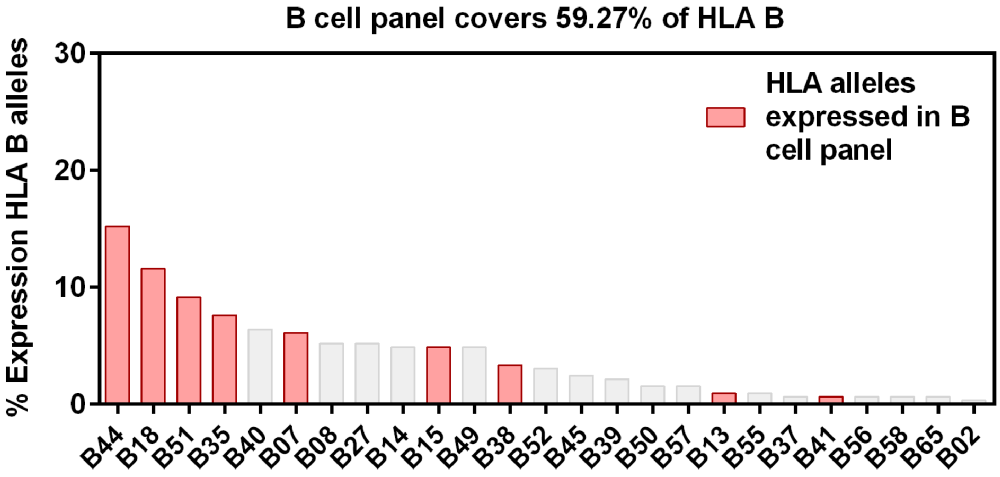


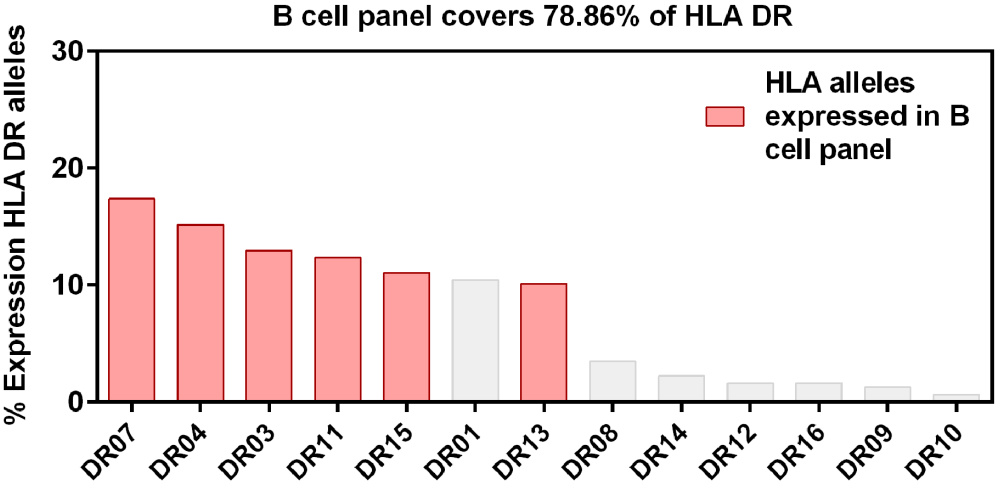
Each responder was tested in duplicate against a panel of six previously frozen B-cell stimulators (**Table s1**) with and without the addition of IL15 (1 ng/mL, Biolegend). PBMCs plus medium alone or PHA served as negative and positive controls, respectively.

B cell lines were obtained from Dr. Heeger (Icahn School of Medicine at Mount Sinai, New York, US)[26]. Briefly, B cells were isolated and expanded from a panel of from 6 distinct donor spleen cells or PBMC. B cells have been expanded in vitro by culturing them with cytokines (IL-2 and IL-4) and CD40L transfected fibroblasts. In order to confirm that B cell lines represented donor HLA repertoires in our cohort of patients, the expression of common HLA A, B and DR alleles was studied in this cohort regarding B cell lines HLA typing. As shown in **Figure S2**, B cell panel HLA alleles represented 61.95%, 59.27% and 78.86% of expression over all HLA A, HLA B and HLA DR alleles expressed in our donor cohort, respectively. Importantly, besides covering a great proportion of our donor HLA repertoire, the HLA type of the B cell lines represented the most frequent alleles of our donor cohort population.

Figure S2. Percentage of expression of HLA A, B and DR alleles and coverage to donor HLA repertoires provided by B cell lines. Expression of B cell panel HLA alleles represented 61.95%, 59.27% and 78.86% of expression over all HLA A, HLA B and HLA DR alleles expressed, respectively. Red bars represent HLA alelles that were in common with the B cell lines.







Spot quantification

The spots for d-sp ELISPOT were quantified using the AID® ELISPOT reader 4th generation (Autoimmun Diagnostika, Strassberg, Germany) and for PRT using the ImmunospotS4 Core Analyzer (CTL, Shaker Heights, OH, USA) by two independent researchers and averaged. We determined mean numbers of d-sp ELISPOTs per $3X10^5$ responder PBMCs from triplicate wells. Spots detected in control wells without stimulators were subtracted from the total number of spots.

To determine d-sp ELISPOT positivity, we used the previously published threshold of ≥25 IFN-γ spots/$3x10^5$ PBMC[14]. This choice was supported by an analysis aimed at identifying the best cut-off point (data not shown), that compared the Akaike information criterion between regression models for BPAR which differed for the selected d-sp ELISPOT positivity cutpoint.

We analyzed PRT and PRT +IL15 as continuous variables represented by the median number of spots/$3x10^5$ PBMCs against the 6 B-cell lines. We defined PRT positivity against a B cell line as ≥40 spots/$3x10^5$ B cells, and ≥50 spots/$3x10^5$ for PRT +IL15 (approximate lower bound of the upper quintile of median PRT and PRT+IL15, respectively). Patients were classified as belonging to the category of "positive" PRT when PRT ELISPOTs were positive against at least one of the six B-cell lines, or to the "negative" PRT category when PRT ELISPOTs were negative against all the six B-cell lines.

Circulating anti-HLA antibodies

Screening for circulating anti-HLA class I and II alloantibodies was done in all patients prior to transplantation and in a subset of 117 patients (69%) at least once after transplantation according to serum availability, using single-antigen

flow bead assays on a Luminex platform (Lifecodes, a division of Immucor, Stamford, CT). All beads showing a normalized mean fluorescence intensity of >1500 MFI were considered positive if (mean fluorescence intensity/[mean fluorescence intensity lowest bead]) > 5.

Statistical analyses

All analyses were performed using the statistical package Stata Statistical Software package, Release 15.0. (StataCorp, College Station, TX). A two-tailed p-value less than 0.05 was regarded as statistically significant.

Estimates were expressed as differences between positive vs. negative d-sp ELISPOT, differences between positive vs negative PRT (and PRT+IL15), or as changes per one unit standard deviation of the continuous variables of d-sp ELISPOTs, median PRT, and median PRT+IL15, which were approximately 50, 25, and 30 IFN-γ spots/3x10^5 PBMCs for d-sp ELISPOT, PRT and PRT+IL15, respectively. Linearity of the continuous variables was tested using fractional polynomials.

We estimated the association between baseline recipients' characteristics and the mean number of IFN-γ spots of d-sp ELISPOT, PRT and PRT+IL15 using gamma regression via generalized linear models with robust standard errors, due to the non-normal distribution with long right tails of the dependent variables

We used the Kaplan–Meier method to estimate the crude probability of uncensored and death-censored graft survival and Cox regression models to examine the multivariable-adjusted relationship between d-sp ELISPOT, PRT, PRT+IL15 and graft failure. Logistic regression models, with the statistical

inference based on the likelihood ratio test, were used to estimate multivariable-adjusted odds ratios of BPAR and de novo DSA associated with d-sp ELISPOT and PRT (and PRT+IL15). Because of the virtual absence of BPAR-outcomes in patients receiving thymoglobulin induction, all the analyses were repeated in the subset of patients not receiving thymoglobulin induction.

For the analysis of 48-months longitudinal changes of eGFR and of Log(proteinuria) from baseline, set at 3 months post-transplantation, we fitted repeated measures linear mixed models using restricted maximum likelihood to take into account of the presence of unbalanced data (i.e. not all patient had the eGFR measured at each time point). All the reported hypothesis tests for the fixed effects were based on a small-sample adjustment[36]. We checked normality distribution assumption by inspecting histograms and standardized normal probability plot of residuals, and homogeneity of variance assumption by inspecting residuals-vs-fitted plots. We verified model fitting by inspecting observed-vs-fitted-values plots, and observed-vs-fitted-individual-eGFR-trajectories plots.

All multivariable-adjusted regression models included, whenever applicable, the following characteristics: baseline eGFR, baseline 24h-proteinuria, recipient and donor age, living (vs deceased) donor, cold ischemia time, thymoglobulin induction (indicator variable 1 if yes, 0 if otherwise), re-transplantation, pre-transplant HLA antibodies (indicator variable 1 if cPRA >5%, 0 if otherwise), HLA A/B and HLA DR mismatch, glomerulonephritis as primary renal disease (indicator variable 1 if yes, 0 if otherwise), dialysis vintage, and prednisone withdrawal.

We used the *margins* Stata command to calculate crude and adjusted means, crude and adjusted effects and 95% confidence intervals, as predicted by the previously fitted regression model.

RESULTS

Patients

The study included 168 patients who were followed-up for a median (interquartile range) period of 45 (37-61) months. Patients were mainly Caucasian and recipients of cadaveric donors (**Table 1**). Only a minority of recipients were at increased immunological risk either because of re-transplantation (13%) or because of pre-formed HLA circulating antibodies (12%). Most patients received induction therapy with thymoglobulin (24%) or basiliximab (65%). Maintenance immunosuppression was based on calcineurin inhibitors, MMF, and steroids. Steroid withdrawal was undertaken in 57% of the subjects at 3 months after transplant. Donor-specific ELISPOT and PRT (±IL15) data were available for all the patients.

Donor-specific ELISPOT and PRT

Donor-specific ELISPOT was positive in 81 (48%) patients, while 71 (42%) and 81 (48%) patients had a positive PRT and a PRT+IL15 against at least one of the six B-cell lines, respectively. The number of spots was of the same order of magnitude across d-sp ELISPOTs, PRT, and PRT+IL15 (50° percentile: 23, 18 and 26 IFN-γ spots/$3x10^5$ PBMCs, respectively), but d-sp ELISPOT had the largest variability among the three assays as judged by its interquartile range (**Table 1**).

Median d-sp ELISPOTs were correlated, to a lower extent, with PRT and PRT+IL15 (rho=0.18, P=0.021 and rho=0.19, P=0.016, respectively, **Figure 1A-B**), while PRT and PRT+IL15 were highly correlated with each other (rho=0.96, P<0.001, **Figure 1C**). Median PRT values were significantly lower

compared to median PRT+IL15 values (P<0.001; **Figure 2**). There was no statistically significant association between patients classified as d-sp ELISPOT positive and patients classified as PRT or PRT+IL15 positive (P=0.64, and P=0.44, respectively), while there was a strong association between positive PRT and positive PRT+IL15 patients (kappa coefficient of agreement: 0.66; P<0.001). The frequencies of PRT and PRT+IL15 spots did not differ between patients with a positive or negative d-sp ELISPOT (data not shown).

Figure 1. Correlation between d-sp ELISPOT (as number of spots) and median (*i.e.*, median number of spots against the six B cell lines) PRT (**A**) and PRT+IL-15 (**B**) (rho=0.18, P=0.021 and rho=0.19, P=0.016, respectively) Correlation between median PRT and median PRT IL-15 (rho =0.96, P<0.001) (**C**).

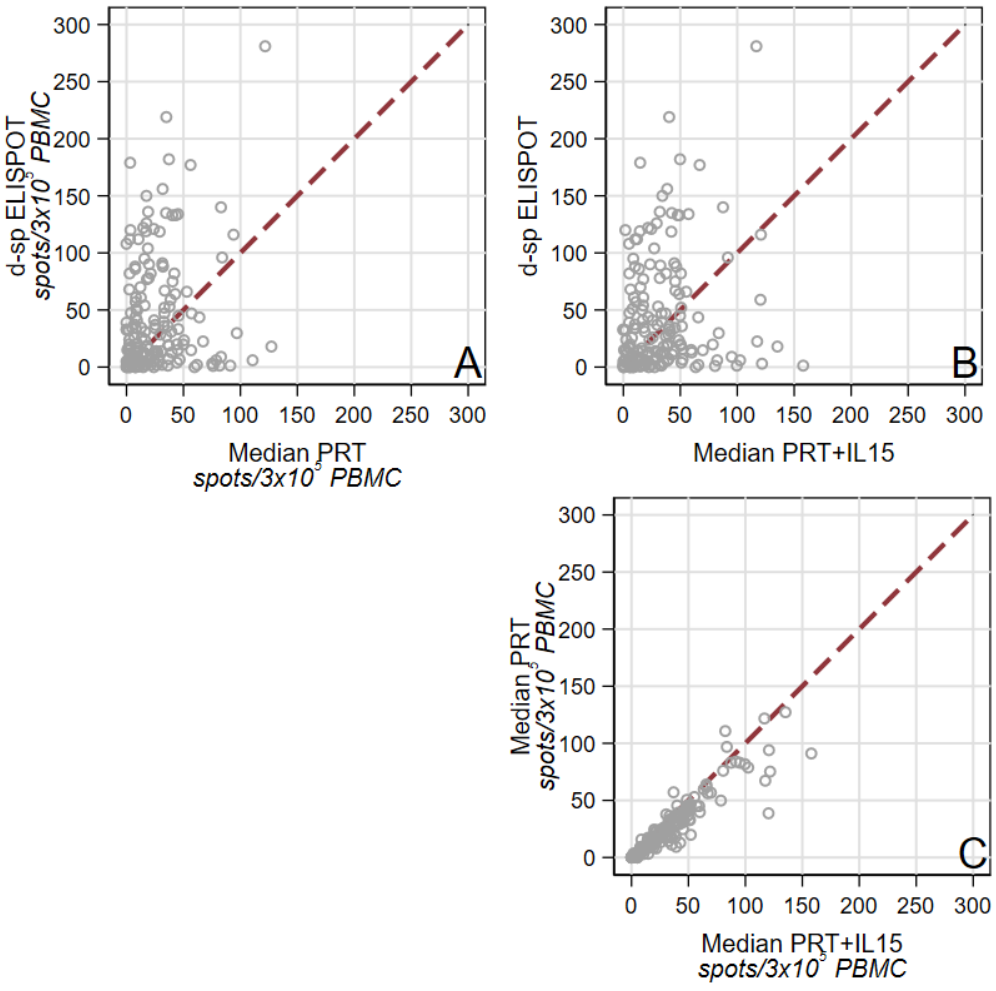


Figure 1

Figure 2. Median PRT and median PRT+IL5 (as number of spots) for each of the 168 patients. Dotted lines connect data belonging to the same patients. The number of spots was larger with median PRT+IL15 compared to median PRT (P<0.001 by Wilcoxon matched-pairs signed-ranks test).

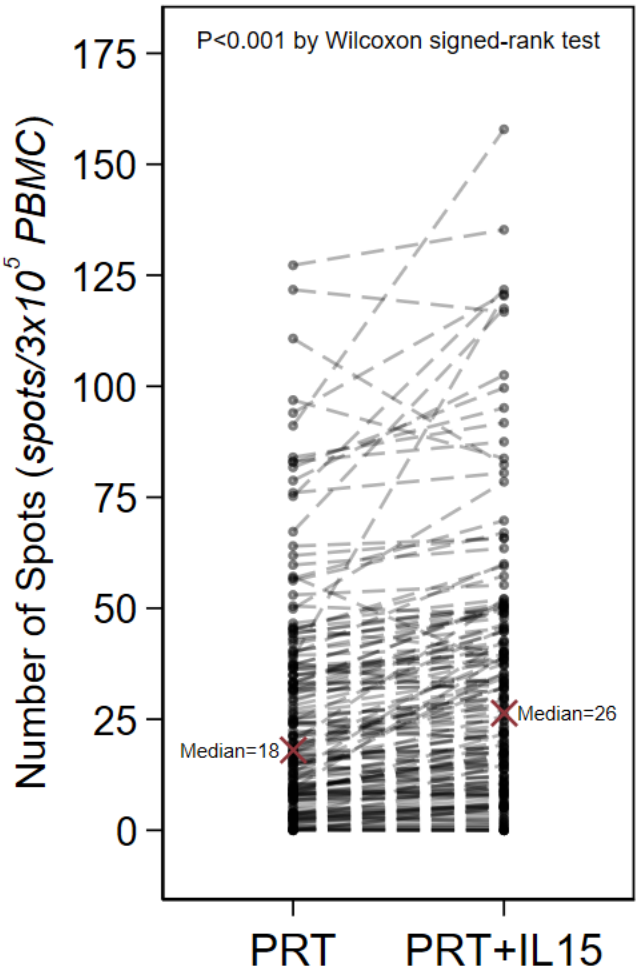


Figure 2

Clinical outcomes of the study cohort

During the follow-up period, nine patients returned to dialysis and six died with a functioning graft (48-months graft survival: 90.2% [95%CI: 84.2 to 94.1]; 48-months death censored graft survival: 94.6% [89.8 to 97.1]). Three- and 48-

month estimated eGFR were 51.1 and 45.6mL/min/1.73m^2, respectively (48-month decrease in eGFR: -5.4mL/min/1.73m^2 [95%CI: -7.9 to -3.0; P<0.001]). At 48 months after transplant, median proteinuria was 16 mg/24h and no patient had proteinuria above 0.5g/24h.

Fifty-seven (42.9%) and two (5.7%) of the deceased donor and living donor recipients respectively, developed DGF. Fifteen patients developed BPAR (8.9% [95%CI: 5.4 to 14.3]), including one case of antibody-mediated rejection. Among 117 patients with available Luminex SAB assessment after transplantation, *de novo* DSAs were detected in 11 cases (9.4%).

Impact of pre-transplant d-sp ELISPOT and PRT assays on main graft outcomes

Donor-specific ELISPOT, PRT and PRT+IL15 were not associated with the main baseline clinical and epidemiological characteristics including number of previous transplants, type of ESRD, pre-transplant sensitization, donor and recipient age and gender, ethnicity, type of transplant or the number of HLA mismatches (**Table 2**).

Unexpectedly, patients who underwent steroid withdrawal had significantly lower pre-transplant d-sp ELISPOTs compared to patients maintained on steroids (**Table 2**). There was no association between pre-transplant d-sp ELISPOT, PRT or PRT+IL15 and DGF (data not shown).

Donor-specific ELISPOT positivity was associated with a significantly higher risk of BPAR (12/81[15%] vs. 3/87 [3%] for d-sp ELISPOT positive vs. negative, P=0.013 respectively; adjusted Odds Ratio, aOR: 4.20 [95%CI: 1.06 to 21.73; P=0.041], **Table 3**). A positive pre-transplant d-sp ELISPOT predicted BPAR

with a negative and positive predictive value of 96% (95%CI: 94 to 99%), and 15% (95%CI: 9 to 20%), respectively. Conversely, there was no association between positive PRT or PRT+IL15 and BPAR (BPAR risk in positive vs negative patients: 6/71 [8%] vs 9/97 [9%], P=0.54; and 6/81 [7%] vs 9/87 [10%], P=0.59 for PRT and PRT+IL15, respectively). When expressed per one standard deviation unit increase in the number of spots, the aOR of BPAR was 1.79 (95%CI: 1.02 to 3.10: P=0.042) for d-sp ELISPOT, and 1.06 (0.50 to 1.95; P=0.87) and 0.95 (0.42 to 1.82; P=0.88) for median PRT and median PRT+IL15, respectively (**Table 3**). **Figure 3** shows the adjusted predicted risk of BPAR (i.e. proportion of patients developing BPAR) according to the number of spots of d-sp ELISPOT, median PRT, and median PRT+IL15, with superimposed the histograms of the actual data distribution of the assay test results in the study population. Increased d-sp ELISPOT spot frequencies were associated with four-time increased risk of BPAR (from zero to 150 spots BPAR risk increased from approximately 5 to 20%), while increased median PRT and median PRT+IL15 spots were not associated with increased BPAR risk.

Figure 3. Risk of BPAR (i.e. proportion of patients developing BPAR) according to the number of IFN-γ spots for d-sp ELISPOT (**A**), median PRT (**B**), and median PRT+IL15 (**C**). The solid line represents the risk of BPAR, the dashed lines represent the upper and lower 95% confidence intervals. The risk of BPAR significantly increased with the number of spots of d-sp ELISPOT (P=0.042), whereas it did not increase with the number of spots of Median PRT and Median PRT+IL15 (P=0.87 and P=0.88, respectively). The superimposed histograms report the frequency distribution of the data values in the study population. The spread of the data values was larger with d-sp ELISPOT compared to Median PRT and Median PRT+IL15. Outside the range of the actual data distribution the risk of BPAR is not reported because it would otherwise represent an inaccurate extrapolation of the BPAR risk estimates. The plotted risk of BPAR is adjusted for recipient and donor age, living (vs deceased) donor, cold ischemia time,

Thymoglobulin induction, re-transplantation, pre-transplant HLA antibodies, HLA A/B AND HLA DR mismatch, glomerulonephritis as primary renal disease, dialysis vintage, and prednisone withdrawal (*i.e.*, Model 2 in Table 3). Every covariate was set to the mean value in the study population, the indicator variate prednisone withdrawal was set to zero (*i.e.*, no withdrawal).

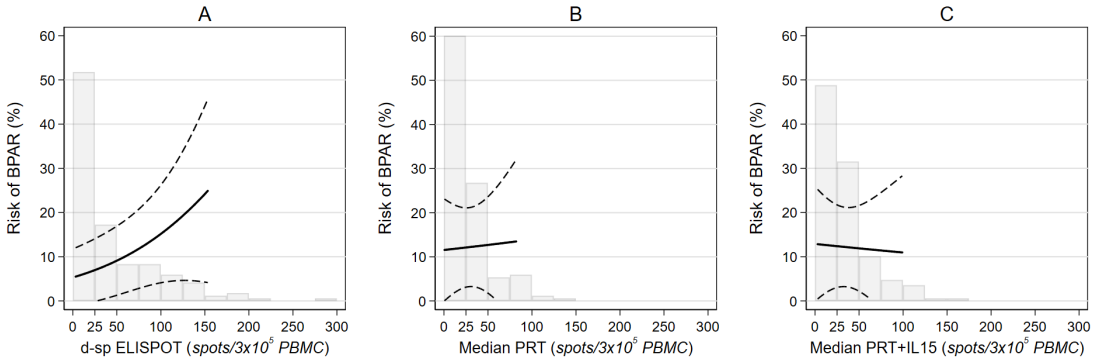


Figure 3

The association between d-sp ELISPOT and BPAR became stronger after excluding the 41 patients who received thymoglobulin induction. In patients with positive d-sp ELISPOT not induced with thymoglobulin, the incidence of BPAR was 12/66 (18%) vs 2/61 (3%) in patients with negative d-sp ELISPOT (P=0.009; aOR 7.97 [1.52 to 68.88; P=0.012], **Table 3**). When expressed per one standard deviation unit increase in the number of spots, the aOR of BPAR increased to 1.89 (1.07-3.37; P=0.029) for d-sp ELISPOT (**Table 3**), whereas the aOR remained unchanged for median PRT and for median PRT+IL15 (aOR 1.12 [0.51-2.23; P=0.75], and 1.02 [0.44-2.06; P=0.96], respectively).

There was no association between pre-transplant d-sp ELISPOT, PRT, or PRT+IL15 and *de novo* DSA (*de novo* DSA risk in positive vs negative patients: 4/65 [6%] vs 7/52 [13%], P=0.21; 4/49 [8%] vs 7/68 [10%], P=0.76; 6/56 [11%] vs 5/61 [8%], P=0.76 for donor-reactive d-sp ELISPOT, PRT, or PRT+IL15, respectively). Similarly, the analysis based on spots considered as a

continuous variable yielded non-significant findings (data not shown). Pre-transplant sensitization (i.e. cPRA>5%) was the single statistically significant risk indicator for development of *de novo* DSA (incidence of *de novo* DSA in sensitized patents was 3/8 [37%] and 8/109 [7%] in non-sensitized patients, P=0.027). Although sensitized patients had a numerically higher increased risk of BPAR compared to non-sensitized patients (2/12 [17%] vs 13/156 [8%]), the difference was not statistically significant (P=0.29).

Graft function progression and survival

After adjusting for 3-month eGFR and for baseline clinical and demographic characteristics, median PRT and median PRT+IL15, but not d-sp ELISPOT, were significantly associated with a sharper decline of 3-48months eGFR (**Figure 4**). The eGFR decline increased by -3.4mL/min/1.73m^2 (95%CI: -5.8 to -1.1; P=0.005) and by -2.8 mL/min/1.73m^2 (-5.2 to -0.3; P=0.037) per one standard deviation unit increase in the number of spots for median PRT and for median PRT+IL15, respectively (**Figure 4**). However, positive PRT and PRT+IL15 were not significantly correlated with 48-month eGFR decline (difference in 48 months eGFR decline between positive and negative patients: -3.9 mL/min/1.73m^2 [-8.5 to +0.7; P=0.096] and -2.3 [-7.0 to +2.3; P=0.32] for PRT and PRT+IL15, respectively) (**Figure 4**). There was no relationship between any of the assays studied and 24h-proteinuria at 48 months after transplant (data not shown).

Being positive or negative for both the d-sp ELISPOT and the PRT (- or + IL15) assay did not provide any prognostic advantage in predicting BPAR or eGFR change beyond every single assay used alone (data not shown). Crude and adjusted analyses showed no significant association between and any of the assays studied and uncensored or death-censored graft failure (data not shown).

Figure 4. Fitted means of 48-months eGFR decline from multiple regression models for repeated measures (see text) in patients with positive and negative d-sp ELISPOT (**A**), positive and negative PRT (**B**), positive and negative PRT+IL15 (**C**), and in hypothetical patients having number of IFN-γ spots equal to the 0°, 80°, 90°, and 95° centile of the study population for d-sp ELISPOT (**D**), for median PRT (**E**), and for median PRT+IL15 (**F**). The 48-months eGFR decline did not differ significantly when comparing positive vs negative assays, but it did differ when examining the relation with the numerical variable number-of-spots of median PRT (**E**) and of median PRT+IL15 (**F**). According to multiple regression models, the 48-months eGFR declined by -3.4mL/min/1.73m^2 (95%CI: -5.8 to -1.1; P=0.005) and by -2.8 mL/min/1.73m^2 (-5.2 to -0.3; P=0.037) per one standard deviation unit increase in the number of IFN-γ spots of median PRT and median PRT+IL15, respectively. Panels **D**, **E**, and **F** report the fitted 48-months eGFR decline of hypothetical patients having number of IFN-γ spots equal to the 0°, 80°, 90°, and 95° centile of the study population to provide a visual appraisal of the fitted relation mentioned above between the number of IFN-γ spots and eGFR decline.

Dots represent predicted means from the fitted multiple regression models for repeated measures, vertical bars represent 95% confidence intervals. Regression models were adjusted for 3-month eGFR, recipient and donor age, living (vs deceased) donor, cold ischemia time, Thymoglobulin induction, re-transplantation, pre-transplant HLA antibodies, HLA A/B AND HLA DR mismatch, glomerulonephritis as primary renal disease, dialysis vintage, and prednisone withdrawal.

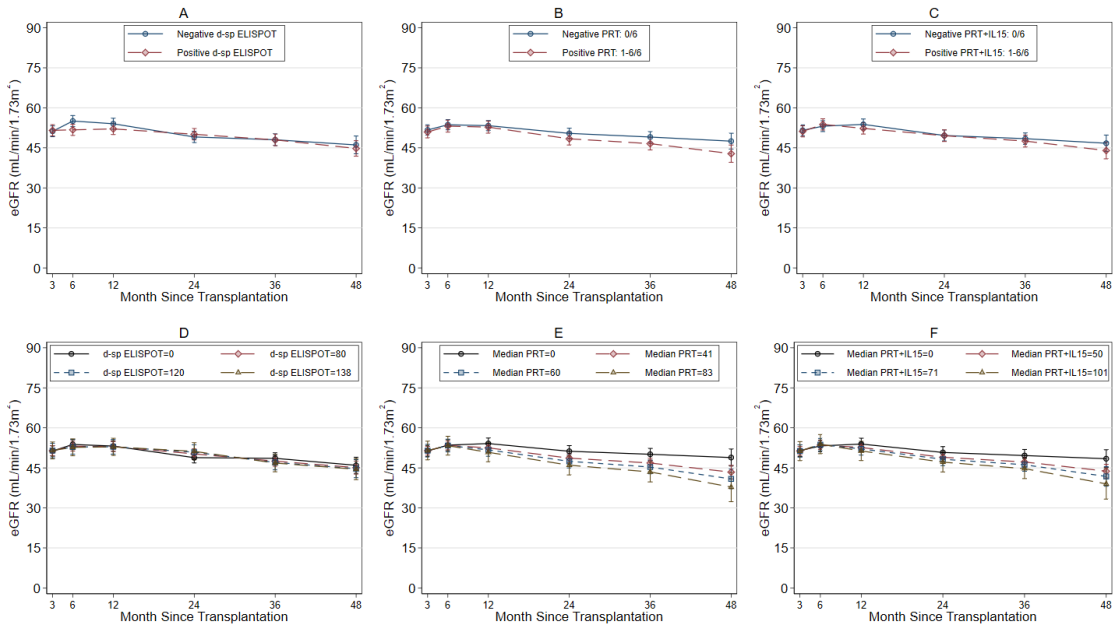

A
Negative d-sp ELISPOT
Positive d-sp ELISPOT
B
Negative PRT: 0/6
Positive PRT: 1-6/6
C
Negative PRT+IL15: 0/6
Positive PRT+IL15: 1-6/6
D
d-sp ELISPOT=0
d-sp ELISPOT=80
d-sp ELISPOT=120
d-sp ELISPOT=138
E
Median PRT=0
Median PRT=41
Median PRT=60
Median PRT=83
F
Median PRT+IL15=0
Median PRT+IL15=50
Median PRT+IL15=71
Median PRT+IL15=101
eGFR (mL/min/1.73m^2)
Month Since Transplantation

Figure 4

DISCUSSION

Our large study of deceased and living kidney transplant recipients shows that positive d-sp ELISPOT, but not PRT, identifies patients at increased risk of BPAR after transplantation, whereas patients with high pre-transplant PRT display significant graft function loss over a 4-year follow-up period. Addition of IL15 did not increase the predictive power of the PRT assay.

Previous studies testing the predictive power value of d-sp ELISPOT or PRT in kidney transplant recipients prior to transplantation largely included small cohorts, have reported a disparity of results and did not formally compare the two assays, while our data from a large series of transplanted individuals allowed to assess the potential complementary characteristics of both assays.

Recent works have shown a strong association between positive pre-transplant d-sp ELISPOT and increased risk of BPAR after transplantation, particularly TCMR, and especially, in patients not receiving T-cell depleting induction therapies[8,14,22]. Our current findings confirm and expand previous evidence in a larger cohort of patients, where the relationship between pretransplant d-sp ELISPOT and a higher risk of BPAR was mainly driven by patients not induced with Thymoglobulin.

Despite the statistically significant association between pre-transplant d-sp ELISPOT and higher risk of BPAR, only 15% of patients with positive test developed the event. On the other hand, amongst subjects with negative assay, virtually no one developed BPAR (only 3%) thus, highlighting the high negative predictive value of the assay enabling an accurate capacity to identify those

patients at low immunological risk that could eventually benefit of receiving less immunosuppression.

We did not find any association between a positive pre-transplant d-sp ELISPOT and worse allograft function progression after transplantation. Nonetheless, while some studies have showed such correlation, particularly among patients not receiving T-cell induction therapy[14,24], some other groups have not been able to find such association transplantation[8,13,15,20,37] but have conversely found a consistent relationship between worse kidney allograft function progression and the d-sp ELISPOT when assessed after transplantation suggesting a much close illustration of the on-going anti-donor T-cell alloimmune response of transplant patients.

To the best of our knowledge, the current study is the largest work testing the association between pre-transplant PRT and graft outcomes. In contrast with previous few smaller studies[26], we did not detect a relationship between PRT and BPAR. A previous study identified an inverse nonsignificant trend between pre-transplant PRT and eGFR. The large sample size of our cohort and long-term follow-up, allowed us to find a significant association between the frequency of pre-transplant PRT and PRT+IL15 and eGFR decline at 4 years. This association suggests that patients with a broad pre-transplant alloreactive T-cell repertoire, expressed by means of high PRT, may be at increased risk of the formation of crossreactive T and B cells with allo- and auto-reactive specificities that may lead to progressive but smoldering subclinical allograft damage. Unfortunately, lack of surveillance graft biopsies or measurement of circulating auto- or allo-antibodies in a significant fraction of patients prevented us from confirming this hypothesis.

Adding IL15 to the PRT did not increase the predictive power of the assay, indicating that the circulating $CD8^{+}CD28^{-}$ Tmem measured before engraftment do not play a major role in the pathogenesis of subsequent allograft injury, at least in patients on calcineurin inhibitor-based immunosuppression. This is consistent with our preliminary data suggesting that the number of these cells before transplant predicted acute rejection only in kidney transplant recipients receiving costimulation blockade-based immunosuppression with CTLA4Ig (Cravedi P, Gandolfini I, Donadei C, et al. Pre-Transplant Panel Reactive T Cells (PRT) with IL-15 as a Risk-Stratifier of Acute Rejection in Kidney Transplant Patients on Belatacept Therapy. American Transplant Congress. Chicago, May 2017. Abstract). Further studies, however, are needed to define the utility of PRT+IL15 in predicting graft outcomes in kidney transplant recipients.

We found no relationship between any of the pre-transplant assays and the development of DSA. While missing data on DSA development may have prevented us from detecting such relationship, these data are in agreement with our 2017 recent study[8] showing that only post-transplant, but not pre-transplant d-sp ELISPOT can inform on the risk of developing DSA. This data together with the absence of association with graft function progression over time highly suggest that monitoring anti-donor T-cell alloreactivity after kidney transplantation may be particularly useful to gain more insight about the alloimmune state of transplant patients after having received the initial high burden of induction immuosuppression and thus, more accurately indicate how such immune state may progressively impact on long-term allograft outcomes.

We acknowledge that our study has some limitations. Firstly, it was a single-center, retrospective study. However, main aim of this study was to perform the first large comparison between the two main assays to measure T-cell alloreactivity that could be implemented in clinical practice in the short term. In addition, the use of only six B-cell lines might have restricted the broad allogenic repertoire of the transplant recipients evaluated in this study and therefore, might have prevented a more granular differentiation between patients, as it occurs with the PRA assays. However, the findings of the current study set the basis for subsequent investigations to test the predictive power of PRT based on a more extensive panel of B-cell lines.

Due to the limited amount of PBMC available, we could not perform d-sp ELISPOT in the presence of IL15 and thus, future studies will be important in assessing are warranted to test the utility of such assay in predicting graft outcomes.

In conclusion, our findings confirm and further expand previous evidence showing that measuring alloreactive Tmem before transplantation by d-sp ELISPOT or PRT allows to predict relevant immune-mediated transplant outcomes that would not be forecasted by current standard clinical and immunological evaluations. Present findings do not support the use of IL15 in the PRT assay, but further studies are needed to define its utility, especially in patients not receiving calcineurin inhibitor immunosuppression. Our data set the basis of prospective studies formally testing the hypothesis that tailoring immunosuppression based on the joint use of d-sp ELISPOT and PRT improves patients' outcomes.

REFERENCES

1. Menon MC, Murphy B. Maintenance immunosuppression in renal transplantation. *Curr Opin Pharmacol.* 2013; 13(4): 662-671.

2. Kidney Disease: Improving Global Outcomes Transplant Work G. KDIGO clinical practice guideline for the care of kidney transplant recipients. *Am J Transplant.* 2009; 9 Suppl 3: S1-155.

3. Montero N, Perez-Saez MJ, Pascual J, et al. Immunosuppression in the elderly renal allograft recipient: a systematic review. *Transplant Rev (Orlando).* 2016; 30(3): 144-153.

4. Abramowicz D, Cochat P, Claas FH, et al. European Renal Best Practice Guideline on kidney donor and recipient evaluation and perioperative care. *Nephrol Dial Transplant.* 2015; 30(11): 1790-1797.

5. Mourad G, Garrigue V, Squifflet JP, et al. Induction versus noninduction in renal transplant recipients with tacrolimus-based immunosuppression. *Transplantation.* 2001; 72(6): 1050-1055.

6. Meier-Kriesche HU, Arndorfer JA, Kaplan B. Association of antibody induction with short- and long-term cause-specific mortality in renal transplant recipients. *J Am Soc Nephrol.* 2002; 13(3): 769-772.

7. Cravedi P, Heeger PS. Immunologic monitoring in transplantation revisited. *Curr Opin Organ Transplant.* 2012; 17(1): 26-32.

8. Crespo E, Cravedi P, Martorell J, et al. Posttransplant peripheral blood donor-specific interferon-gamma enzyme-linked immune spot assay

differentiates risk of subclinical rejection and de novo donor-specific alloantibodies in kidney transplant recipients. *Kidney Int.* 2017; 92(1): 201-213.

9. Angeletti A, Cravedi P. Looking into the Graft without a Biopsy: Biomarkers of Acute Rejection in Renal Transplantation. *Contrib Nephrol.* 2017; 190: 181-193.

10. Behnam Sani K, Sawitzki B. Immune monitoring as prerequisite for transplantation tolerance trials. *Clin Exp Immunol.* 2017; 189(2): 158-170.

11. Gandolfini I, Harris C, Abecassis M, et al. Rapid Biolayer Interferometry Measurements of Urinary CXCL9 to Detect Cellular Infiltrates Noninvasively After Kidney Transplantation. KIR. 2017; In press.

12. Heeger PS. T-cell allorecognition and transplant rejection: a summary and update. *Am J Transplant.* 2003; 3(5): 525-533.

13. Crespo E, Bestard O. Biomarkers to assess donor-reactive T-cell responses in kidney transplant patients. *Clin Biochem.* 2016; 49(4-5): 329-337.

14. Crespo E, Lucia M, Cruzado JM, et al. Pre-transplant donor-specific T-cell alloreactivity is strongly associated with early acute cellular rejection in kidney transplant recipients not receiving T-cell depleting induction therapy. *PLoS One.* 2015; 10(2): e0117618.

15. Nickel P, Presber F, Bold G, et al. Enzyme-linked immunosorbent spot assay for donor-reactive interferon-gamma-producing cells identifies T-cell presensitization and correlates with graft function at 6 and 12 months in renal-transplant recipients. *Transplantation.* 2004; 78(11): 1640-1646.

16. Kim SH, Oh EJ, Kim MJ, et al. Pretransplant donor-specific interferon-gamma ELISPOT assay predicts acute rejection episodes in renal transplant recipients. *Transplant Proc.* 2007; 39(10): 3057-3060.

17. Heeger PS, Greenspan NS, Kuhlenschmidt S, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol.* 1999; 163(4): 2267-2275.

18. Hricik DE, Rodriguez V, Riley J, et al. Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. *Am J Transplant.* 2003; 3(7): 878-884.

19. Hricik DE, Poggio ED, Woodside KJ, et al. Effects of cellular sensitization and donor age on acute rejection and graft function after deceased-donor kidney transplantation. *Transplantation.* 2013; 95(10): 1254-1258.

20. Bestard O, Cruzado JM, Lucia M, et al. Prospective assessment of antidonor cellular alloreactivity is a tool for guidance of immunosuppression in kidney transplantation. *Kidney Int.* 2013; 84(6): 1226-1236.

21. Kim Y, Park KH, Chung BH, et al. Pretransplant IFN-gamma ELISPOT assay as a potential screening test to select immunosuppression protocols for patients receiving basiliximab induction therapy. *Transl Res.* 2012; 160(3): 230-236.

22. Augustine JJ, Poggio ED, Heeger PS, Hricik DE. Preferential benefit of antibody induction therapy in kidney recipients with high pretransplant frequencies of donor-reactive interferon-gamma enzyme-linked immunosorbent spots. *Transplantation.* 2008; 86(4): 529-534.

23. Hricik DE, Formica RN, Nickerson P, et al. Adverse Outcomes of Tacrolimus Withdrawal in Immune-Quiescent Kidney Transplant Recipients. *J Am Soc Nephrol.* 2015; 26(12): 3114-3122.

24. Hricik DE, Augustine J, Nickerson P, et al. Interferon Gamma ELISPOT Testing as a Risk-Stratifying Biomarker for Kidney Transplant Injury: Results From the CTOT-01 Multicenter Study. *Am J Transplant.* 2015; 15(12): 3166-3173.

25. Sawinski D, Uribarri J, Peace D, et al. 25-OH-vitamin D deficiency and cellular alloimmunity as measured by panel of reactive T cell testing in dialysis patients. *Am J Transplant.* 2010; 10(10): 2287-2295.

26. Poggio ED, Augustine JJ, Clemente M, et al. Pretransplant cellular alloimmunity as assessed by a panel of reactive T cells assay correlates with acute renal graft rejection. *Transplantation.* 2007; 83(7): 847-852.

27. Poggio ED, Clemente M, Hricik DE, Heeger PS. Panel of reactive T cells as a measurement of primed cellular alloimmunity in kidney transplant candidates. *J Am Soc Nephrol.* 2006; 17(2): 564-572.

28. Pawlik A, Florczak M, Masiuk M, et al. The expansion of CD4+CD28- T cells in patients with chronic kidney graft rejection. *Transplant Proc.* 2003; 35(8): 2902-2904.

29. Baeten D, Louis S, Braud C, et al. Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. *J Am Soc Nephrol.* 2006; 17(1): 294-304.

30. Mou D, Espinosa J, Lo DJ, Kirk AD. CD28 negative T cells: is their loss our gain? Am J Transplant. 2014; 14(11): 2460-2466

31. Traitanon O, Gorbachev A, Bechtel JJ, et al. IL-15 induces alloreactive CD28(-) memory CD8 T cell proliferation and CTLA4-Ig resistant memory CD8 T cell activation. *Am J Transplant.* 2014;14(6): 1277-1289.

32. Dedeoglu B, Litjens NHR, Klepper M, et al. CD4+ CD28null T cells are not alloreactive unless stimulated by interleukin-15. Am J Transplant. 2017. In press.

33. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* 2009;150(9): 604-612.

34. Haas M, Sis B, Racusen LC, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant.* 2014;14(2): 272-283.

35. Bestard O, Crespo E, Stein M, et al. Cross-validation of IFN-gamma Elispot assay for measuring alloreactive memory/effector T cell responses in renal transplant recipients. *Am J Transplant.* 2013;13(7): 1880-1890.

36. Chapter “Small sample inference for fixed effects” in “Mixed. Multilevel mixed effects linear regression, Stata Reference Manual, Release 15. 2017, Stata Press Publication StataCorp LLCCollege Station, Texas

37. Näther BJ, Nickel P, Bold G, et al. Modified ELISPOT technique -highly significant inverse correlation of post-Tx donor-reactive IFNgamma-producing cell frequencies with 6 and 12 months graft function in kidney transplant recipients. Transpl Immunol. 2006; 16(3-4):232-7.

TABLES

Table 1. Demographic and clinical characteristics of the study population

Number of subjects		168
Living Donor	*%*	35 (20.8)
Donor Age	*yrs*	57.7 (16.0)
Recipients		
Age	*Yrs*	56.2 (13.4)
Males	*%*	108 (64.3)
Caucasian Race	*%*	159 (94.6)
Dialysis vintage	*months*	30.6 (6.9-44.9)
Primary Renal disease		
Glomerular	*%*	34 (20.2)
Vascular	*%*	16 (9.5)
Tubulo-interstitial	*%*	20 (11.9)
Diabetes	*%*	20 (11.9)
ADPKD	*%*	28.(16.7)
Others and unknown	*%*	50 (29.8)
CIT (deceased donor)	*hours*	19.1 (4.3)
HLA-I mm	*number*	2.7 (2-3)
HLA-II mm	*number*	1.0 (1-1)
Re-transplant	*%*	22 (13.1)
cPRA>5%	*%*	12 (7.1)
Thymoglobulin	*%*	41 (24.4)
Basiliximab	*%*	109 (64.9)
Tacrolimus	*%*	150 (89.3)
Cyclosporine	*%*	18 (10.7)
Steroid withdrawal	*%*	96 (57.1)
d-sp ELISPOT	*spots/3x10^{5}PBMC*	23.0 (6-65)
Median PRT	*spots/3x10^{5}PBMC*	18.1 (7.9-36.8)
Median IL-15 PRT	*spots/3x10^{5}PBMC*	26.4 (10.4-45.4)

Continuous variables are reported as mean (standard deviation) or median (interquartile range), categorical variables as number (percentage). PRT, Panel Reactive T-cell ELSPOT; d-sp ELISPOT, Donor-specific ELISPOT; cPRA, calculated Panel Reactive Antibody; DSA, Donor-specific antibodies; eGFR, estimated GFR (CKD-EPI formula). Median PRT and median PRT+IL15, median number of spots against the six B-cell lines.

Table 2. Association between recipients' pre-transplant characteristics and pre-transplant number of spots of each assay.

	Difference in d-sp ELSPOT number of spots/3x10^{5}PBMC (95%CI; P value)	**Difference in median PRT number of spots/3x10^{5}PBMC (95%CI; P value)**	**Difference in median PRT+IL15 number of spots/3x10^{5}PBMC (95%CI; P value)**
Age≥60yrs vs <60yrs	+2 (-13 to +17; P=0.79)	+2 (-5 to +10; P=0.55)	+2 (-7 to +11; P=0.69)
Male vs female	+2 (-15 to +18; P=0.84)	+1 (-7 to +10; P=0.75)	+1 (-9 to +11; P=0.84)
Dialysis vintage≥5yrs vs <5yrs	-11 (-34 to +13; P=0.37)	+1 (-12 to +14; P=0.92)	+2 (-13 to +18; P=0.77)
Glomerulonephritis vs other primary renal diseases	+8 (-11 to +27; P=0.41)	+5 (-6 to +16; P=0.36)	+6 (-7 to +19; P=0.37)
Living vs deceased donor	-5 (-22 to +13; P=0.60)	+2 (-7 to +11; P=0.61)	+6 (-6 to +18; P=0.34)
Re-transplantation vs first transplant	-8 (-34 to +18; P=0.55)	+1 (-14 to +16; P=0.88)	-3 (-18 to +11; P=0.64)
cPRA > 5% per <5%	-1 (-18 to +16; P=0.89)	-1 (-18 to +16; P=0.89)	-7 (-20 to +7; P=0.34)
Thymoglobulin	-14 (-29 to +1; P=0.073)	-2 (-11 to +8; P=0.75)	-2 (-12 to +8; P=0.69)
Cyclosporine vs tacrolimus	-13 (-33 to +8; P=0.22)	+3 (-11 to +16; P=0.72)	+4 (-11 to +20; P=0.56)
Steroid withdrawal	-26 (-41 to -11; P=0.001)	+2 (-6 to +10; P=0.62)	+6 (-3 to +14; P=0.21)

Difference (95 percent confidence interval and P vale) of the number of spots of each pre-transplant assay between dichotomous categories defined by pre-transplant recipients' characteristics. 95%CI, 95 percent confidence interval; cPRA, calculated Panel-Rective Antibody. Median PRT and median PRT+IL15, median number of spots against the six B-cell lines.

Table 3. Crude and adjusted Odds Ratio of BPAR associated with d-sp ELISPOT

	Crude Analysis OR (95% CI; P value)	**Adjusted Analysis, Model 1 OR (95% CI; P value)**	**Adjusted Analysis, Model 2 OR (95% CI; P value)**
Positive vs negative d-sp ELISPOT			
Whole population	4.87 (1.48-21.99; P=0.008)	3.70 (1.02-17.84; P=0.046)	4.20 (1.06-21.73; P=0.041)
Not receiving Thymoglobulin	6.56 (1.69-43.36; P=0.005)	5.32 (1.25-37.01; P=0.022)	7.87 (1.52-68.88; P=0.012)
Number of d-sp ELISPOTs			
Whole population	1.91 (1.25-2.99; P=0.004)	1.75 (1.08-2.84; P=0.024)	1.79 (1.02-3.10; P=0.042)
Not receiving Thymoglobulin	1.95 (1.24-3.17; P=0.004)	1.81 (1.09-3.06; P=0.022)	1.89 (1.07-3.37; P=0.029)

OR, Odds, Ratio; 95%CI, 95 percent Confidence Interval; BPAR, Biopsy-prove acute rejection; d-sp ELISPOT, donor-specific ELISPOT

The Odds Ratio associated to the number of d-sp ELISPOT is expressed per one standard deviation unit increase (i.e. 50 spots/$3x10^5$ PBMC)

Model 1, adjusted for recipient and donor age, Thymoglobulin induction (whole population only), and prednisone withdrawal

Model 2, adjusted for recipient and donor age, living (vs deceased) donor, cold ischemia time, Thymoglobulin induction (whole population only), re-transplantation, pre-transplant HLA antibodies, HLA A/B AND HLA DR mismatch, glomerulonephritis as primary renal disease, dialysis vintage, and prednisone withdrawal.

S1 Table. HLA typing of the six B cell lines used as stimulators in the PRT assay.

B cell line	**A-1**	**A-2**	**B-1**	**B-2**	**C-1**	**C-2**	**DRB1-1**	**DRB1-2**	**DRB3**	**DQA1-1**	**DQA1-2**	**DQB1-1**	**DQB1-2**
B1	A*30	A*34	B*15 (72)	B*44	C*04	C*04	DRB1 *11	DRB1 *15:03	DRB3 *02	DQA1 *01	DQA1 *05	DQB1 *03:19 (7)	DQB1 *06
B2	A*03	A*03	B*44	B*44	C*05	C*16	DRB1 *04	DRB1 *11	DRB3 *02	DQA1 *03:01	DQA1 *05	DQB1 *03 (7)	DQB1 *03(8)
B3	A*02	A*30	B*18	B*42	C*05	C*17	DRB1 *03 (17)	DRB1 *15:03	DRB3 *02	DQA1 *01	DQA1 *05:01	DQB1 *02	DQB1 *06
B4	A*03	A*24	B*07	B*35	C*04	C*07	DRB1 *07	DRB1 *13	DRB3 *03:01	DQA1 *01	DQA1 *02:01	DQB1 *03(9)	DQB1 *06
B5	A*11	A*26	B*15 (62)	B*51	C*04	C*14	DRB1 *03 (17)	DRB1 *11	DRB3 *02	DQA1 *05	DQA1 *05	DQB1 *02	DQB1 *03(7)
B6	A*03	A*30	B*13	B*38	C*06	C*12	DRB1* 07	DRB1* 13	DRB3* 01	DQA1 *01	DQA1 *02:01	DQB1 *02	DQB1 *06

HLA typing of the 6 B cell lines used as stimulators in the PRT+/- IL15 assay. Each allele is reported in a separate column.

CHAPTER 3

Post-transplant non-invasive diagnosis of cell-mediated rejection and its treatment response:

Rapid Bio-Layer Interferometry Measurements of Urinary CXCL9 to Detect Cellular Infiltrates Noninvasively after Kidney Transplantation

Ilaria Gandolfini, MD[1,2], Cynthia Harris[1], Michael Abecassis, MD[3], Lisa Anderson[1], Oriol Bestard, MD, PhD[4], Giorgia Comai, MD[5], Paolo Cravedi, MD, PhD[1], Elena Cremaschi, MD[2], J. Andrew Duty, PhD[6], Sander Florman, MD[7], John Friedewald, MD[3], Gaetano La Manna MD[5], Umberto Maggiore, MD[2], Thomas Moran, PhD[6], Giovanni Piotti, MD[2], Carolina Purroy, MD[1], Marta Jarque, MD[4], Vinay Nair, MD[7], Ron Shapiro, MD[7], Jessica Reid-Adam, MD[8], Peter S. Heeger, MD[1,7]

[1]Translational Transplant Research Center, Icahn School of Medicine at Mount Sinai, NY, NY USA; [2]Kidney and Kidney-Pancreas Unit, Department of Nephrology, Parma University Hospital, Parma, Italy; [3]Comprehensive Transplant Center, Feinberg School of Medicine, Northwestern University, Chicago IL, USA; [4]Kidney Transplant Unit, Bellvitge University Hospital, IDIBELL, UB, Barcelona, Spain; [5]Department of Experimental, Diagnostic, Specialty Medicine, Nephrology, Dialysis, and Renal Transplant Unit, S. Orsola University Hospital, Bologna, Italy; [6]Department of Microbiology, Icahn School of Medicine at Mount Sinai, NY, NY USA; [7]Recanati Miller Transplant Institute, Icahn School of Medicine at Mount Sinai, NY, NY USA, [8]Division of Pediatric Nephrology, Icahn School of Medicine at Mount Sinai, NY, NY USA.

INTRODUCTION

Despite reduction of acute cellular rejection (ACR) rates since the 1990s,[1, 2] ACR continues to affect long-term kidney allograft survival negatively.[3, 4] A few experienced centers perform follow-up biopsies to assess efficacy of anti-rejection therapy, but this practice is impractical, risky and therefore not done routinely in most transplant centers. Noninvasive monitoring tools capable of rapidly assessing intragraft inflammation could guide therapeutic decision-making following treatment for rejection and thereby potentially improve graft function and patient health.

Urinary chemokines are among candidate biomarkers for detecting kidney allograft inflammation.[5, 6] CXCL9 is an interferon-gamma (IFNγ)-induced, T cell chemoattractant chemokine produced by monocyte/macrophages, endothelial cells and renal parenchymal cells[7]. Results of single center studies[8-14] showed that measurements of urinary CXCL9 (uCXCL9) can differentiate ACR from most other causes of acute post-transplant kidney dysfunction in the absence of infection. Findings from Clinical Trials in Organ Transplantation (CTOT)-01 (NCT01974999), a prospective, multicenter, observational study of 280 kidney transplant recipients (KTRs) showed that uCXCL9 (by ELISA) at a threshold of ≥200 pg/ml, diagnosed ACR at the time of an acute elevation of serum creatinine (negative predictive value, NPV:92%, positive predictive value, PPV:67%).[12] Excluding subjects with BK virus (BKV) or urinary tract infection (UTI) increased the PPV to >80%.[12] In a follow-up tacrolimus withdrawal trial (CTOT-09, NCT01517984), serial ELISA uCXCL9 measurements detected ACR 3-30 days prior to clinical presentation.[14] The 12-24 hour requisite turn-around time for ELISAs is not ideal for “real-time” implementation of therapeutic changes based on assay results. Herein we report an alternative, automated, “point-of-care” uCXCL9 assay that can be performed in <1 hour. In addition to confirming

that this innovative technology can diagnose ACR noninvasively, we provide proof-of-concept that serial uCXCL9 measurements following therapy of ACR could be employed to guide subsequent clinical decision-making.

MATERIALS AND METHODS

Samples and Patients

Aliquots of stored BKV-negative urine samples were obtained from 2 multi-center, prospective, observational kidney transplant studies, CTOT-01[12] and CTOT-08 (www.ctot.org, NCT01289717). These samples (n=86) were used to compare BLI and ELISA (**Figure 1**).

We also prospectively collected serial urine samples and clinical data from 46 kidney transplant recipients with for cause biopsies followed at four institutions (Bellvitge University Hospital, IDIBELL, UB, Barcelona, Spain, Mount Sinai Hospital, NY, NY, USA; Parma University Hospital, Parma, Italy, S. Orsola University Hospital, Bologna, Italy). We also analyzed urine from 10 BKV-negative subjects with stable serum creatinine and no intragraft infiltrates at 6-month surveillance biopsies. Consenting subjects were enrolled at the time of biopsy. The initial urine sample was obtained prior to the biopsy and prior to any anti-rejection treatment.

Inclusion criterion was acute increase in serum creatinine >30%. Patients with pure antibody mediated rejection[15] as reported by the local pathologist were excluded. Immunosuppression varied by site but generally included induction with anti-thymocyte globulin (ATG) or anti-CD25 mAb and maintenance immunosuppression with a calcineurin inhibitor, mycophenolic acid ± corticosteroids. Therapeutic interventions were made at the discretion of the site investigators and were not dictated by study. Therapy of ACR included steroids, ATG, and/or intravenous immunoglobulin (IVIg). Serum creatinine values were determined at each hospital laboratory and the information was collected from subjects' hospital records. Surveillance studies for viral infections including BK polyoma virus were

performed according to local practice at each participating site. Bacterial (e.g. urinary) and BK polyoma virus infections were routinely tested for in patients with acute renal allograft dysfunction as per local standard of care. Included patients were negative for BK virus infection. The enrollment and sample/data collection was performed following IRB approval at each site. All patients signed informed consent.

Laboratory studies

Urine samples for chemokines were centrifuged at 2000*g* for 30min at 4C within 4 hours of collection. The supernatant was divided into aliquots and frozen at -80C.

Urine ELISA for CXCL9

Frozen aliquots of urine supernatant were diluted (1:1) in 0.05% Tween-20/0.4% bovine serum albumin in phosphate buffered saline, pH 7.2-7.4, and tested by ELISA for CXCL9 (R&D Systems, Minneapolis, MN) as reported [12, 14].

Urine CXCL9 detection by Bio-layer Inferometry

Samples were run on OctetRED96 using Octet Data Acquisition software (8.2) and analyzed using Data Analysis software (8.2), continually monitoring wavelength. Assays were performed in black 96-well plates. All steps used a shake speed of 1000rpms unless otherwise specified. All wells contained 200µL of fluid. Streptavidin-(SA) conjugated sensor tips (Fortebio, cat #18-5019) were incubated in phosphate buffered saline (PBS), pH 7.2-7.4 containing 0.025% Tween-20/0.4% bovine serum albumin (BSA) for 10 min. and then incubated with 20µg/ml (in above buffer) biotinylated mouse anti human CXCL9 (clone B8-11, BD Pharmingen) for 10 min. Following a 30 second wash the tips were exposed for 30 min to urine supernatants diluted 1:1 with PBS/Tween/BSA or to recombinant CXCL9 (62.5pg/ml-500pg/mL-, R&D Sys. cat#DY392) diluted in PBS/Tween/BSA to calculate a

standard curve. Urine samples obtained from healthy subjects (IRB approved collection of urine from normal volunteers at Mount Sinai Hospital) served as a negative control. Following an additional 30 second wash, the sensor tips were exposed to HRP-conjugated anti-human CXCL9 clone B8-6 (5 μg/ml in PBS/Tween/BSA for 15 min. The tips were washed in stable peroxide buffer (DAB substrate kit, Thermo, cat #PI-34065) for 30 seconds on a shaker at 200rpms and then exposed to metal enhanced DAB diluted into peroxide buffer for 15 min.

Statistical methods

Data are summarized using descriptive statistics for categorical (counts/percentages) and continuous (mean and standard deviations) variables. To assess the agreement between BLI and standard ELISA measurements of CXCL9, we used the Lin's concordance correlation coefficient (the concordance correlation coefficient combines measures of both precision and accuracy to determine how far the observed data deviate from the line of perfect concordance) and Bland and Altman's 95% limits-of-agreement in urine samples from 86 kidney transplant patients with various biopsy diagnoses. Bland-Altman's 95% limits of agreement were additionally computed after exclusion of values of CXCL9 above 500pg/mL, because close agreement between BLI and ELISA with respect to such large CXCL9 values is not relevant for the purpose of clinical decisions making. The intra-plate assay coefficient of variation (%CV) was calculated based on the wavelength shift for samples of recombinant CXCL9 diluted in both PBS/Tween/BSA and urine from healthy subjects, four replicates (n=4) for each dilution on the same plate. The average of the %CVs at each dilution is reported as the intra-assay %CV. The inter-plate %CV is calculated based on the mean %CV of the different dilutions, with four replicates on one plate, repeated three times over a period of one week. Differences in CXCL9 values were analyzed by Mann-

Whitney test (Prism, GraphPad software, La Jolla CA) . A two-tailed p-value <0.05 was considered to be statistically significant.

RESULTS

To shorten the time required to detect uCXCL9, we used Bio-Layer Interferometry (BLI), a methodology in which binding of a ligand to a fiber optic sensor tip induces a real time, detectable wavelength shift in the returning beam of light (Δλ) that correlates with the quantity of bound ligand[16]. BLI has been used to screen monoclonal antibody (mAb) binding affinities among other indications[16]. We adapted the technology to detect CXCL9 (**Figure 1A**) by attaching an anti-CXCL9 mAb to commercially available sensor tips, exposing the tips to CXCL9 and amplifying the signal with a second, horseradish peroxidase (HRP)-conjugated anti-CXCL9 mAb followed by addition of a metal-enhanced, HRP substrate. Using pre-coated sensor tips, the assay can be completed in <1 hour (**Figure 1A**). We measured CXCL9 by ELISA and BLI in 86 urinary samples from patients with various biopsy diagnoses.

A Bland-Altman plot (**Figure 1B**) analysis showed good agreement between the two assays for values below 500pg/ml, the average difference between ELISA and BLI being -66pg/mL (standard deviation 41) and the 95% limits of agreement being -147 to +14pg/mL. Depiction of the same data using a scatter plot comparison of ELISA and BLI results (**Figure 1C**) showed a Lin's concordance correlation coefficient 0.78, BLI is more sensitive than ELISA), and a 100% concordance of positive vs. negative results based on the threshold of 200 pg/ml.

Specificity controls using independent samples showed that the BLI CXCL9 assay does not cross-react with CXCL10 (**Figure 1D**). BLI has the same limit of sensitivity as reported for commercial ELISAs (~35 pg/ml, see data sheet, R&D catalogue #DY392) with intra- and inter-plate CVs <5% and <12%, respectively (not shown).

Figure 1.

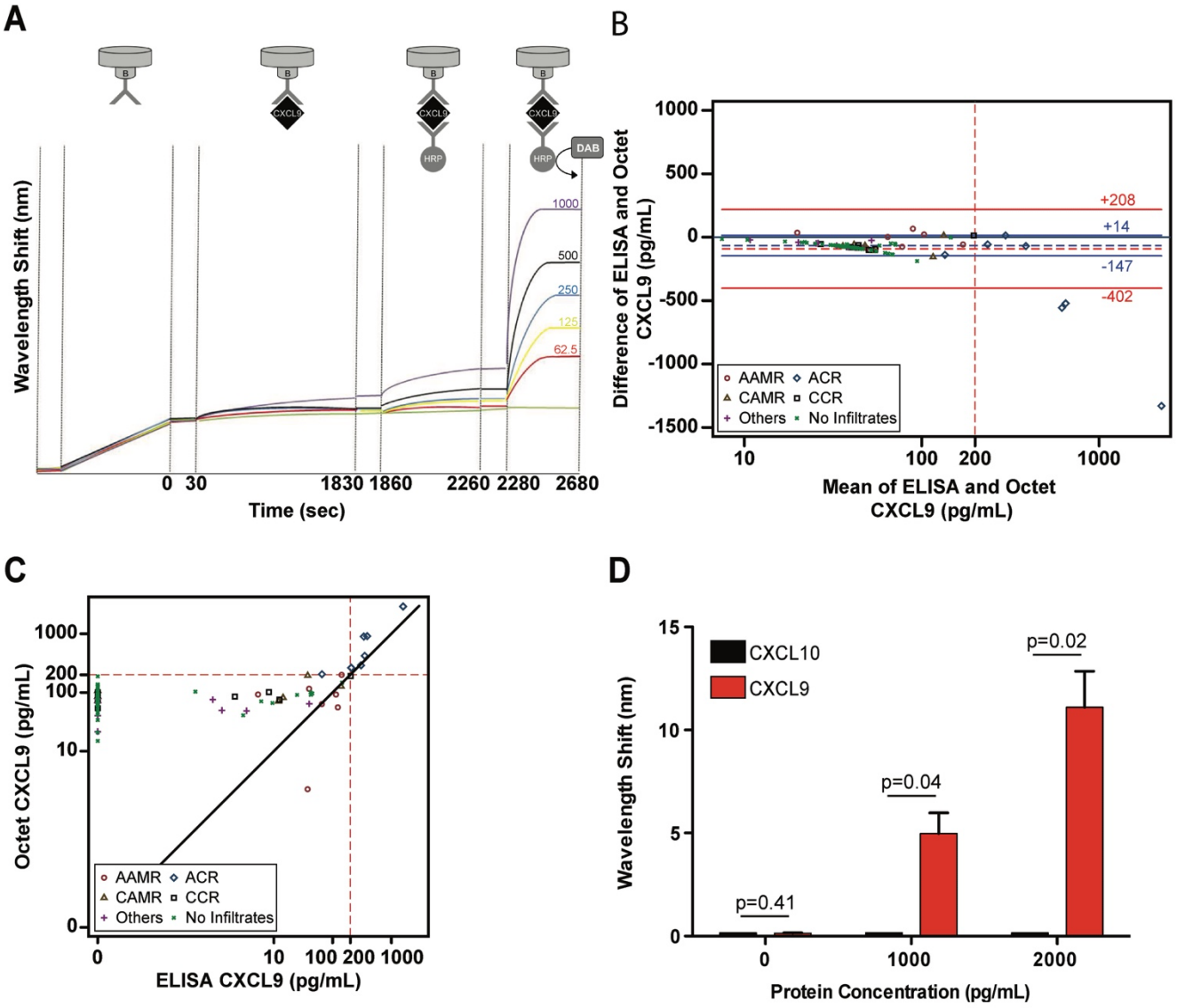


Figure 1. Bio-layer Interferometry rapidly detects uCXCL9. A. Schematic representation of assay (top) with primary readout depicting light wave length shift with each step (Y axis) over time (X axis) using 62.5-1000 pg/ml of recombinant CXCL9 (each colored line is a different concentration) as a standard curve. B. Bland-Altman plot of CXLC9 as measured by standard ELISA vs. BLI. The X-axis is on a logarithm scale to allow visualization of the numerical range close to the positivity threshold of the assays (i.e., 200pg/mL). Symbols represent different diagnoses (see legend in the plot region; AAMR: acute antibody mediated rejection, CAMR: chronic antibody mediated rejection, ACR: acute cellular rejection, CCR, chronic cellular rejection). Horizontal dashed lines represent the mean difference of ELISA and BLI results, horizontal solid lines represent the 95% limits of agreement, which are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences. Red lines include all the data points; blue lines are drawn after exclusion of mean values

>500pg/mL. The numeric values of the limits of agreement are reported above or below the respective lines. C. Scatter plot of CXCL9 results as performed by ELISA vs. BLI (logarithm scale). The solid line passing through the origin with a 45 degrees angle represents the line of perfect concordance between ELISA and BLI. Dashed lines passing through 200pg/mL divide the plot into four quadrants. Note that all results lie in the lower left quadrant or in the upper right quadrant, both representing areas of agreement between the two methods. Points within the upper left quadrant would represent instances where BLI results are positive (i.e >200pg/mL) but standard ELISAs are negative (i.e. <200pg/L), while points within the lower right quadrant would represent instances where BLI results are negative and ELISA results are positive (note there are no points in either of these 2 quadrants). D. BLI-ELISA for CXCL9 does not detect recombinant CXCL10. Each bar is mean of 3 replicate values; p values show reflect unpaired t-tests. Assay was repeated with similar results. Statistical comparisons performed by t-test.

We then measured uCXCL9 by BLI in a different set of samples obtained from 56 BKV-negative KTRs. This new set included 22 samples from subjects with a $\geq$30% increase serum creatinine and biopsy-proven ACR $\geq$Banff 1A (**Table 1**). Urinary CXCL9 was >200 pg/ml in all of these samples (**Figure 2**). In contrast, uCXCL9 was <100 pg/ml ($p<0.01$ vs. ACR) in urine samples from 10 BKV-negative KTRs with stable serum creatinine and a normal 6-month surveillance biopsy (**Table 1** and **Figure 2**). Urine CXCL9 values in samples from BKV-negative subjects (n=9) with an acute rise in serum creatinine due to calcineurin inhibitor toxicity and/or volume depletion, but without mononuclear cell infiltrates, were all 100-200 pg/ml ($p<0.01$ vs. ACR or vs. normal, **Figure 2**). CXCL9 measurements in prospectively collected urine from BKV-negative subjects with for-cause biopsies showing biopsy-proven borderline rejection (n=15 **Table 1**) were higher than those biopsies without infiltrates ($p<0.01$, **Figure 2**); 7/15 contained $\geq$200 pg/ml CXCL9 and 8 showed <200 pg/ml (**Fig 2**).

Figure 2.

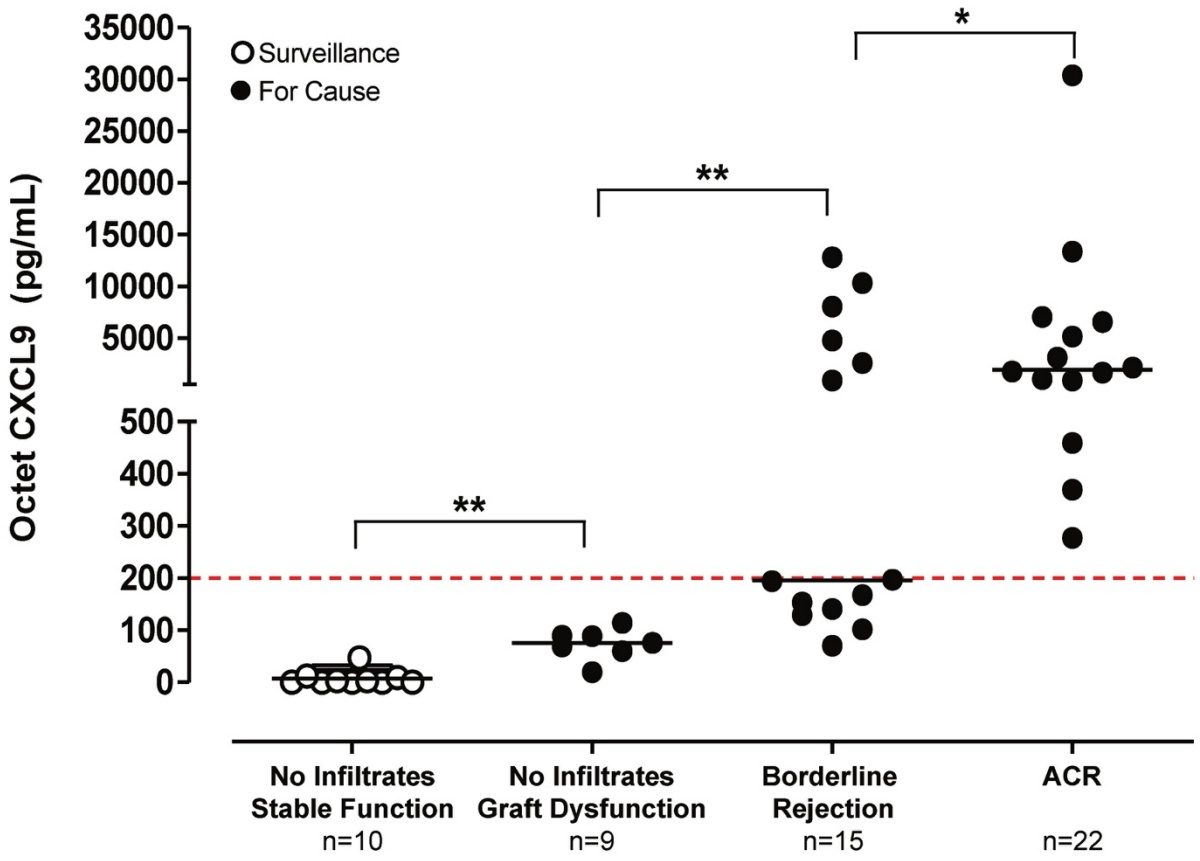
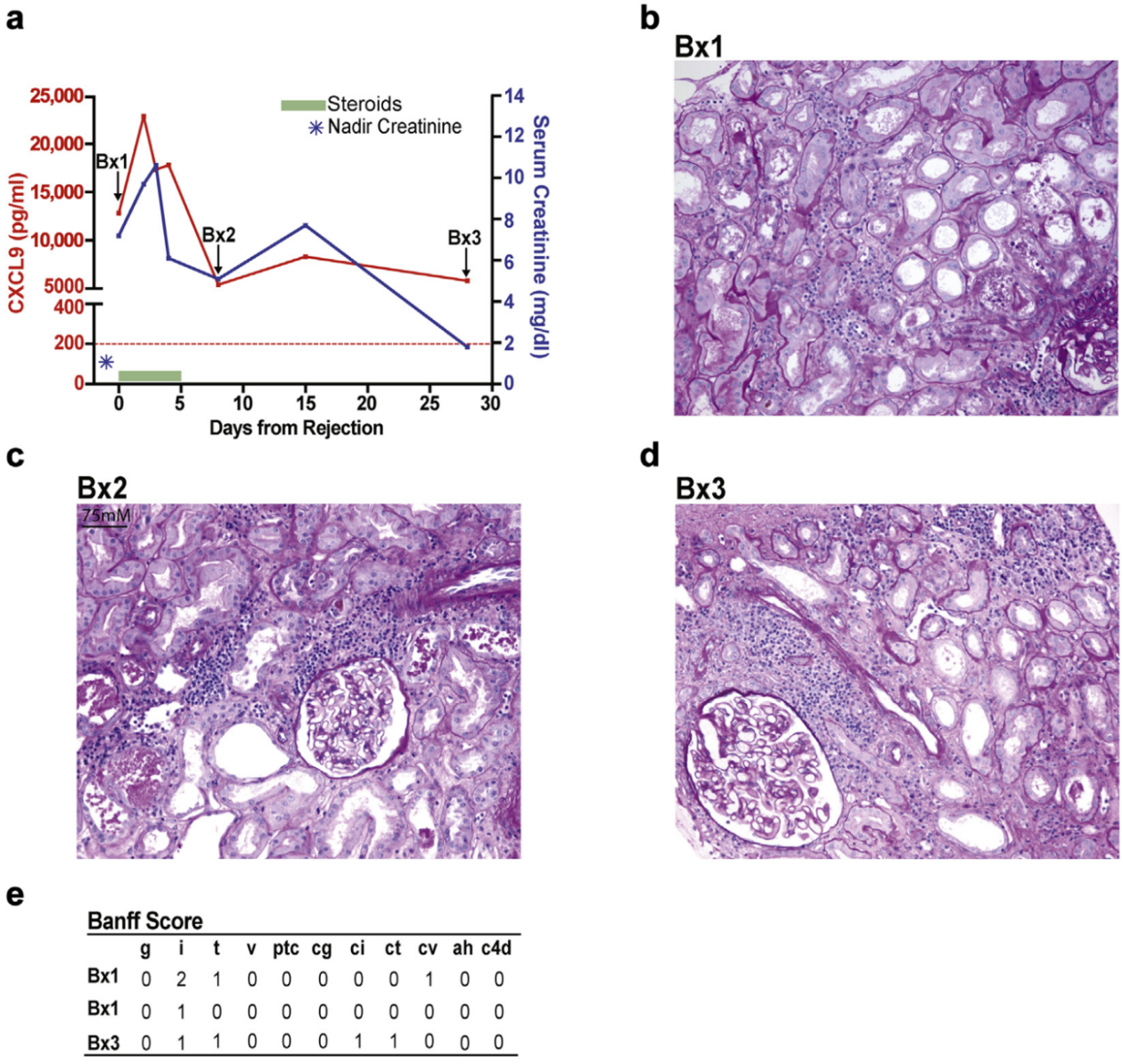


Figure 2. BLI uCXCL9 detects intragraft infiltrates in kidney transplant recipients. We analyzed 56 samples from kidney transplant recipients with surveillance (empty circles, n=10) or for-cause (full circles; >30% increase in serum creatinine) biopsies . Subjects are stratified according to the presence of stable graft function and no graft infiltrates (in surveillance biopsies, n=10), or acute graft dysfunction and no graft infiltrates (n=9), borderline rejection (n=15), or acute cellular rejection (ACR) (in for-cause biopsies, n=22). Dotted red line is drawn at the 200 pg/ml threshold for CXCL9 positivity. Horizontal black lines are drawn at the median value for each group. Statistical comparison performed by Mann-Whitney test. *p<0.05; **p<0.01.

We additionally collected and analyzed serial urine samples from 6 BKV-negative KTRs with ACR before and after anti-rejection therapy, and who had follow-up biopsies as part of clinical care. The summarized results from one representative subject at 20 days after

transplant are depicted in **Fig 3**. The subject's baseline serum creatinine was 1.0 mg/dl and increased to 7.2 mg/dl coincident with an episode of biopsy-proven acute cellular rejection. Steroid pulses reduced serum creatinine levels to <2 mg/dl, but follow-up biopsies on d7 and d28 after the initial diagnostic biopsy showed intragraft infiltrates associated with persistently elevated levels of uCXCL9.

Figure 3.



	g	i	t	v	ptc	cg	ci	ct	cv	ah	c4d
Bx1	0	2	1	0	0	0	0	0	1	0	0
Bx1	0	1	0	0	0	0	0	0	0	0	0
Bx3	0	1	1	0	0	0	1	1	0	0	0

Figure 3. Persistently elevated uCXCL9 after treatment for ACR detects subclinical intragraft infiltrates despite a progressive decline in serum creatinine. A. Clinical course depicting changes in serum creatinine and

uCXCL9 (day 0 is the date of the biopsy, 20 days posttransplant). Colored horizontal bars depict time during which each drug was administered (key: upper right panel A). Blue star represents the nadir serum creatinine within the initial 6 mo posttransplant. Red dashed line is drawn at the 200 pg/ml threshold for CXCL9 positivity. B-D. Representative PAS stained sections of biopsy 1 (Bx1), 2 (Bx2), and 3 (Bx3) depicted in panel A showing areas of mononuclear cell infiltration in each biopsy. E. Quantitative Banff scores as read by the local pathologist for the 3 biopsies.

In 2 additional subjects (**Fig 4A-B**), anti-rejection therapy resulted in uCXCL9 <200 pg/ml and absence of mononuclear infiltrates in follow-up biopsies. In 3 cases (**Fig 4B-E**), persistent uCXCL9 >200 pg/ml despite therapy for ACR was associated with intragraft infiltrates regardless of changes in serum creatinine.

Figure 4.

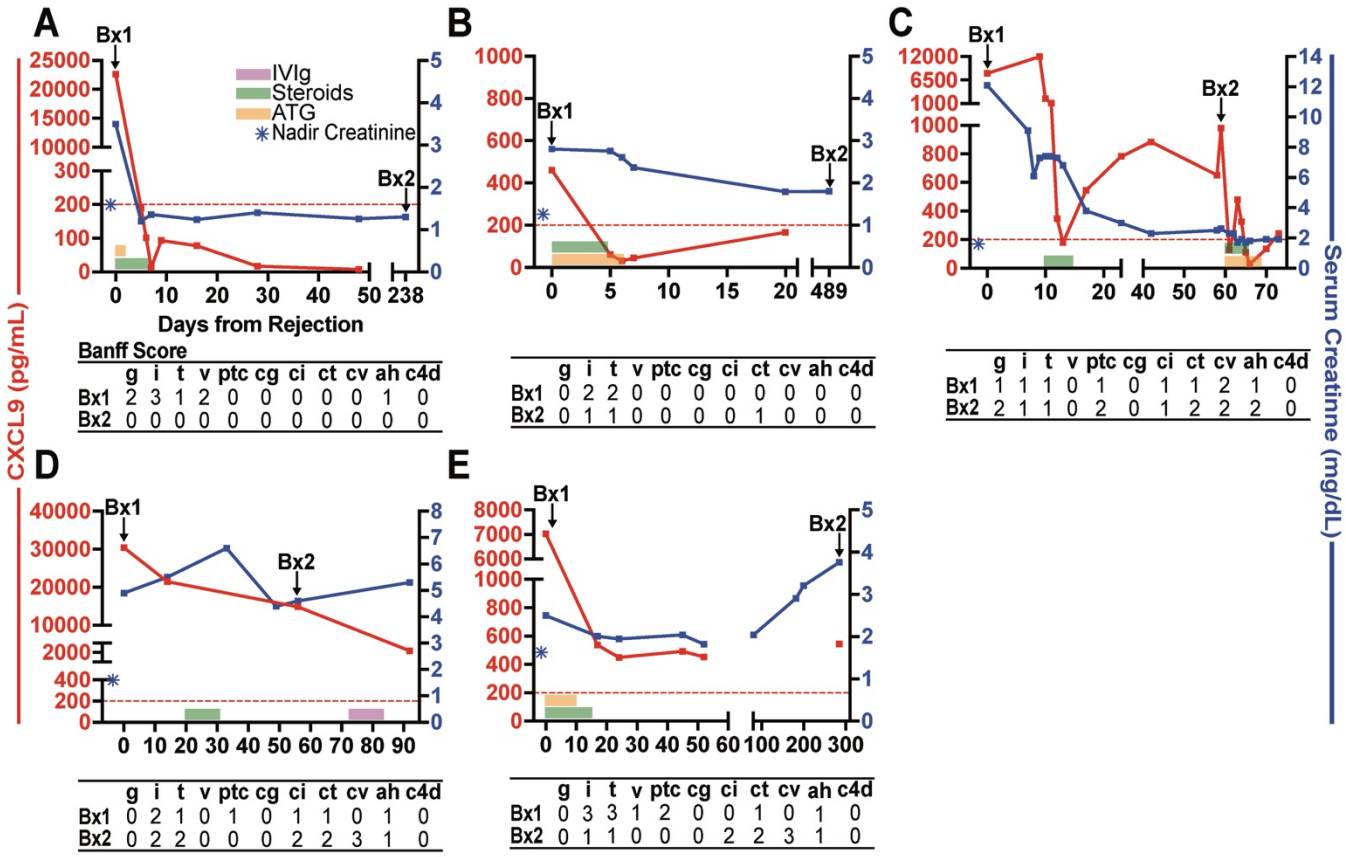


Figure 4. Changes in serum creatinine and uCXCL9 in 5 BKV-neg, DSA-neg subjects with ACR and follow-up biopsies to monitor treatment efficacy. Tables below each panel depict Banff scores of each of the biopsies. Dotted red line is drawn at the 200 pg/ml threshold for CXCL9 positivity.

DISCUSSION

We demonstrate that BLI and ELISA can similarly detect uCXCL9, but BLI is considerably faster and largely automated. These advantages could permit implementing BLI-based uCXLC9 testing in a clinical transplant practice. While BLI methods are straightforward, the currently available detection device has a complex and expensive interface designed for broader use. Nonetheless, modifications could simplify the interface lower costs to accommodate practicing physicians.

We show that in BKV-negative KTRs with acute graft dysfunction, BLI-measured uCXCL9 $\geq$200 pg/ml detected Banff grade $\geq$1A ACR. Approximately 50% of subjects with borderline/suspicious rejection also had uCXCL9 >200 pg/ml with the remainder falling in the normal range. As a) histological diagnoses in kidney transplantation are subject to sampling bias and inter-reader variation and b) the significance of borderline rejection remains controversial,[17, 18] one testable hypothesis arising from these observations is that elevated uCXCL9 measurements are better indicators of ongoing pathological inflammation than serum creatinine or histological evidence of suspicious/borderline rejection. While UTI and BKV increase uCXCL9, routine BKV monitoring and urinalysis together with rapid CXCL9 diagnostics could guide clinical decision-making noninvasively, a hypothesis that is also testable.

We acknowledge that none of the tested subjects in this series had pure antibody mediated rejection (ABMR). Other groups have shown associations between ABMR and the chemokine CXCL10 (in the absence of CXCL9).[10, 19] Pilot studies indicate that we can detect CXCL10 by an analogous BLI-based ELISA (not shown), providing feasibility for potentially incorporating both CXCL9 and CXCL10 measurements into clinical care.

We also provide evidence that serial uCXCL9 monitoring after initiating treatment for ACR could be diagnostically informative. In our limited analysis of KTRs with clinically indicated follow-up biopsies, continuous elevation of uCXCL9 detected persistent intragraft cellular infiltrates regardless of serum creatinine. We acknowledge the small numbers of subjects, the inconsistent numbers and timing of biopsies, and the descriptive nature of this case study precludes reaching definitive conclusions regarding the utility of BLI uCXCL9 to guide post-treatment decision making in KTRs with rejection. Nonetheless, these proof-of-concept results provide a foundation to support future controlled studies to test the hypothesis that inclusion of “real-time” BLI uCXCL9 measurements will lower the need for follow-up biopsies, guide decisions to continue/alter anti-rejection therapy and consequently, improve patient outcomes.

References

1. Kramer BK, Klinger M, Vitko S*, et al.* Tacrolimus-based, steroid-free regimens in renal transplantation: 3-year follow-up of the ATLAS trial. *Transplantation* 2012; **94:** 492-498.

2. Tanriover B, Jaikaransingh V, MacConmara MP*, et al.* Acute Rejection Rates and Graft Outcomes According to Induction Regimen among Recipients of Kidneys from Deceased Donors Treated with Tacrolimus and Mycophenolate. *Clin J Am Soc Nephrol* 2016; **11:** 1650-1661.

3. McDonald S, Russ G, Campbell S*, et al.* Kidney transplant rejection in Australia and New Zealand: relationships between rejection and graft outcome. *Am J Transplant* 2007; **7:** 1201-1208.

4. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. *N Engl J Med* 2010; **363:** 1451-1462.

5. Anglicheau D, Naesens M, Essig M*, et al.* Establishing Biomarkers in Transplant Medicine: A Critical Review of Current Approaches. *Transplantation* 2016; **100:** 2024-2038.

6. Menon MC, Murphy B, Heeger PS. Moving Biomarkers toward Clinical Implementation in Kidney Transplantation. *J Am Soc Nephrol* 2017.

7. Lazzeri E, Romagnani P. CXCR3-binding chemokines: novel multifunctional therapeutic targets. *Curr Drug Targets Immune Endocr Metabol Disord* 2005; **5:** 109-118.

8. Hu H, Aizenstein BD, Puchalski A*, et al.* Elevation of CXCR3-binding chemokines in urine indicates acute renal-allograft dysfunction. *Am J Transplant* 2004; **4:** 432-437.

9. Hauser IA, Spiegler S, Kiss E*, et al.* Prediction of acute renal allograft rejection by urinary monokine induced by IFN-gamma (MIG). *J Am Soc Nephrol* 2005; **16:** 1849-1858.

10. Schaub S, Nickerson P, Rush D*, et al.* Urinary CXCL9 and CXCL10 levels correlate with the extent of subclinical tubulitis. *Am J Transplant* 2009; **9:** 1347-1353.

11. Jackson JA, Kim EJ, Begley B*, et al.* Urinary chemokines CXCL9 and CXCL10 are noninvasive markers of renal allograft rejection and BK viral infection. *Am J Transplant* 2011; **11:** 2228-2234.

12. Hricik DE, Nickerson P, Formica RN*, et al.* Multicenter validation of urinary CXCL9 as a risk-stratifying biomarker for kidney transplant injury. *Am J Transplant* 2013; **13:** 2634-2644.

13. Kim SC, Page EK, Knechtle SJ. Urine proteomics in kidney transplantation. *Transplant Rev (Orlando)* 2014; **28:** 15-20.

14. Hricik DE, Formica RN, Nickerson P*, et al.* Adverse Outcomes of Tacrolimus Withdrawal in Immune-Quiescent Kidney Transplant Recipients. *J Am Soc Nephrol* 2015; **26:** 3114-3122.

15. Haas M, Sis B, Racusen LC*, et al.* Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant* 2014; **14:** 272-283.

16. Dysinger M, King LE. Practical quantitative and kinetic applications of bio-layer interferometry for toxicokinetic analysis of a monoclonal antibody therapeutic. *J Immunol Methods* 2012; **379:** 30-41.

17. Becker JU, Chang A, Nickeleit V*, et al.* Banff Borderline Changes Suspicious for Acute T Cell-Mediated Rejection: Where Do We Stand? *Am J Transplant* 2016; **16:** 2654-2660.

18. Ozluk Y, Blanco PL, Mengel M*, et al.* Superiority of virtual microscopy versus light microscopy in transplantation pathology. *Clin Transplant* 2012; **26:** 336-344.

19. Ho J, Rush DN, Karpinski M*, et al.* Validation of urinary CXCL10 as a marker of borderline, subclinical, and clinical tubulitis. *Transplantation* 2011; **92:** 878-882.

Table 1. Baseline demographic and clinical characteristics of study subjects.

Characteristics	Overall (n=56)	No Infiltrates* (n=10)	No Infiltrates (n=9)	Borderline (n=15)	ACR (n=22)
Donors					
Deceased; n (%)	30 (53.6)	2 (20.0)	5 (55.6)	11 (73.3)	12 (54.6)
Male; n (%)	22 (39.3)	3 (30.0)	2 (22.2)	6 (40.0)	11 (50.0)
Age (*years*)	37.5 ± 15.6	34.9 ± 11.5	32.9 ± 15.2	41.0 ± 14.9	38.3 ± 18.5
Race					
Black or African American; n (%)	10 (17.9)	3 (30.0)	3 (33.0)	2 (13.3)	2 (9.1)
Other race; n (%)	27 (48.2)	5 (50.0)	5 (55.6)	9 (60.0)	8 (36.4)
Unknown or not reported; n (%)	19 (33.9)	2 (20.0)	1 (11.1)	4 (26.7)	12 (54.5)
Recipients					
Male; n (%)	42 (75.0)	6 (60.0)	6 (66.7)	12 (80.0)	18 (81.8)
Age (*years*)	45.4 ± 18.3	45.6 ± 9.8	45.8 ± 21.7	47.1 ± 19.7	44.0 ± 19.8
Race					
Black or African American; n (%)	19 (33.9)	5 (50.0)	2 (22.2)	5 (33.3)	7 (31.8)
Other race; n (%)	27 (48.2)	5 (50.0)	2 (22.2)	8 (53.3)	12 (54.5)
Unknown or not reported; n (%)	10 (17.9)	0 (0.0)	5 (55.6)	2 (13.3)	3 (13.6)
Induction					
Yes	52 (92.9)	8 (80.0)	8 (88.9)	15 (100.0)	21 (95.5)
No	4 (7.1)	2 (20.0)	1 (11.1)	0 (0.0)	1 (4.5)
Time after transplant**	183 (70-211)	191 (186-197)	167 (79-211)	125 (28-190)	120 (76-597)

Variables are expressed as mean ± SD, median (IQR), or absolute number (percentage). *Patients with stable graft function who underwent surveillance biopsies. All the other patients received a biopsy for cause (serum creatinine increase >30%). **Days from transplant to the date of first graft biopsy.

CHAPTER 4

Post-transplant non-invasive diagnosis of urothelial cancer:
The role of p16 immunohistochemistry on urinary cytology for non-invasive diagnosis of urothelial cancer in the context of BKV infection

Gandolfini Ilaria[1], Alibrandi Sara[1], Oliva Vincenzo[1], Francesco Paolo Pilato[2], Gnetti Letizia[2], Benigno Giuseppe Daniele[1], Salvetti Daniel[1], Vizzini Giuseppe[1,] Italiano Chiara[1,] Delsante Marco[1], Palmisano Alessandra[1], Fiaccadori Enrico[1], Maggiore Umberto[1]

[1] Division of Nephrology, Department of Medicine and Surgery, University of Parma, Parma, Italy. [2] Division of Pathology, Department of Medicine and Surgery, University of Parma, Parma, Italy.

Introduction

Kidney transplant recipients are at increased risk for urothelial cancer compared to the general population[1]. This phenomenon can be related to increased incidence of classical urothelial risk factors (i.e. smoking) that can be found in the chronic kidney disease

population, previous treatment with urothelial toxic drugs (i.e. cyclophosphamide) and reduced immune surveillance. Recent studies hypothesized the potential role of BKV in the genesis of urothelial cancer in these patients [2-5].

BKV is a single strain DNA polyomavirus firstly isolated in 1971 in a kidney transplant recipient with ureteral stenosis[6].

The viral genome is divided in 3 regions: early genes, late genes, and non-coding genes. The early genes, large and small t-antigen (T-Ag and t-Ag), are crucial for the viral DNA replication, late genes are coding for capsid protein (VP1-3) while non-coding regions are regulating gene transcription[7].

BKV infects asymptomatically most (80-90%) of the human population during childhood through oral or respiratory transmission[8] and subsequently remains lifelong latent in the epithelium of the urinary tract[9]. In the context of a kidney transplantation, BKV can reactivate following immunosuppression in previously asymptomatic infected patients or transmitted with the graft [10,11].

BKV reactivation can be detected as urinary viral DNA in up to one third of kidney transplant recipients under current standard immunosuppression, it is generally asymptomatic, and it is related to the immunosuppressive burden, with the highest incidence within the first 3-6 months after transplantation[12,13].

Persistent high levels of urothelial viral reactivation are associated with an increased risk of ascending infection in the renal tubules of the renal graft, leading to polyomavirus associated nephropathy (PVAN), afflicting 3% to 10% of renal transplant recipients[14-16].

PVAN is characterized by viral cytopathic alterations of the renal tubular cells and secondary interstitial inflammation and tubulitis, leading to graft loss in half of the cases[17-19]. The shedding of altered tubular or urothelial cells, with increased nucleus/cytoplasmatic ratio and basophilic nuclear inclusion, can be found in the urine as a marker of BKV infection[20].

These cells are called ‘decoy cells’ for their resemblance to cancer cells, and for decades the concomitant positivity of BKV-DNA in urine and plasma or the positive immunohistochemistry for BKV T-Ag in those cells have been used in the differential diagnosis from urothelial cancers in kidney transplant recipients[21].

However recent animal models demonstrated a potential oncogenic role of BKV through the interaction between viral large and small T-antigen and various oncogenes (p53 and pRB) with subsequent deregulation of cell cycle, transition to S phase and increased risk of mutations accumulation[10,22-24].

The role of BKV in human cancer is still controversial[25-27],[28] however more and more studies reported renal and urothelial cancer that stains positive for T-Ag supporting the hypothesis of a potential oncogenic role for this virus also in humanbeings[2-5]. Some authors argued that BKV could infect the whole urothelium, cancer included, without having a role into the oncogenic pathway[28]. Although some recent papers showed that the surrounding urothelium stained positive for both T-Ag and structural viral protein, VP1, while the urothelial cancer stained positive only for T-Ag, suggesting a loss of permissive infection in the mutated cells[28-31]. Moreover, these cancer cells stained positive for oncosuppressor p16, as a marker of pRB inactivation, similar to what happen in the oncogenesis of cervical cancer, induced by another Polyomavirus, the Human Papillomavirus (HPV)[32]. In this context the p16 staining is currently used on cervical cytology in order to increase the negative predictive value of Papanicolau-test in the context of high-risk HPV genotypes[33].

Starting from the evidence of a p16 staining in BKV-positive urothelial cancers, our Pathology Department started to apply the same cytology technique of the cervical cytology to urinary cytology in the context of BKV-decoy cells since 2014 in order to differentiate BKV-infection from urothelial cancer cells.

The first aim of the study is to analyze the prevalence of urothelial cancer in our population and correlate it with the prevalence of BKV infection, the second aim of the study is to

investigate the sensitivity and specificity of immunohistochemistry p16 in the diagnosis of urothelial cancer in the context of BKV infection in kidney transplant recipients.

Methods

This is a single center retrospective study conducted at the Nephrology Unit of the University Hospital of Parma, Italy. The study was approved by the local Ethical Committee and in accordance with the current guidelines of the World Medical Association (revised Declaration of Helsinki).

The Study Population

We included all adult patients who underwent a kidney transplant at the Kidney and Pancreas Transplantation Nephrology Unit, University of Parma, Italy, between 30-11-2010 and 30-03-2020, and who consent to participate this retrospective study.

The study included all kidney transplant recipients who develop a BKV infection after transplant with at least one urinary cytology analyzed for BKV and p16 staining.

Clinical data

Clinical data were extracted from electronical and paper clinical charts to collect the following parameters: sex, age, native nephropathy, transplant vintage, dialysis vintage, type of transplant, type of donor, immunosuppressive therapy, delayed graft function. Data on regular follow-up visits (at 14 days, 1, 2, 3, 6, 12, 18 months and yearly thereafter): incidence of rejection, urinary tract infections, cytomegalovirus (CMV) infection, BKV-DNA on urine, plasma and blood, s-creatinine, urinary sediment and urinary cytology with BKV and p16 staining (in case of BKV positivity), cystoscopy examinations and diagnosis of urothelial cancer.

Statistical analysis

The study population have been analyzed through descriptive statistics for categorical variables: absolute, relative and cumulative frequencies. Pearson-Chi square for categorical variables and unpaired T-test was used to analyze the data.

Results

The study population

The study included 147 kidney transplant recipients followed at the Kidney and Pancreas Transplantation Nephrology Unit, University of Parma, Italy,

The patients characteristics are reported in **Table 1**: 71% of the patients were male with a mean age of 55 ±13 years. 89% received a cadaveric donor transplant, the incidence of delayed graft function of 18%. Native nephropathy was Autosomal Dominant Polycystic Kidney Disease (ADPKD) in 16% of the patients, hypertensive nephropathy in 5%, diabetic nephropathy in 6%, IgA Nephropathy in 6%. 70% received basiliximab as induction therapy, while almost all of the patients (99%) received the triple immunosuppressive therapy based on tacrolimus, mycophenolate mofetil and steroids as maintenance therapy.

The prevalence of urothelial cancer in the study population was 3.4%: 4 patients were diagnosed with bladder cancer and one patient with distal ureteral cancer.

BKV and urinary cancer

In line with the current literature, the prevalence of BKV detection on urine was 35%, while 15% of the patients had BKV-DNA detected on plasma and 14% on blood (**Figure 1**).

Figure 1. BKV-DNA positivity on different biological samples.

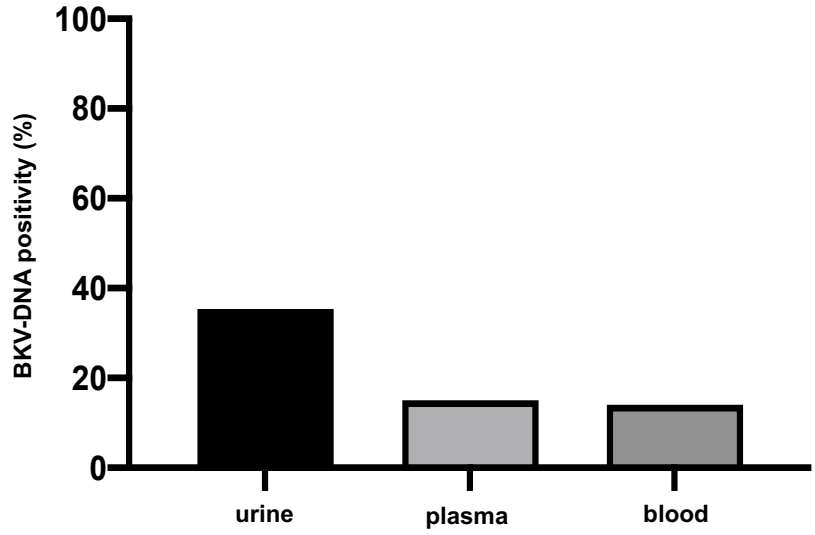


Figure 1. BKV-DNA was detected in 35% of the patients on urine, 15% on plasma and 14% on blood samples.

CMV replication was detected in 15% of the patient population.

The patients with diagnosis of urothelial cancer had a more persistent BKV infection, expressed as number of positive urinary detection at follow-up visits, compared to the patients who did not develop urothelial cancer (p=0.0001) (**Figure 2**).

Figure 2. Number of urinary BKV-DNA detections according to the diagnosis of urothelial cancer.

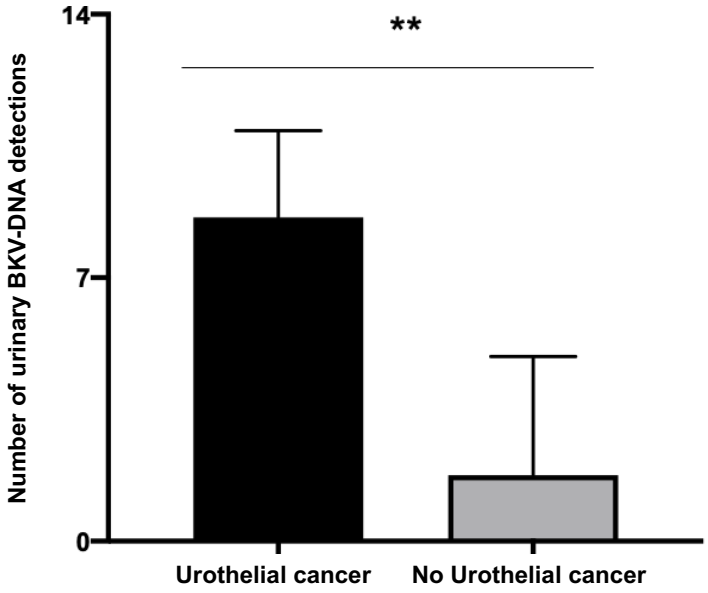


Figure 2. The patients with the diagnosis of urothelial cancer had increased number of BKV-DNA urinary detections at follow-up visits, when compared to the patients who did not develop urothelial cancer. ** p=0.0001

The patients with diagnosis of urothelial cancer had a higher prevalence of BKV-DNA plasmatic detection, when compared to the patients who did not develop urothelial cancer (p=0.002) (**Figure 3**).

Figure 3. Percentage of patients with BKV-DNA detection on plasma samples according to the diagnosis of urothelial cancer

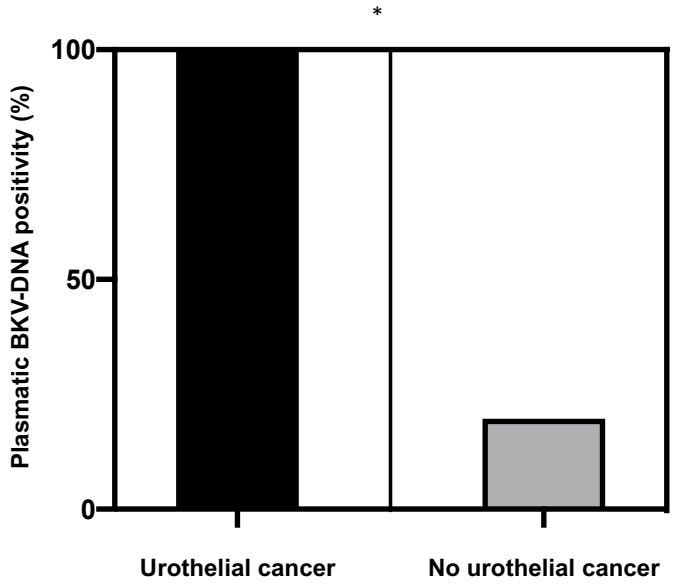


Figure 3. The patients with diagnosis of urothelial cancer had a higher prevalence of BKV-DNA plasmatic detection, when compared to the patients who did not developed urothelial cancer (p=0.002)

No difference was detected in the incidence of CMV infection between patients with diagnosis of urothelial cancer when compared to the patients who did not develop urothelial cancer (**Figure 4**).

Figure 4. Prevalence of CMV positivity according to the diagnosis of urothelial cancer.

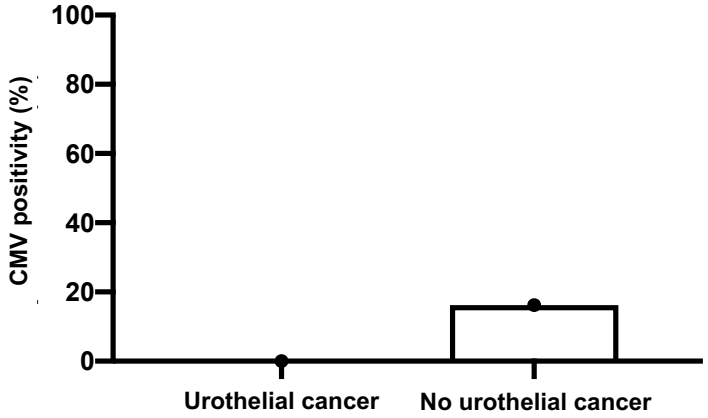
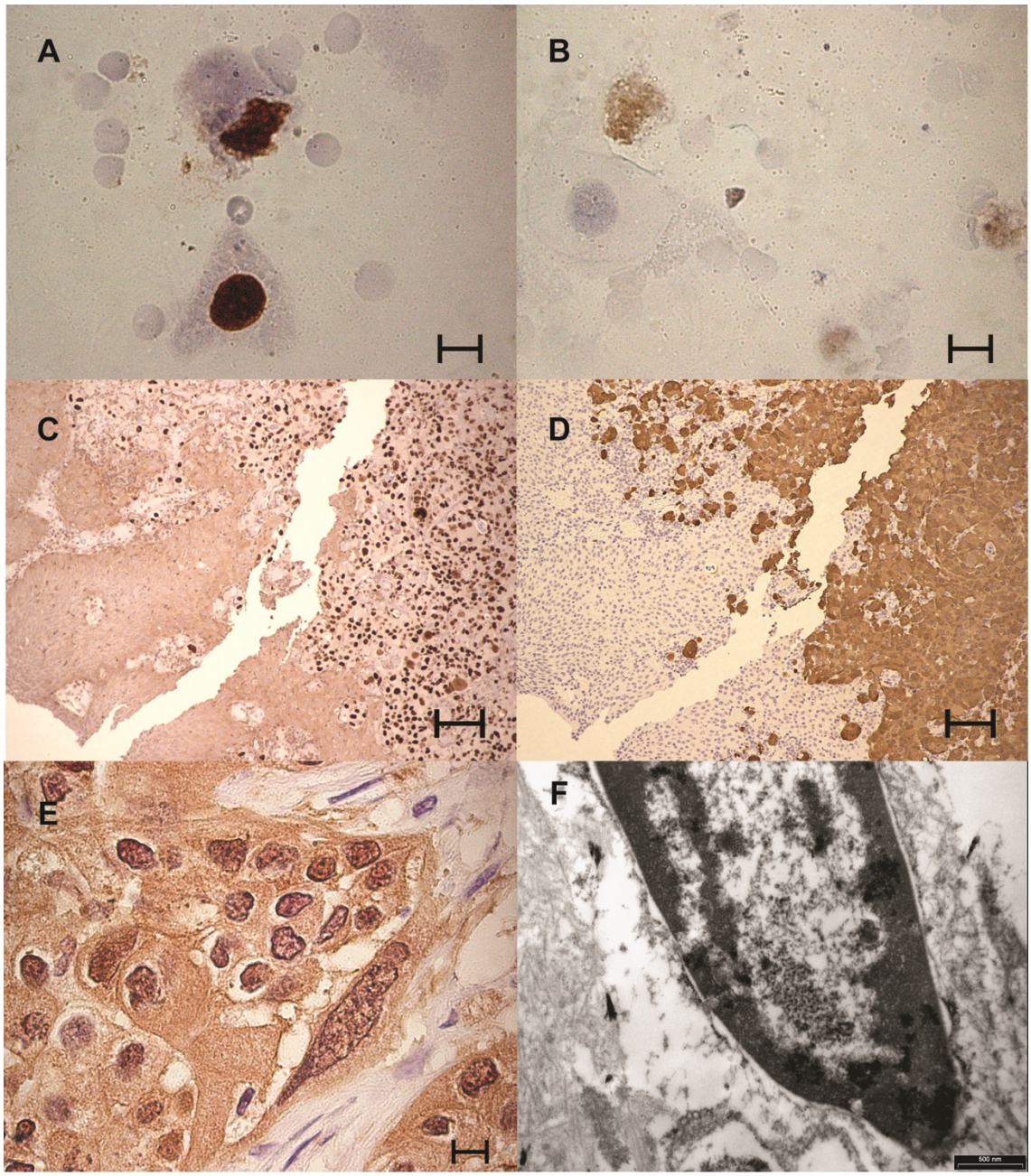


Figure 4. No difference was detected in the prevalence of CMV replication (pp65 positivity on peripheral leukocytes) according to the diagnosis of urothelial cancer (p=ns).

Non-invasive techniques for the diagnosis of urothelial cancer during BKV infection

4 over 5 patients with diagnosis of urothelial cancer stained positive for BKV and p16 immunohistochemistry both on cancer tissue and urinary sediment (**Figure 5**).

Figure 5 – Urinary cytology and urothelial cancer pathology with BKV and p16 immunohistochemistry .



A urinary cytology, 100X: immunohistochemistry for BKV TAg. **B** urinary cytology, 100X: positive and negative immunohistochemistry for p16 on urothelial cells. **C,D** immunohistochemistry on bladder tissue, 10X: positive BKV-Tag and p16 on urothelial cancer, negative surrounding urothelium **E** colocalization of dual immunohistochemistry on bladder tissue, 100X: nuclear staining for BKV TAg and cytoplasmic staining for p16 in both epithelial and mesenchymal component of a sarcomatoid bladder cancer. **F** electronic microscopy on bladder tissue: 40 nm viral particles inside the cancer cells.

Urinary BKV-DNA detection had the highest sensitivity (100%), but poor specificity (66%) for urothelial cancer in the context of BKV infection. Urinary cytology had both poor sensitivity and specificity, while the combination of urinary BKV-DNA detection and immunohistochemistry for p16 on urinary cytology allowed to reach a specificity of 99% for the diagnosis of urothelial cancer in this context. (**Table 2**).

Discussion

During BKV infection the non-invasive diagnosis of urothelial cancer can be difficult because of the presence of BKV-induced cytopathic alteration on ‘decoy cells’. These cells have been considered a reassuring finding for many decades, however recent studies supporting an oncogenic role of BKV in the urothelial cancer, have pushed the whole transplant community to a more invasive approach for urothelial cancer monitoring in the context of BKV infection, with some centers performing follow-up cystoscopies in one third of the kidney transplant recipients.

Starting from our BKV-positive urothelial cancers that stained positive per BKV Tag and p16 oncosuppressor gene, our Pathology Laboratory started performing p16 immunohistochemistry on urinary citology since 2014. This approach has been used in the context of another Polyomavirus, for the non-invasive diagnosis of high-risk genotype HPV-induced cervical cancer in which the conventional Papanicolau-test has been implemented with p16 immunohistochemistry.

In our retrospective study we found that patients with diagnosis of urothelial cancer had a BKV persistent infection with increased viral replication (higher plasmatic detection) compared to the patients who did not develop urothelial cancer. Some could argue that BKV infection could be a marker of excessive immunosuppression, for this reason we analyzed the incidence of CMV reactivation in these patients and we found no difference between the patients with or without urothelial cancer, suggesting a specific oncogenic role for BKV.

We further analyzed the sensitivity and specificity of different non-invasive techniques for the diagnosis of urothelial cancer in the context of BKV infection and we found that the best results were reported from the concomitant use of BKV-DNA urinary detection and p16 immunohistochemistry on urinary cytology.

The main limitations of our study are the limited number of patients with urothelial cancer in the context of BKV infection and the retrospective nature of the study. For this reason, we

hope for larger multicentric prospective studies to confirm our initial findings. If these results will be confirmed the transplant community will have an easy, cheap, repeatable non-invasive biomarker for the early diagnosis of urothelial cancer in kidney transplant recipients also in the context of BKV infection.

REFERENCES

1. Birkeland SA, Storm HH, Lamm LU, et al. Cancer risk after renal transplantation in the Nordic countries, 1964-1986. Int J Cancer 1995;60:183-9.
2. Muller DC, Ramo M, Naegele K, et al. Donor-derived, metastatic urothelial cancer after kidney transplantation associated with a potentially oncogenic BK polyomavirus. J Pathol 2018;244:265-70.
3. Prom A, Jorgenson M, Alagusundaramoorthy S, Parajuli S. Persistent BK polyomavirus-DNAemia may warrant cystoscopy to rule out urologic carcinoma: A case report and review of the literature. Transpl Infect Dis 2020;22:e13316.
4. Yan L, Salama ME, Lanciault C, Matsumura L, Troxell ML. Polyomavirus large T antigen is prevalent in urothelial carcinoma post-kidney transplant. Hum Pathol 2016;48:122-31.
5. Abend JR, Jiang M, Imperiale MJ. BK virus and human cancer: innocent until proven guilty. Semin Cancer Biol 2009;19:252-60.
6. Gardner SD, Field AM, Coleman DV, Hulme B. New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet 1971;1:1253-7.
7. Seif I, Khoury G, Dhar R. BKV splice sequences based on analysis of preferred donor and acceptor sites. Nucleic Acids Res 1979;6:3387-98.
8. Pahari A, Rees L. BK virus-associated renal problems--clinical implications. Pediatr Nephrol 2003;18:743-8.
9. Knowles WA, Pipkin P, Andrews N, et al. Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40. J Med Virol 2003;71:115-23.

10. Tognon M, Corallini A, Martini F, Negrini M, Barbanti-Brodano G. Oncogenic transformation by BK virus and association with human tumors. Oncogene 2003;22:5192-200.

11. Lin PL, Vats AN, Green M. BK virus infection in renal transplant recipients. Pediatr Transplant 2001;5:398-405.

12. Gupta M, Miller F, Nord EP, Wadhwa NK. Delayed renal allograft dysfunction and cystitis associated with human polyomavirus (BK) infection in a renal transplant recipient: a case report and review of literature. Clin Nephrol 2003;60:405-14.

13. Manzano Sanchez D, Jimeno Garcia L, Manzano Sanchez D, et al. Renal Function Impairment in Kidney Transplantation: Importance of Early BK Virus Detection. Transplant Proc 2019;51:350-2.

14. Brochot E, Descamps V, Handala L, et al. BK polyomavirus in the urine for follow-up of kidney transplant recipients. Clin Microbiol Infect 2019;25:112 e1- e5.

15. Madden K, Janitell C, Sower D, Yang S. Prediction of BK viremia by urine viral load in renal transplant patients: An analysis of BK viral load results in paired urine and plasma samples. Transpl Infect Dis 2018;20:e12952.

16. Elfadawy N, Yamada M, Sarabu N. Management of BK Polyomavirus Infection in Kidney and Kidney-Pancreas Transplant Recipients: A Review Article. Infect Dis Clin North Am 2018;32:599-613.

17. Hirsch HH. Polyomavirus BK nephropathy: a (re-)emerging complication in renal transplantation. Am J Transplant 2002;2:25-30.

18. Hirsch HH, Vincenti F, Friman S, et al. Polyomavirus BK replication in de novo kidney transplant patients receiving tacrolimus or cyclosporine: a prospective, randomized, multicenter study. Am J Transplant 2013;13:136-45.

19. Vasudev B, Hariharan S, Hussain SA, Zhu YR, Bresnahan BA, Cohen EP. BK virus nephritis: risk factors, timing, and outcome in renal transplant recipients. Kidney Int 2005;68:1834-9.

20. Drachenberg CB, Papadimitriou JC, Hirsch HH, et al. Histological patterns of polyomavirus nephropathy: correlation with graft outcome and viral load. Am J Transplant 2004;4:2082-92.

21. Hirsch HH. BK virus: opportunity makes a pathogen. Clin Infect Dis 2005;41:354-60.

22. Bulut Y, Ozdemir E, Ozercan HI, et al. Potential relationship between BK virus and renal cell carcinoma. J Med Virol 2013;85:1085-9.

23. Papadimitriou JC, Randhawa P, Rinaldo CH, Drachenberg CB, Alexiev B, Hirsch HH. BK Polyomavirus Infection and Renourinary Tumorigenesis. Am J Transplant 2016;16:398-406.

24. Harris KF, Christensen JB, Imperiale MJ. BK virus large T antigen: interactions with the retinoblastoma family of tumor suppressor proteins and effects on cellular growth control. J Virol 1996;70:2378-86.

25. Chen CH, Wen MC, Wang M, et al. High incidence of malignancy in polyomavirus-associated nephropathy in renal transplant recipients. Transplant Proc 2010;42:817-8.

26. Roberts IS, Besarani D, Mason P, Turner G, Friend PJ, Newton R. Polyoma virus infection and urothelial carcinoma of the bladder following renal transplantation. Br J Cancer 2008;99:1383-6.

27. Wang HH, Liu KL, Chu SH, Tian YC, Lai PC, Chiang YJ. BK virus infection in association with posttransplant urothelial carcinoma. Transplant Proc 2009;41:165-6.

28. Alexiev BA, Drachenberg CB, Papadimitriou JC. Polyomavirus-cystitis associated with in situ and invasive urothelial carcinoma in a heart transplant recipient: evidence suggesting sequential progression/evolution from infection to carcinoma. Transplantation 2015;99:e3-4.

29. Buch MH, Liaci AM, O'Hara SD, Garcea RL, Neu U, Stehle T. Structural and Functional Analysis of Murine Polyomavirus Capsid Proteins Establish the Determinants of Ligand Recognition and Pathogenicity. PLoS Pathog 2015;11:e1005104.

30. Kenan DJ, Mieczkowski PA, Burger-Calderon R, Singh HK, Nickeleit V. The oncogenic potential of BK-polyomavirus is linked to viral integration into the human genome. J Pathol 2015;237:379-89.

31. Lavien G, Alger J, Preece J, Alexiev BA, Alexander RB. BK Virus-Associated Invasive Urothelial Carcinoma With Prominent Micropapillary Carcinoma Component in a Cardiac Transplant Patient: Case Report and Review of Literature. Clin Genitourin Cancer 2015;13:e397-9.

32. Munger K, Baldwin A, Edwards KM, et al. Mechanisms of human papillomavirus-induced oncogenesis. J Virol 2004;78:11451-60.

33. Shain AF, Kwok S, Folkins AK, Kong CS. Utility of p16 Immunohistochemistry in Evaluating Negative Cervical Biopsies Following High-risk Pap Test Results. Am J Surg Pathol 2018;42:69-75.

TABLES

Table 1. The population characteristics

Patients (nr)		**147**
Age*, years*		55.04 +/- 13.65
Sex*, male*		105 (71.43%)
Dialysis vintage*, years*		4.68 +/- 3.63
Native nephropathy		
	ADPKD	24 (16.32%)
	Diabetic nephropathy	9 (6.12%)
	IgA Nephropathy	8 (5.44%)
	Hypertensive Nephropathy	13 (8.84%)
	Interstitial Nephropathy	7 (4.76%)
	Chroni**c** glomerulonephritis	4 (4.08%)
	Other	82 (55.78%)
Delayed graft function		27 (18.37%)
Donor		
	Cadaveric	131 (89.12%)
	Living	18 (12.45%)
Induction Therapy		
	Basiliximab	102 (69.39%)
	Thymoglobuline	43 (29.25%)
Maintenance therapy		
	Tacrolimus	146 (99.32%)
	Mycophenolate Mofetil	144 (97.96%)

mTOR-inhibitors	2 (1.36%)
steroids	146 (99.32%)

Categorical variables are expressed as mean+/- standard deviation, non-categorical variables are expressed as number and percentage. ADPKD, Autosomic Dominal Polyccystic Kidney Disease; mTOR, mammalian Target Of Rapamycin.

Table 2. Sensitivity and specificity for the diagnosis of urothelial cancer

Non-invasive Technique	sensitivity	specificity
Urinary BKV-DNA	100%	66%
Plasmatic BKV-DNA	100%	79%
Blood BKV-DNA	80%	79%
Conventional Urinary Cytology	66%	79%
Urinary Cytology + BKV staining	80%	79%
Urinary Cytology + p16 staining	80%	96%
Urinary Cytology + p16 staining**+** Urinary BKV-DNA	80%	99%

Table 2. Urinary BKV-DNA detection is characterized by the highest sensitivity (100%) and lowest specificity. The conventional urinary cytology has both suboptimal sensitivity and specificity in this context.
While the association between p16 staining on urinary cytology and BKV-DNA urinary detection increases the specificity for urothelial cancer up to 99%.

CHAPTER 5

GENERAL DISCUSSION AND FUTURE PROSPECTIVES

Kidney transplantation is the treatment of choice for end-stage renal disease, however in order to avoid rejection, kidney transplant recipients should undergo a lifelong immunosuppression with subsequent increased risk of infections and cancers.

It is therefore crucial to find biomarkers that are able to stratify the patients according to their risk of developing rejection in order to minimize the therapy as much as possible in the low-risk group and tailored the immunosuppression in the high-risk population.

The ideal biomarker should be cheap, quick, and available at the time of the transplant to assess the appropriate therapy for every patient but also it should be easily repeatable after transplant to follow up the immunological status and avoid graft biopsies and other invasive tests.

While the detection of anti-HLA alloantibodies had been a milestone for the non-invasive diagnosis of antibody-mediated rejection, non-invasive biomarker to stratify patients at risk for T cell-mediated rejection are lacking.

The findings of my first study confirm and further expand previous evidence showing that measuring alloreactive memory T cells before transplantation by donor-specific ELISPOT or

PRT allows to predict relevant immune-mediated transplant outcomes that would not be forecasted by current standard clinical and immunological evaluations. In fact, while positive donor-specific ELISPOT identifies patients at increased risk of acute rejection after transplantation, high pre-transplant PRT display significant graft function loss over a 4-year follow-up period. Our data set the basis of prospective studies formally testing the hypothesis that tailoring immunosuppression based on the joint use of donor-specific ELISPOT and PRT improves patients' outcomes.

The gold standard for TCMR diagnosis after kidney transplantation is the pathological evaluation of kidney biopsy. This procedure is, however, invasive, and poorly repeatable.
Noninvasive monitoring tools capable of rapidly assessing intragraft inflammation could help early diagnosing and treating rejection, thereby potentially improve graft function and patient health. Urinary chemokines, like CXCL9, are among candidate biomarkers for detecting kidney allograft inflammation. CXCL9 is an interferon-gamma-induced, T cell chemoattractant chemokine produced by monocyte/macrophages, endothelial cells and renal parenchymal cells. Previous studies, showed an association between elevated urinary CXCL9 levels and the diagnosis of TCMR, with serial ELISA urinary CXCL9 measurements detected up to 30 days prior to clinical presentation of rejection, emphasizing the potential role of this non-invasive biomarker in the early diagnosis of rejection.
One of the major obstacle for "real-time" implementation of therapeutic changes based on uCXCL9 is the 12-24 hour requisite turn-around time for ELISA tests.
We demonstrated that biolayer interferometry can detect urinary CXCL9 similarly to ELISA, but through a faster and largely automated assay. These advantages could permit implementing BLI-based uCXLC9 testing in a clinical transplant practice.

We also provide evidence that serial uCXCL9 monitoring after initiating treatment for ACR could be diagnostically informative. In our limited analysis of KTRs with clinically indicated

follow-up biopsies, continuous elevation of uCXCL9 detected persistent intragraft cellular infiltrates regardless of serum creatinine. We acknowledge the small numbers of subjects, the inconsistent numbers and timing of biopsies, and the descriptive nature of this case study precludes reaching definitive conclusions regarding the utility of BLI uCXCL9 to guide post-treatment decision making in KTRs with rejection. Nonetheless, these proof-of-concept results provide a foundation to support future controlled studies to test the hypothesis that inclusion of “real-time” BLI uCXCL9 measurements will lower the need for follow-up biopsies, guide decisions to continue/alter anti-rejection therapy and consequently, improve patient outcomes.

The last chapter of my thesis is dealing with another clinical dilemma in kidney transplantation: the non-invasive diagnosis of urothelial cancer in the context of BKV virus infection in kidney transplant recipients.

BKV is a polyomavirus that can often reactivate in immunosuppressed individuals and can be responsible for cytopathic alteration of the urinary tract in up to one third of the kidney transplant recipients. This condition strongly limits the sensitivity and specificity of urinary cytology for non-invasive diagnosis of urothelial cancer in the context of a high-risk population. Moreover, recent studies suggested a role of BKV in altering cell cycle, through p16 and p53 oncogene dysregulation, and potentially inducing urothelial cancer in human and animal models. For this reason, many transplant centers are performing periodically invasive monitoring through cystoscopy these patients.

Starting from our BKV-positive urothelial cancers that stained positive per BKV Tag and p16 oncosuppressor gene, our Pathology Laboratory started performing p16 immunohistochemistry on urinary citology since 2014. This approach has been used in the context of another Polyomavirus, for the non-invasive diagnosis of high-risk genotype HPV-

induced cervical cancer in which the conventional Papanicolau-test has been implemented with p16 immunohistochemistry.

In our retrospective study we found that patients with diagnosis of urothelial cancer had a BKV persistent infection with increased viral replication (higher plasmatic detection) compared to the patients who did not develop urothelial cancer. Some could argue that BKV infection could be a marker of excessive immunosuppression, for this reason we analyzed the incidence of CMV reactivation in these patients and we found no difference between the patients with or without urothelial cancer, suggesting a specific oncogenic role for BKV.

We further analyzed the sensitivity and specificity of different non-invasive techniques for the diagnosis of urothelial cancer in the context of BKV infection and we found that the best results were reported from the concomitant use of BKV-DNA urinary detection and p16 immunohistochemistry on urinary cytology.

The main limitations of our study are the limited number of patients with urothelial cancer in the context of BKV infection and the retrospective nature of the study. For this reason, we hope for larger multicentric prospective studies to confirm our initial findings. If these results will be confirmed the transplant community will have an easy, cheap, repeatable non-invasive biomarker for the early diagnosis of urothelial cancer in kidney transplant recipients also in the context of BKV infection.