ORIGINAL ARTICLE

Immune biomarkers and long-term graft survival: a prospective follow-up of 457 kidney transplant recipients

Michał Ciszek[1], Krzysztof Mucha[1,2], Bartosz Foroncewicz[1], Dorota Żochowska[1], Maciej Kosieradzki[3], Tadeusz Grochowiecki[4], Magdalena Durlik[5], Andrzej Górski[6], Leszek Pączek[1,2]

1 Department of Immunology, Transplantology and Internal Diseases, Medical University of Warsaw, Warsaw, Poland
2 Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland
3 Department of General and Transplant Surgery, Medical University of Warsaw, Warsaw, Poland
4 Department of General, Vascular and Transplant Surgery, Medical University of Warsaw, Warsaw, Poland
5 Department of Nephrology and Transplant Medicine, Medical University of Warsaw, Warsaw, Poland
6 Department of Clinical Immunology, Medical University of Warsaw, Warsaw, Poland

KEY WORDS

antibody-mediated rejection, anti--HLA antibodies, anti-MICA antibodies, immune biomarkers, kidney transplantation

ABSTRACT

INTRODUCTION Antibodies against donor human leukocyte antigens (HLAs) play a significant role in the pathogenesis of antibody-mediated rejection, although their relevance during the late posttransplant period is unknown. A non-HLA polymorphic antigenic system, like major histocompatibility class I chain--related antigen A (MICA), might be another target for antibody responses involved in rejection.

OBJECTIVES We conducted a 7-year prospective study to determine the effect of positivity for anti-HLA and anti-MICA antibodies on kidney graft survival.

PATIENTS AND METHODS A random blood sample was collected from 457 kidney recipients during a regular outpatient visit. Patients who were less than 6 months after transplantation were excluded. Evaluation of anti-HLA (classes I and II) and anti-MICA antibodies was performed with the use of Luminex assays. An outpatient registry was used to monitor kidney function during a 7-year follow-up.

RESULTS A total of 147 patients (32%) had anti-HLA and 88 patients (19%) had anti-MICA antibodies. Graft failure occurred in 67 anti-HLA-positive individuals (46%) as compared to 81 anti-HLA-negative ones (26%) ($P < 0.05$), and in 30 anti-MICA-positive individuals (34%) as compared to 118 anti-MICA-negative ones (32%) ($P = 0.52$). Anti-HLA antibodies were associated with increased incidence of graft failure: it was reported in 200 patients with an estimated glomerular filtration rate of more than 30 ml/min/1.73 m^2 body surface area more than 5 years after transplantation ($P < 0.005$).

CONCLUSIONS Anti-HLA, but not anti-MICA, antibodies in randomly obtained blood samples were the significant predictor of late kidney graft failure and could be a low-cost method enabling identification of patients requiring an individualized posttransplant approach. The results of our study provide an additional rationale for investigating immune biomarkers in certain diseases.

Correspondence to:
Krzysztof Mucha, MD, PhD,
Klinika Immunologii, Transplantologii i Chorób Wewnętrznych,
Warszawski Uniwersytet Medyczny,
ul. Nowogrodzka 59,
02-006 Warszawa, Poland,
phone: +48 22 502 21 27,
e-mail: kjmucha@gmail.com
Received: November 14, 2016.
Revision accepted: February 14, 2017.
Published online: February 15, 2017.
Conflict of interest: none declared.
Pol Arch Intern Med. 2017;
127 (3): 178-183
doi:10.20452/pamw.3937

INTRODUCTION Antibody-mediated rejection (AMR) of kidney grafts has a distinct pathology and worse prognosis in comparison to the T–cell-mediated rejection. The donor-specific anti--human leukocyte antigen (HLA) antibodies (DSAs) have a causative role in the development of AMR.[1] Allograft biopsies performed in a subset of patients with anti-HLA antibodies revealed that 66% had complement C4d deposits (indicating humoral immune responses) in peritubular capillaries as compared to 0% in patients without antibodies.[2] Successful AMR treatment (eg, by antibody-depletion therapy) should aim at DSA removal and inhibition of their production. The presence of DSAs despite antibody-depletion therapy is a negative predictor of graft survival in

kidney transplantation (KTx).[3,4] Moreover, anti-HLA antibodies (without assessing their specificity) were reported to increase the risk of kidney graft failure.[5] According to the current guidelines, DSA levels should be monitored in high-risk patients (ie, desensitized or DSA positive before transplantation) during the first 3 months after KTx. Low-risk patients (nonsensitized, first transplantation) should also be screened for DSAs at least once at 3 to 12 months after transplantation, and a biopsy should be performed if DSAs are detected.[6] However, there are no data regarding the effects of anti-HLA antibodies on the long-term graft survival and kidney function.

Major histocompatibility class I chain-related antigen A (MICA) is a non-HLA polymorphic antigenic system that could potentially be another target for antibody responses and possibly may also be involved in AMR. However, the data regarding the impact of anti-MICA antibodies on acute rejection episodes and graft survival are conflicting.[7-9] Therefore, we conducted a prospective study aimed to determine the effect of positivity for anti-HLA and anti-MICA antibodies on kidney graft survival.

PATIENTS AND METHODS A total of 469 patients at least 6 months after KTx were enrolled. The 12 patients who were not evaluated in our 7-year analysis included 9 patients lost to follow-up, 2 patients with missing HLA data, and 1 individual with no MICA results. The study protocol was approved by the local ethics committee and informed consent was obtained from all participants. The study was performed in accordance with the principles of the Declaration of Helsinki.

Patients were selected randomly during their outpatient visits, so the time between the transplantation and the entry to the study was different for each patient. The blood samples were collected only once at baseline and tested for the presence of anti-HLA class I and II antibodies using Luminex kits (One Lambda, Inc., Canoga Park, California, United States); and anti-MICA antibodies with the use of Luminex assays with MICA*001, *002, *004, *007, *012, *018, *019, and *027 antigens purified from recombinant cell lines and coated on Luminex beads in the Terasaki Foundation Laboratory (Los Angeles, California, United States). Mean fluorescence intensity of more than 1500 was considered positive for MICA 001–027 antigens, except for MICA 019 antigen (positive when >2500). Patients were prospectively followed for a period of 7 years from the time of blood collection. The in-depth clinical and pathological analyses were facilitated by the 14th International HLA and Immunogenetics Workshop Prospective Chronic Rejection Project, in which our center participated. The workshop was designed to assess the effects of anti-HLA and anti-MICA antibodies detected after transplantation on chronic graft failure. Patient death, graft failure, and kidney function test results were the primary outcomes for the entire group of patients and separately for a subgroup of 200 patients who had an estimated glomerular filtration rate (eGFR) of more than 30 ml/min/1.73 m^2 body surface area (BSA) and were at least 5 years after transplantation at enrollment to this study. Kidney function tests were done every 3 months during routine patient visits in our outpatient clinic. Kidney function was estimated with the use of the Modification of Diet in Renal Disease Study equation.

The SAS software (version 9.3, SAS Institute Inc., Cary, North Carolina, United States) was used for statistical analysis. The outcomes in different patient groups were compared by the χ^2 test. Additional analysis determined odds ratios (ORs) using logistic regression models. Wilcoxon rank sum tests were used to compare the results for nonnormally distributed variables. *P* values of less than 0.05 were considered significant. Univariate survival curves were generated for preliminary analysis of factors possibly related to graft survival, and multivariate regression models were used to identify risk factors. Survival curves were generated using the Kaplan–Meier method and compared by the log-rank test. Relative risk levels for each factor were estimated using multivariate regression based on Cox proportional hazards models.

RESULTS Anti-HLA and anti-MICA antibody results and full medical reports were available for 457 kidney transplant recipients including 188 women (41%) and 269 men (59%). The mean age of patients at baseline (day of blood sample collection) was 46 years (range, 19–71 years). The time between KTx and enrollment to this study was different in each patient due to random selection method and ranged from 6 to 304 months (mean, 78 months). Patient characteristics are presented in **TABLE 1**.

Anti-HLA antibodies Anti-HLA antibodies were detected in 147 patients (31%): 61 with both classes I and II, 64 with class I only, and 22 with class II only. Interestingly, the antibodies were found in 122 of 424 patients (29%) after the first transplantation, and in 25 of 30 patients (83%) after the second transplantation ($P < 0.0001$). Both anti-HLA I and anti-HLA II antibodies were detected in 1 recipient of the third transplant. There were more women (79; 54%) than men (68; 46%) who were anti-HLA positive, compared to 109 women (35%) and 199 men (65%) who were anti-HLA negative ($P < 0.0005$). This difference was also significant for those who were anti–HLA-I positive ($P < 0.0001$), but not for those who were anti–HLA-II positive.

The median time between KTx and entry to the study was significantly longer for patients with anti-HLA antibodies than for those without these antibodies (72 vs 57 months; $P < 0.005$) and for those with and without anti–HLA-II antibodies (95 vs 74 months; $P < 0.05$). The same trend

TABLE 1 Characteristics of kidney transplant recipients

Parameter		Anti-HLA antibodies		Anti-MICA antibodies	
		Negative	Positive	Negative	Positive
Sex, female/male, n		109/199[a]	79/68[a]	155/213	33/55
Mean age, y		46	47	47	44
Time after KTx, mo		72	92[b]	78	78
No. of KTx, n	1	303	121[a]	344	81
	2	5	25[a]	23	7
	3	0	1	1	0
PRA test, n (%)	>0%	90 (37)	77 (66)[a]	132 (45)	34 (49)
	>10%	60 (25)	57 (49)[a]	85 (29)	20 (29)
Treatment, n	Cyclosporine	211	80[c]	239	53
	Tacrolimus	58	34	77	15
	Azathioprine	82	41	100	23
	MMF	156	74	177	53
	Rapamycin	24	10	343	80
	Steroids	282	140	26	8

a $P < 0.001$, **b** $P < 0.05$, **c** $P < 0.01$

Abbreviations: HLA, human leukocyte antigen; KTx, kidney transplantation; MICA, major histocompatibility class I chain-related antigen A; MMF, mycophenolate mofetil; PRA, panel reactive antigen

was found for those with and without anti–HLA-I antibodies (85 vs 75 months), although this difference was not significant. Anti-HLA antibodies were detected in 8 of 26 patients (31%) at 6 to 12 months posttransplantation, 52 of 200 patients (26%) at 1 to 5 years, in 41 of 130 patients (32%) at 5 to10 years, and in 46 of 99 patients (46%) at more than 10 years after transplantation ($P = 0.005$). The positive results of the panel reactive antibody (PRA) test before transplantation correlated with the presence of anti-HLA (both class I and class II) antibodies ($P < 0.0001$). No significant correlations were found between the presence of anti-HLA antibodies (either class I and class II together or each one separately) and patient age or number of immunosuppressive drugs.

Immunosuppression with cyclosporine A (CsA) correlated negatively with the presence of anti-HLA antibodies (both anti-HLA I and anti-HLA II): 67 patients (41%) who were anti-HLA positive were treated with CsA compared to 80 patients (28%) who received no CsA ($P = 0.005$). In contrast, patient age and time since KTx were not related to CsA therapy.

Anti-MICA antibodies Anti-MICA antibodies were detected in 88 patients (19%): 81 of 425 patients (19%) after the first graft and in 7 of 30 patients (23%) after the second KTx ($P = 0.44$). No anti-MICA antibodies were detected in 1 patient with the third transplant. The median time between the KTx and enrollment to the study was significantly longer for patients with anti-MICA antibodies than for those without these antibodies (72 vs 57 months; $P < 0.005$).

Anti-MICA antibodies were found in 4 of 26 patients (15%) at 6 to 12 months posttransplantation, in 38 of 201 patients (19%) at 1 to 5 years, in 28 of 130 patients (22%) at 5 to 10 years, and in 18 of 99 patients (21%) at more than 10 years after transplantation. The presence of anti-MICA antibodies was not correlated with PRA test results (any PRA or PRA >10%). No significant correlations were found between the presence of anti-MICA antibodies and patient age, sex, or immunosuppressive drugs (both type and number).

Patient and graft survivals A total of 39 patients died during the 7-year follow-up, of whom 11 (7.5%) were anti-HLA positive compared to 28 (9.0%) who were anti-HLA negative ($P = 0.57$) and of whom 7 (8.0%) were anti-MICA positive compared to 32 (8.7%) who were anti-MICA negative. The presence of anti-HLA antibodies was a significant predictor of graft survival for the entire study population and for the subgroups of patients who were positive for anti–HLA-I and anti–HLA-II antibodies (TABLE 2, FIGURE 1). Death-censored graft failure occurred in 56 patients (38%) who were anti-HLA positive, and only in 53 patients (17%) who were anti-HLA negative (OR, 3.02; 95% CI, 1.92–4.76; $P < 0.0001$).

The presence of anti-MICA antibodies had no effect on 7-year graft survival. Death-censored graft failure occurred in 23 patients (26%) who were anti-MICA positive and in 86 patients (23%) who were anti-MICA negative (OR, 1.08; 95% CI, 0.63–1.85; $P = 0.48$) (TABLE 2, FIGURE 2). In a subgroup of 200 patients with an eGFR of more than 30 ml/min/1.73 m^2 BSA who were enrolled into our study more than 5 years after KTx, there were 18 death-censored graft failures in 72 patients (25%) who were anti-HLA positive compared to 11 of 128 patients (9%) without anti-HLA antibodies ($P < 0.005$). The presence of anti-MICA antibodies was not correlated with death-censored graft failure in this subgroup: 7 of 40 patients (18%) compared to 22 of 160 (1%, $P = 0.6$).

Kidney function There were no differences in the mean serum creatinine concentrations and eGFR at baseline between patients who were anti-HLA positive and those who were anti-HLA negative. Importantly, the mean increase in serum creatinine concentrations at 2, 5, and 7 years was significantly higher in anti–HLA-positive compared to anti–HLA-negative recipients. Such trend could also be observed in patients who were both positive and negative for anti–HLA-I and anti–HLA-II antibodies. There was no significant difference in graft function at baseline and at 2, 5, and 7 years between patients who were positive and negative for anti-MICA antibodies (TABLE 2).

DISCUSSION We found that anti-HLA, but not anti-MICA, antibodies in blood samples randomly obtained from renal transplant recipients were the significant predictor of late kidney graft failure. We confirmed that the presence of anti-HLA antibodies (either class I and II together or each

TABLE 2 Graft function and failure at 2 and 7 years after kidney transplantation

Parameter	Anti-HLA antibodies		Anti-HLA class I antibodies		Anti-HLA class II antibodies		Anti-MICA antibodies	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Cr0, mg/dl, mean	1.63	1.71	1.65	1.68	1.63	1.81	1.67	1.61
	$P = 0.89$		$P = 0.57$		$P = 0.18$		$P = 0.56$	
CrCl, ml/min, mean (SD)	50.6 (18.4)	49.5 (23.9)	50.2 (18.7)	50.4 (24.1)	51.0 (19.2)	46.8 (24.7)	50.2 (20.5)	51.1 (19.7)
	$P = 0.28$		$P = 0.63$		$P = 0.1$		$P = 0.9$	
ΔCr2, mg/dl, mean	0.07	0.16	0.07	0.16	0.08	0.19	0.08	0.15
	$P < 0.05$		$P < 0.05$		$P < 0.005$		$P = 0.68$	
ΔCr7, mg/dl, mean	0.16	0.33	0.17	0.32	0.17	0.4	0.18	0.28
	$P < 0.01$		$P < 0.01$		$P < 0.05$		$P = 0.73$	
DCGF, n (%)	53 (17)	56 (38)	64 (22)	45 (40)	69 (21)	40 (53)	86 (23)	23 (26)
	$P < 0.001$		$P < 0.001$		$P < 0.001$		$P = 0.69$	
GF, n (%)	81 (26)	67 (46)	93 (29)	55 (46)	101 (29)	47 (59)	118 (32)	30 (34)
	$P < 0.001$		$P < 0.001$		$P < 0.001$		$P = 0.52$	

Abbreviations: Cr0, serum creatinine concentration at baseline; CrCl, creatinine clearance at baseline; ΔCr2, change in serum creatinine concentration at 2 years; ΔCr7, change in serum creatinine concentration at 7 years; DCGF, death-censored graft failure; GF, graft failure; others, see **TABLE 1**

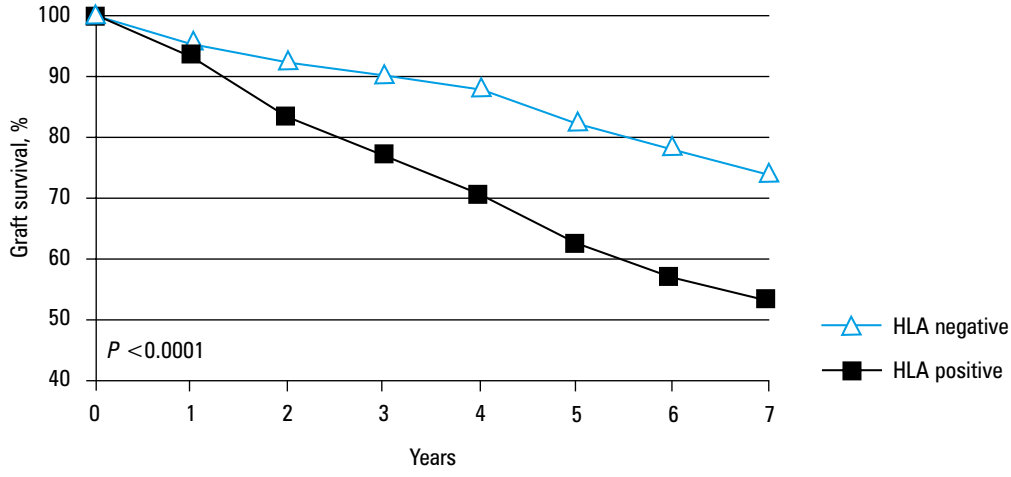

FIGURE 1 Seven-year graft survivals for anti–HLA-positive and anti–HLA-negative patients

class separately) significantly correlated with kidney graft survival and with worsening of kidney function during a 7-year follow-up. Similar results were also shown in a 3-year analysis within the 14th International HLA and Immunogenetics Workshop Prospective Chronic Rejection Project.[5] In another study of a 4-year duration, patients with anti-HLA class I and II antibodies after KTx, either alone or in combination, showed significantly lower graft survival.[10] Accordingly, we previously reported that the presence of anti-HLA or anti-MICA antibodies correlated with late liver transplant rejection.[11]

In our present study, we found that anti-HLA antibodies positively correlated with PRA test results (both >0% and >10%) and with the number of transplants. Moreover, a subgroup analysis of 200 patients with an eGFR of more than 30 ml/min/1.73 m^2 BSA more than 5 years after KTx at enrollment revealed that a relatively inexpensive and rapid anti-HLA test (without assessing specificity of antibodies) can identify patients at risk for late graft loss. Therefore, we plan to implement anti-HLA screening for all kidney transplant recipients in our center. Patients with positive results will undergo further evaluations including DSA testing and kidney biopsy in order to tailor their immunosuppressive treatment. We believe that anti–HLA-antibody titer may also be used as a biomarker of safe immunosuppression minimization in selected patients. On the contrary, positive anti-HLA results might alert to the need for reintroduction of immunosuppressive therapy if detected during or after the weaning procedure.[12,13]

There are conflicting reports regarding the role of anti-MICA antibodies in KTx. In a cohort of 1910 kidney recipients, the presence of anti-MICA antibodies before transplantation was associated with episodes of acute rejection and inferior 1-year graft survival. This finding was even more evident in kidney transplant recipients with good HLA matching.[8] Death-censored graft survivals at 1 and 4 years posttransplantation were 10% to 15% lower, respectively, in a group of patients with anti-MICA antibodies but without anti-HLA antibodies compared to patients without any of these antibodies. Furthermore, findings from

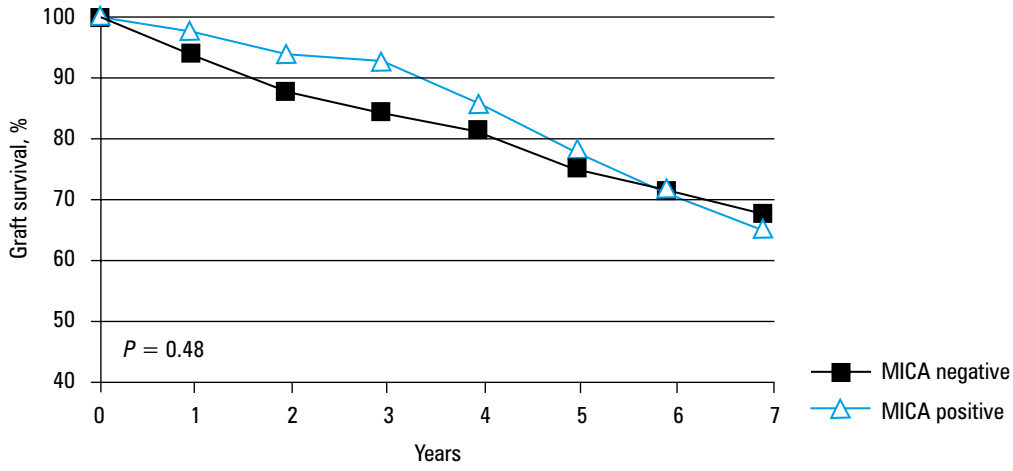

FIGURE 2 Seven-year graft survivals for anti–MICA-positive and anti–MICA-negative patients

a study of 40 living unrelated donor kidney recipients supported the view that monitoring of anti-HLA and anti-MICA antibodies early after transplantation has predictive value for early and late allograft dysfunction and that the presence of these factors is detrimental to graft function and survival.[14] In contrast, Lemy et al[9] found no association between the presence of anti-MICA antibodies at 1 year posttransplantation with 4-year death-censored graft survival in a group of 779 kidney transplant recipients. It was also reported that the presence of anti-MICA antibodies was an important predictor of graft failure only when HLA class I antibodies were detected.[15] In our study population, the presence of anti-MICA antibodies did not correlate significantly with graft survival or kidney function. Moreover, their presence was not associated with positive results of the PRA test.

One of the study limitations was screening performed only once and at different time points after KTx. However, we did prove that even random anti-HLA assessments more than 6 months posttransplantation may indicate progressive graft failure and could consequently affect therapeutic decisions. The second weakness was the fact that we assessed anti-HLA antibodies in general, but not DSAs. It is known that patients who were positive for anti-HLA antibodies should be tested for the presence of DSA because non-DSA probably does not increase the risk of AMR.[16] In a large group of kidney transplant recipients, it was observed that AMR or AMR overlapping with T–cell-mediated rejection was diagnosed in most patients with de novo DSA who underwent renal biopsy due to deterioration of graft function, whereas T–cell-mediated rejection was diagnosed mostly in DSA-negative recipients. It is known that prompt diagnosis of any rejection and appropriate immunosuppressive treatment are crucial to ameliorate further graft destruction.[17,18] Unfortunately, the single-antigen Luminex assays were unavailable when our study was initiated. Still, their costs limit repeating DSA Luminex study with an anti-C1q run with samples from the frozen sera. Interestingly, the presence of anti-HLA antibodies was correlated with the number of transplants and positive PRA test results before surgery. This suggested to us the effects of pretransplant alloantibodies (probably other than DSAs) that were not detected during crossmatch testing. Moreover, the percentage of patients who were positive for anti-HLA antibodies tended to increase with time, which may indicate recipients' actual humoral reactions to donor antigens rather than their previous immunization.[19]

Obviously, it would be extremely interesting to know more clinical details of the patients, such as a history of rejection episodes (acute or chronic), biopsy-proven reasons for the graft loss, as well as the number and type of blood transfusions. However, this was not the aim of the study and the retrospective character of data analysis did not permit a thorough clinical evaluation. On the other hand, our results, along with a great relevance of antibodies to graft rejection and survival, necessitate such detailed analysis in future studies. Also, the data on previous pregnancies would be of great value, particularly because more women were anti-HLA positive.

We found that anti-HLA antibodies were detected less frequently in patients treated with CsA in comparison with the remaining transplant recipients. Therefore, the exact analysis of the effect of immunosuppression on anti-HLA antibodies would also be of interest. We evaluated the immunosuppressive drugs only at the time of testing, although we previously reported that immunosuppression changes may significantly impact the outcomes.[20,21] The mentioned shortcomings result from the origin of the patient data source. The evaluation of retrospective registry data revealed that clinical information was not complete.

Biomarkers have emerged as essential tools for treatment and diagnosis of diseases since the variability of disease behavior, the cost and diversity of treatments, and the related impairment of quality of life have given rise to a need for a personalized approach. High-throughput technology platforms in proteomics and genomics have accelerated the development of potential biomarkers.[22-24] Our finding that anti-HLA antibodies may serve as the biomarkers

of the long-term kidney outcome after KTx provides rationale for further investigations, particularly in the field of immune monitoring in certain diseases.

In conclusion, the presence of anti-HLA, but not anti-MICA, antibodies in randomly acquired blood samples obtained at different times after KTx may predict inferior graft survival. We suggest testing for anti-HLA antibodies in all kidney transplant recipients to select those who require a more aggressive approach. Further studies are necessary to link our findings to specific clinical details.

Acknowledgments We wish to thank Dr. Paul Terasaki and Dr. Mikki Ozawa for their help with experimental procedures. Luminex assays for anti-HLA and anti-MICA antibodies were provided by Terasaki Foundation Laboratory, Los Angeles, California, United States.

Contribution statement MC, MK, TG, and LP designed the study. MC and DŻ performed the study. MC, DŻ, MD, and AG collected data. MC, KM, BF, and LP prepared the manuscript.

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