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CLINICAL RELEVANCE OF HLA ANTIBODIES IN RENAL TRANSPLANTATION

ABSTRACT OF PHD THESIS

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Contents of the PhD thesis

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PhD thesis abstract

Chronic kidney disease (CKD) is a worldwide public health problem associated with a relatively poor prognosis of survival, with higher frequency in the adult population, and lower in children and adolescents (Warady and Chadha, 2007). CKD includes all grades or stages of renal function decline that can range from at-risk to mild, moderate and severe kidney injury (Chen et al., 2019).

According to the Kidney Disease Quality Outcomes Initiative (Levin and Stevens, 2014), chronic kidney disease is associated with a glomerular filtration rate (GFR) less than 60 ml/min/1. 73 m2 that persists for a longer period of timeTo prevent worsening of the disease or the development of complications associated with the inability of the kidneys to eliminate toxins accumulated in the body (treatment-resistant hypertension, uremic encephalopathy, hydroelectrolyte imbalances associated with a high risk of death), dialysis (peritoneal/hemodialysis) or renal transplantation is performed to replace renal function.

Worldwide, the most common cause of chronic renal failure is diabetic nephropathy (Vallon and Komers, 2011) in which hyperglycemia causes hyperfiltration with damage to the nephrons, with formation of glycolysis end products in the glomerular basement membrane and extracellular matrix of the nephron, and increased production of cytokines (such as IL1 and TNF) and growth factors (such as growth factor beta (TGF β) and vascular endothelial growth factor (VEGF)) (Toth-Manikowski and Atta, 2015). As a result of the release of all the above-mentioned factors, 3 major histological changes can occur, such as glomerular basement membrane thickening, mesangial expansion, and glomerular sclerosis, each of which causes or is associated with an irreversible loss of nephron function (Lin and Susztak, 2016; Pourghasem et al., 2015).

The 2nd most common cause of chronic renal failure is hypertensive nephropathy in which persistent hypertension will cause thickening of the renal arterial walls (Stompór and Perkowska-Ptasińska, 2020). Thickening of the renal arterial walls is associated with a reduction in blood flow to the glomeruli, which will cause activation of the renin-angiotensin-aldosterone system (Fountain and Lappin, 2022). The activated renin-angiotensin-aldosterone system induces a further increase in blood pressure which will cause

further thickening of the arterial walls which may lead to ischaemic necrosis of the nephrons (Fountain and Lappin, 2022).

Vascular (such as renal artery stenosis, renal artery embolism, hypertensive nephrosclerosis, or renal vein thrombosis) or glomerular (such as membranous nephropathy, Alport syndrome, or membranoproliferative glomerulonephritis) diseases and untreated or unresponsive acute renal failure are other common causes of chronic renal failure (Kang and Park, 1987).

Dialysis can promote some complications, such as calcification of coronary arteries that can even lead to cardiovascular disease (Volodarskiy et al., 2016). These complications may be the consequence of activation of the patient's immune system (granulocytes, IgG antibodies and complement system, release of cytokines such as IL-1) against the semi-permeable membranes used (Volodarskiy et al., 2016).

Complications associated with dialysis but also the need to reduce treatment costs and frequent day hospitalization of patients makes renal transplantation the method of choice for renal function replacement.

Kidney transplantation is a surgical procedure that involves replacing a "defective" kidney with a healthy kidney from a donor (living, usually a close relative of the recipient, or from a brain-dead donor). The transplanted kidney is usually grafted lower than the normal anatomical position, especially in the right iliac fossa (due to easier vascular approach). This is the most common transplant and has a very high success rate.

The discovery and understanding of the immune system in general, and the HLA system and immunosuppressive drugs in particular, has made kidney transplantation the most successful form of solid organ transplantation. In 2011, M Tonelli et al find that cardiovascular risk and mortality risk were lower among kidney transplant patients compared to dialysis patients (Tonelli et al., 2011). The observation was made following a meta-analysis in which nearly 2 million patients (transplant and dialysis) from 110 studies published in MEDLINE and EMBASE databases were included (Tonelli et al., 2011).

According to the National Transplant Agency ("Annual Statistics," n.d.), at the end of 2021, 4918 Romanian patients with chronic kidney failure were on the waiting list for a

living/brain-dead donor kidney transplant. Of these, 480 patients with chronic kidney disease were enrolled in 2021 alone.

Currently, biomarkers such as serum and urine urea and creatinine do not allow the determination of risk of developing chronic kidney disease, they are used to establish the diagnosis of chronic kidney disease and monitor response to different types of treatment. To correct this shortcoming, a number of studies have focused on immunological mechanisms of disease occurrence in general and chronic kidney disease in particular. This research has identified human leukocyte antigen (HLA) class I and class II genes as possible risk factors for certain diseases such as autoimmune diseases but has failed to identify the exact mechanisms of their association (Rich et al., 2012).

Several drugs with different immunological mechanisms have been developed to promote renal transplantation from brain-dead non-identical HLA donors or living donors and to prevent hyperacute, acute or chronic renal allograft rejection and organ failure. Immunosuppressive drugs can, however, cause short- and long-term diabetes, de novo neoplasia, bacterial or viral infections in transplant patients (Hussain and Khan, 2021).

Even after adjustment of immunosuppressants and development of new drugs (Kalluri and Hardinger, 2012), the presence of different reported 5-year graft survival rates of renal allografts after transplantation (de Sousa et al., 2018; Phillpott et al., 2022; Zecher et al., 2017) may still be related to either HLA antibody-induced humoral rejection (Zhang, 2018) associated with small vessel injury (Halloran et al., 2014) or cellular rejection.

The lack of data on the incidence of anti HLA antibodies in patients on the waiting list for renal transplantation, the role of de novo anti HLA antibodies in the occurrence of rejection, the frequency of rejection, the level of blood immunosuppressants associated with de novo anti HLA antibodies, graft function and survival, the use of HLA epitopes to identify the risk of anti HLA antibodies as well as the effects of the presence of this type of antibodies among renal transplant patients in Romania were the reasons for the choice of the research topic.

The formulation of the research hypothesis took into account some of the observations highlighted in previous studies, adapted to the studied population and to the practice of the transplant center from which the patients included in the research were recruited.

Taking into account the above-mentioned aspects, in our PhD research we aimed to evaluate for the first time the presence and importance of anti HLA Ac in a sample of renal transplant patients and to make a contribution to the field, providing an answer to some of the uncertainties reported in previous studies.

In the general part of the PhD work we described the HLA genes - types, structure and functions, association with various diseases; mechanisms of appearance (T lymphocyte alloreduction by direct, indirect, and semi-direct pathways; activation of B lymphocytes and their transformation into plasma cells producing anti HLA and non-HLA antibodies) and their relevance in renal graft survival. The classes of immunosuppressants administered to post renal transplant patients in Romania and the types of rejection that can occur post renal transplant according to the Banff classification were also presented.

The major objective (clinical relevance of anti HLA antibodies) and a series of specific objectives (association of tacrolimus-type immunosuppressant levels with the occurrence of anti HLA antibodies, link between epitopes or CREGs and anti HLA antibodies, link between anti HLA antibodies and 5-year graft survival and identification of HLA genes associated with risk of chronic kidney disease) were established.

According to the proposed objectives the special part was structured into 2 studies. In the first study, the presence of pre- and post-transplant anti HLA antibodies, the dynamics of their evolution, and the effects of their presence in 347 patients who underwent a renal transplant procedure in a single transplant center were evaluated. The second study focused on the evaluation of HLA genes associated with the risk of developing chronic kidney disease.

The presence of preformed and de novo HLA antibodies was retrospectively analyzed in serum samples before transplantation and 1 to 5 years after transplantation. All recipients had an HLA gene and ABO blood group matched donor. None of the recipients had preformed DSA.

Class I and Class II HLA antibodies were screened with LABScreen Mixed™12, lot 023 (One Lambda, Inc., Canoga Park, CA, USA) on FlexMap3d (Luminex Corporation, USA). Positive screening was followed by HLA antibody identification with LS1A04 lot 013 (for Class I) and LS2A01 lot 014 (for Class II) kits from One Lambda (One Lambda, Inc., Canoga Park, CA, USA). Both screening and identification of HLA class I and class II

antibodies were performed following the manufacturer's instructions, and sample data were collected using xPONENT software version 4.3 (Luminex Corporation, USA) (Tait, 2016) and analyzed with HLA Fusion version 4.3 (One Lambda, Inc., Canoga Park, CA, USA). Based on a mean fluorescence intensity cutoff value of 1500, an anti HLA antibody was considered positive or negative. The identified anti HLA antibodies were then compared to the donor HLA, which allowed us to identify DSA (i.e., donor-specific antibodies) and non-DSA (i.e., non-donor-specific antibodies). We were also able to specify whether the HLA antibodies identified were preformed (formed before any kidney transplant) or de novo (arising after a kidney transplant). For categorical and continuous variables, the Fisher exact test or Chi-square test was used to compare the characteristics of renal transplant patients in groups differentiated by the presence or absence of pre-transplant HLA antibodies. Bonferroni correction was also applied for multivariate analysis.

Blood tacrolimus levels were determined using Tacrolimus Reagent kits and a chemiluminescent magnetic microparticle immunoassay (Architect i2000, Abbot).

The variability of tacrolimus blood levels was estimated by calculating the CV using the following equation: CV (%) = (standard deviation/mean concentration) × 100 (Mo et al., 2019). Cut-off values for the minimum level of intra-patient variability of tacrolimus at 1 to 5 years post-transplant for predicting HLA antibody production were determined using receiver operating curve (ROC) analysis.

Acute or chronic graft rejection were identified by kidney biopsies that were analyzed based on Banff criteria (Roufosse et al., 2018). Transplant survival data were recorded and stored until graft loss. Kaplan Meier analysis was used to plot renal graft survival rates and the log-rank test was used to compare groups. $p < 0.05$ was considered statistically significant.

At 5 years after transplantation, 48 (15%) kidney transplant patients had de novo anti HLA antibodies, the percentage of patients immunized post renal transplantation being comparable to that reported by Terasaki and Ozawa (Terasaki and Ozawa, 2004). Twenty-two (6.87%) patients had de novo anti HLA class I antibodies. De novo anti HLA class II antibodies were observed in 37 (13.07%) patients.

Antibody-mediated rejection was observed in 10 (20.83%) of the 48 patients in the de novo anti HLA antibody group and in 7 (25.93%) of the 27 patients in the pre-existing

anti HLA antibody group. T-cell-mediated rejection was diagnosed in 13 (48.15%) of the 27 patients in the pre-existing anti HLA group and in 19 (39.58%) of the 48 patients in the de novo anti HLA group.

The association of Tacrolimus blood levels with de novo HLA antibody production and rejection was studied. The ROC test was used to determine the cut-off value of Tacrolimus blood levels. Only Tacrolimus blood levels less than 4.6 ng/mL were associated with de novo HLA antibody positivity. The area under the ROC curve (used to determine cut-off values of 4.6 ng/mL was 0.85, with a 95% confidence interval of 0.80 to 0.88 and $p<0.05$. Tacrolimus blood levels ($p=0.2376$) were not associated with allograft rejection rate (Mărunţelu et al., 2022).

Another risk factor identified in this PhD study is mismatch at the HLA epitope level. A higher number of 18 mismatches at the HLA epitope level increases the risk of de novo anti HLA antibodies, which will negatively influence renal graft survival. The number of mismatches was determined using the HLA Matchmaker program (created and developed by René Duquesnoy and his team), based on existing mismatches at the epitope level between donor and recipient HLA antigens (Daniëls et al., 2018). Because the HLA Matchmaker program allows epitope studies only based on HLA genotypes determined at the high-resolution level, and because in our transplant center all kidney transplant donors and recipients are genotyped at the low-resolution level, HLA genes obtained at the low-resolution level were converted to HLA genes at the high-resolution level based on the frequency of HLA genes in the population (according to http://www.allelefrequencies.net/).

We also examined the possible association of donor age, donor type and recipient age, de novo HLA antibodies with renal rejection in both groups. In patients without previous sensitization, only donor age (OR=1.07, $p= 0.001$) and donor type= "cadaveric donor" ($p<0.0001$) were associated with higher allograft rejection rates, while recipient age ($p= 0.048$), after applying Bonferroni correction, did not remain statistically significant. In patients with previous sensitization, only donor type= "living" ($p<0.001$) was associated with higher allograft rejection rates, while donor age ($p=.06$) and recipient age ($p=.481$) did not influence post-transplant renal graft outcome (Mărunţelu et al., 2022).

In patient groups without prior anti HLA antibodies, graft loss-censored renal graft survival rates were 95.8% vs 98.9% (1 year), 89.6% vs 97.1% (2 years), 85.4% vs 95.2% (3

years), 81.3% vs 92.6% (4 years), and 77.1% vs 90.8% (5 years). The graft survival rate was significantly lower in recipients with de novo anti HLA antibodies at 5 years after transplantation (p=.004, hazard ratio= 4.0251) (Mărunţelu et al., 2022).

In the groups of patients with preformed anti HLA antibodies, the renal graft survival rates censored on rejection were 96.3% (1 year), 92.6% (2 years), 88.9% (3 years 77.8% (4 years) and 74.1% (Mărunţelu et al., 2022).

Comparing both groups of patients, it was observed that there was a significant difference in the 5-year survival distributions after renal transplantation (p=.0286) (Mărunţelu et al., 2022).

Only 4 patients with previous anti HLA antibodies and 8 patients without previous HLA sensitization develop donor-specific HLA antibodies (DSA) after kidney transplantation. In our patients, between non-DSA and DSA there was no significant difference in 5-year post renal transplant survival distributions (p=.754) (Mărunţelu et al., 2022).

To perform the HLA gene association study with chronic renal failure, 2199 patients with chronic renal failure (with or without renal transplantation) were retrospectively enrolled together with 2786 healthy donors from the National Voluntary Hematopoietic Stem Cell Donor Registry with demographic data (such as age and sex) and known HLA genotypes.

Based on human genomic DNA extracted from blood collected on Ethylene-Diamino-Tetra-Acetic Acid (EDTA) using the QIAamp® DNA Blood Mini Kit (Qiagen), HLA class I genotypes (HLA-A, -B and -C) and HLA class II (HLA-DRB1, -DQB1 and -DP) genotypes were genotyped by primer-specific sequence polymerase chain reaction (PCR-SSP, low resolution) using HLA-Ready Gene kits (Innotrain Diagnostik GmbH).

Frequencies of all HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1 and HLA-DPB1 alleles and genotypes, and demographic data were analyzed in patients with chronic kidney disease and healthy donors using Microsoft Excel from the Office 365 package (Microsoft).

$\chi 2$ test or Fisher's test was performed in SPSS version 21.0 (IBM) to identify groups of HLA alleles, genotypes and haplotypes that may be associated with an increased risk of

chronic kidney disease. The $\chi 2$ test was used when the expected value was greater than 5, and Fisher's test was used when the expected value was less than 5. Odds ratios with 95% confidence intervals (95% CI) were also calculated in SPSS to determine the strength of each association of the allele, genotype or haplotype studied with the risk of chronic kidney disease.

Estimation of HLA haplotype frequencies was performed using the estimation-maximization algorithm of Arlequin 3.5 software (Swiss Institute of Bioinformatics).

To avoid the risk of misidentification of allele, genotype or haplotype associations with chronic kidney disease risk, the Bonferroni correction formula for multiple testing was applied for all $p<.050$ values.

The results obtained in the framework were similar to those presented in the literature, but also revealed differences specific to Romanian transplant patients.

More than 59% of patients were male, and more than 70% were aged between 26 and 45 years. No significant difference was observed between patients and controls when analysing the age and gender distribution.

In each cluster analyzed, 19 alleles from locus A, 29 alleles from locus B, 13 alleles from locus C, 15 alleles from locus DRB1, 5 alleles from locus DQB1 and 12 alleles from alleles from locus DPB1 were identified.

After applying Bonferroni correction, we found that certain groups of HLA alleles were associated with chronic kidney disease: HLA-B*40 (OR = 1.4661, $p \leq .001$, pc ≤ 001), HLA-C*12 (OR = 1.6966, $p \leq .001$, $pc \leq .001$), HLA-C*15 (OR = 1.8005, $p \leq .001$, $pc \leq .001$), HLA-DRB1*14 (OR = 1.2868, p = .002, pc = .04) and HLA-DPB1*02 (OR = 1.498, p = .004, pc = .048) (Mărunţelu et al., 2021).

Doctoral research revealed several allele combinations that remained or were not significantly and positively associated with chronic kidney disease after Bonferroni correction: A*01.11 (OR = 1.73, p = .004, pc = .2437), A*03.32 (OR = 2.33, p = .003, pc = .2121), C*12.- (OR = 2.51, $p < .001$, $pc < .001$), DRB1*04,- (OR = 2.242; p = 0.006, pc = 0.7002), DRB1*04,14 (OR = 2.44; p = 0.004, pc = 0.3596) and DRB1*14,- (OR = 2.191; p = 0.007, pc=..042) (Mărunţelu et al., 2022).

Using the estimation-maximization algorithm included in the Arlequin software (version 3.5), we analyzed all HLA- A, -B, -C, -DRB1, -DQB1 and -DPB1 haplotypes combinations of two, three, four, five and six loci found in Romanian patients with chronic kidney disease. To determine the haplotypes that were significantly positively associated with chronic kidney disease in our patients, Bonferroni correction was performed by multiplying the P-value obtained by the number of haplotypes identified for each locus combination (e.g. 1000 haplotypes identified for the A-C loci combination): A*01-C*15 (p = .0003, pc=.030) and A*02-C*12 (p = 0.0005, pc=.0486) (Mărunţelu et al., 2022).

The results of the HLA gene study indicate that several groups of HLA alleles and genotypes are strongly associated with chronic kidney disease in the Romanian population.

One of the personal contributions is the identification of new risk factors not previously described in association with the occurrence of anti HLA antibodies in Romanian patients. By analyzing the association between tacrolimus blood level, in patients treated with tacrolimus calcineurin inhibitor) and the development of anti HLA antibodies, a minimum blood tacrolimus level of 4.6 ng/ml associated with a lower risk of developing antibodies was identified. Another important observation was related to the significant impact of de novo anti HLA antibodies in decreasing graft function at 5 years post-transplant.

Our study demonstrated that graft survival at 1 year is 98.5%, decreasing to approximately 90%. The second study aimed to identify HLA genes that may be associated with an increased risk of renal graft disease but also with possible renal graft dysfunction. Another personal contribution is the identification of HLA genes that may be associated with an increased risk of developing and progressing to stage 5 renal failure requiring renal replacement therapy or even renal transplantation. Choosing kidney donors who do not carry genes that predispose to chronic renal failure could improve the long-term prognosis of kidney transplants, given that there are pathologies that can recur post-transplant and damage the transplanted organ.

The major limitations of the studies are the small number of patients and the short follow-up time. As a result, changes in transplant procedures and immunosuppression over time may lead to an overestimation of survival in our study (Graham et al., 2022). Other limitations are the kits used for HLA antibody analysis, which are suitable for identifying the target protein but lack the ability to quantify antibody levels and the MFI threshold value,

which may differ from values used in other laboratories. However, this is the first report of the association between immunosuppressive therapy and de novo anti HLA antibody production after transplantation and graft survival in the Romanian population.

As chronic kidney disease is still a relatively common condition, it is not possible to exclude this in the control population. Therefore, some subjects would have been diagnosed with CKD during their lifetime.

Future studies should address the limitations mentioned above and verify whether increasing the number of patients and follow-up time will confirm our findings. Prospects for further research during the post-doctoral period are to conduct a multicenter study including all renal transplant patients.

We believe that this research could be extended to determine whether selecting kidney donors without HLA allele clusters or genotypes susceptible to the development of chronic renal failure could lead to higher renal allograft survival rates in our patients. At the same time, studying HLA epitopes to determine the maximum number of miss-matches associated with a low risk of de novo anti HLA antibody development as well as the level of pro-inflammatory cytokines in antibody-positive patients may be future research topics and would allow nephrologists to better manage kidney transplant patients to improve their clinical status but also to prevent chronic graft dysfunction as long as possible.

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