Impact of Covid-19 on HLA antibody profile in renal transplant recipients

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Abbreviations

ADP- adenosine 5′-diphosphate

anti-HLA- Antibodies against human leukocyte antigens

BKV- BK virus

BMI- Body mass index

CKD-EPI- Chronic Kidney Disease Epidemiology Collaboration equation

CMV- Cytomegalovirus

COVID-19- Novel coronavirus disease 2019

DNA- Deoxyribonucleic acid

DSA- Donor-specific antibodies

EBV- Epstein-Barr virus

eGFR- Estimated glomerular filtration rate

F- Female

HLA- Human leukocyte antigens

IQR- Interquartile range

IVIg- Intravenous immunoglobulin

MFI- Mean fluorescence intensity

MHC- Major histocompatibility complexes

MM- Class I and class II mismatch

mRNA- Messenger ribonucleic acid

PRA- Panel reactive antibodies

RT-PCR- Real-time reverse transcriptase polymerase chain reaction

SARS-CoV-2- Severe acute respiratory syndrome coronavirus 2

TNF- tumor necrosis factor

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Summary

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Exposure to different tissues such as in organ transplantation, pregnancy, or blood transfusions, can result in the production of antibodies against human leukocyte antigens (HLA). It has been demonstrated that the presence of anti-HLA donor-specific antibodies (DSA) among renal transplant recipients is a substantial risk factor for graft deterioration. The novel coronavirus disease 2019 (COVID-19) brought on by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been hypothesized to cause an unusual immunological dysregulation that can in some populations, such as kidney transplant recipients, lead to a number of complications. The outcomes of 321 kidney transplant recipients who had COVID-19 illness were assessed in this prospective observational cohort study. Additionally, after an acute COVID-19 infection, factors influencing the development of HLA *de novo* DSA and non-DSA specificities were assessed. Logistic regression analysis was used to analyze the independent factors associated with the development of anti-HLA *de novo* DSA and non-DSA. A stepwise multivariable logistic regression was used to assess the association between potential risk factors and the development of anti-HLA *de novo* DSA and non-DSA specificities after COVID-19, adjusting for known confounders. The variables evaluated were acute COVID-19 characteristics (i.e., presentation, need for hospitalization), demographic characteristics (i.e., age, gender, and primary renal disease), clinical characteristics (i.e., various comorbidities), and post-COVID-19 sequelae. Anti-HLA *de novo* DSA developed in 18,7% of patients, and they were more likely to be female. Anti-HLA class I antibodies developed *de novo* in 84 (26,3%) patients, while anti-HLA class II antibodies developed *de novo* in 83 (25,9%) patients. There was an increased prevalence of certain anti-HLA class II antibodies. The development of DSA, HLA-DQ, and HLA-DR was predicted by the history of graft rejection. Obesity appears to serve a protective role against the emergence of *de novo* DSA. *De novo* DSA and HLA-DR formation was positively linked with intravenous immunoglobulin use, CMV-hyperimmune globulin use, and decreased doses of immunosuppression during acute infection. Better allograft function during acute disease was a protective factor against the formation of HLA-DQ and HLA-DR. Positive predictors of *de novo* DSA development were graft biopsy and reactivation of EBV after infection. In conclusion, these findings suggest that the SARS-CoV-2 virus has an immunomodulatory effect and may be associated with an increase in mortality in this population. Further research with long-term follow-up is required.

KEYWORDS: COVID-19, renal transplantation, SARS-CoV-2, anti-HLA, DSA

Sažetak

Utjecaj bolesti COVID-19 na anti-HLA protutijela u primatelja bubrežnog presatka

Marina Kljajić

Antitijela protiv antigena humanog leukocita (engl. Human Leukocyte Antigen, HLA) nastaju nakon izlaganja stranim HLA antigenima koji u organizam mogu dospjeti nakon transplantacije organa, trudnoće ili transfuzije krvi. Istraživanja su pokazala da da je prisutnost anti-HLA donorskih antitijela (engl. Donor Specific Antibodies, DSA) značajan čimbenik rizika za odbacivanje bubrežnog presatka. Pretpostavlja se da nova bolest koronavirusa 2019. (COVID-19) uzrokovana teškim akutnim respiratornim sindromom koronavirus 2 (SARS-CoV-2) može uzrokovati imunološku promjenu koja u određenim populacijama poput primatelja bubrežnog presatka uzrokuje razne komplikacije. U ovoj prospektivnoj kohortnoj studiji opisani su ishodi 321 primatelja bubrežnog presatka nakon bolesti COVID-19. Nadalje, procijenjeni su čimbenici koji utječu na razvoj HLA *de novo* DSA i ne-DSA nakon akutne infekcije COVID-19. Logistička regresijska analiza korištena je za analizu neovisnih čimbenika povezanih s razvojem anti-HLA *de novo* DSA i ne-DSA. Za procjenu povezanosti između potencijalnih čimbenika rizika i razvoja anti-HLA *de novo* DSA i specifičnosti izvan DSA nakon COVID-19 korištena je „stepwise“ logistička regresija, prilagođavajući se poznatim čimbenicima zabune. Procijenjene varijable uključivale su demografske karakteristike (tj. dob, spol, primarnu bolest bubrega), kliničke karakteristike (tj. različite komorbiditete), akutne karakteristike COVID-19 (tj. kliničku prezentaciju, potrebu za hospitalizacijom) i komplikacije nakon bolesti COVID-19. Anti-HLA *de novo* DSA razvila su se u 18,7% bolesnika od kojih je većina bila ženskog roda. Anti-HLA klasa I antitijela razvila su se *de novo* u 84 (26,3%) bolesnika, dok su anti-HLA klasa II antitijela razvijena *de novo* u 83 (25,9%) bolesnika. Zabilježena je veća prevalencija određenih antitijela protiv HLA klase II među pacijentima. Povijest odbacivanja presatka bila je prediktivni čimbenik za razvoj DSA, HLA-DQ i HLA-DR. Pretilost se pokazala zaštitnim čimbenikom protiv razvoja *de novo* DSA. Smanjene doze imunosupresivnih lijekova te primjena intravenskog imunoglobulina i CMV-hiperimunog globulina tijekom akutne infekcije našli su se u pozitivnoj korelaciji s razvojem *de novo* DSA i HLA-DR. Bolja funkcija presatka tijekom akutne bolesti bila je zaštitni faktor protiv razvoja HLA-DQ i HLA-DR. Pozitivni prediktori razvoja *de novo* DSA bili su biopsija presatka i reaktivacija EBV-a nakon COVID-19 infekcije. Zaključno, ovi rezultati ukazuju na imunomodulatorni učinak virusa SARS-CoV-2 te mogu biti povezani s povećanom smrtnošću u ovoj populaciji. Potrebna su daljnja istraživanja s dugoročnim praćenjem.

KLJUČNE RIJEČI: COVID-19, bubrežni presadak, SARS-CoV-2, anti-HLA antitijela, DSA

Introduction

Human leukocyte antigens (HLA) are genes in major histocompatibility complexes (MHC) that encode proteins responsible for regulating our immune system and distinguishing between "own" and "foreign" antigens (1). In accordance with the functions and characteristics of their genetic products, HLA are separated into three regions on the short arm of chromosome 6 and categorized as class I, class II, and class III (2,3). Class I HLA are present on the surface of almost all nucleated cells and are involved in the presentation of endogenous peptides to responding CD8+ T Cells (2,3). On the other hand, HLA class II molecules are restricted to antigen-presenting cells and involved in exogenous peptide presentation to CD4+ helper T Cells. Instead of HLA molecules, the class III region encodes immune regulatory molecules such as tumor necrosis factor (TNF), complement factor C3, C4, and C5, and heat shock proteins (2,3). Antibodies against human leukocyte antigens (anti-HLAs) are produced after exposure to different tissue during an organ transplantation, pregnancy, or blood transfusions (3). They can be divided into class I and class II anti-HLAs. Anti-HLA donor-specific antibodies (DSA), directed specifically against the donor's HLA antigens, have been demonstrated to be an important risk factor for graft rejection or loss in renal transplantation (4). According to studies, between 4% to more than 50% of transplant patients have anti-HLA DSA (5). It is therefore advisable to frequently check the anti-HLA antibody profiles of kidney transplant recipients (6). The majority of anti-HLA antibodies develops 6 months after the transplantation. Class I anti-HLA antibodies are usually the first ones to appear, followed by Class II (7). Understanding the mechanism of HLA antibody-mediated graft damage is essential given the close correlation between the presence of HLA antibodies and reduced transplant function and survival. Complement activation is the main component of acute and hyperacute graft rejection (8). Hyperacute rejection happens within hours after transplantation and is mediated by preformed anti-HLA antibodies in pre-sensitised patients (8). This type of rejection nowadays occurs rarely because of pre-transplant crossmatch tests and desensitization protocols. During the crossmatch test, donor lymphocytes are mixed with recipient serum in order to prove/disprove the presence of the recipient’s antibodies that react with the donor HLA on lymphocytes (9). If anti-HLA DSAs develop after the transplantation, their binding to donor cells activates the complement cascade and promotes neutrophil infiltration, which leads to microvascular injury and, consequently, graft dysfunction and failure (8). This antibody-mediated acute rejection is characterized by immunologic evidence of renal injury and evidence of circulating anti-HLA DSA (9). Another type of acute rejection is acute T cell-mediated rejection, and occurs when the recipient's lymphocytes are activated because of antigen-presenting cells directly, indirectly, or both recognizing foreign donor antigens in the transplanted organ. This activation and infiltration of T cells cause damage to the allograft (9). In addition to the features of acute rejection, cellular proliferation, apoptosis, and development of vascular lesions also occur in the setting of chronic rejection due to intracellular signaling triggered by agonistic activation of HLA I, HLA II, endothelial, or epithelial cell surface markers (8). The main risk factor for chronic rejection is

noncompliance with immunosuppressive therapy, and when this is mediated by newly developed anti-HLA DSA, it is referred to as chronic antibody-mediated graft rejection (9).

Allograft dysfunction has been linked to several viral infections, including Epstein-Barr virus (EBV), Herpes simplex, varicella zoster, and cytomegalovirus (CMV). It has been postulated that these infections stimulate the development of anti-HLA antibodies via T-cell cross-reactivity (10–12).

The Novel coronavirus disease 2019 (COVID-19) pandemic has had a significant global impact on kidney transplant recipients. They have had serious outcomes (hospitalization, intensive care unit admission, and death) from COVID-19–related disease both during the acute infection and in the post-COVID follow-up (13–16). Moreover, many transplantation surgeries were delayed or cancelled, which impacted the management and care of wait-listed patients (17). Increased mortality from COVID-19 disease among kidney transplant recipients is explained by a higher incidence of concomitantly present illnesses such as diabetes mellitus, hypertension, iatrogenic-induced immunosuppression and cardiovascular diseases, which are all known risk factors for the development of severe form of the disease (17). Chronic immunosuppressive therapy decreases T and B- cell function and further contributes to the worse outcomes in the transplant population (17). Kidney graft rejection following COVID-19 disease has been described in the literature (18–20). Vasquez-Jimenez et al. (18) performed kidney graft biopsies in 20 kidney transplant patients 4 weeks after recovery from COVID-19 disease and demonstrated histological signs of graft rejection among 14 (70%) patients. It has been postulated that COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes unusual immunologic dysregulation and triggers allosensitivity in kidney transplant recipients by the formation of HLA *de novo* DSA and non-DSA specificities.

The aim of this study was to investigate factors affecting the development of HLA *de novo* DSA and non-DSA specificities after acute COVID-19 infection in kidney transplant recipients, which may complicate the post-transplant period with graft rejection.

Hypothesis

It has been postulated that the Novel coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes unusual immunologic dysregulation and triggers allosensitivity in kidney transplant recipients by the formation of HLA *de novo* DSA and non-DSA specificities.

Objectives

The aim of this study was to:

- Evaluate the likelihood of development of anti-HLA *de novo* DSA and non-DSA specificities after COVID-19 infection in renal transplant recipients considering their demographic characteristics (i.e., age, gender, primary kidney disease), clinical characteristics (i.e., different comorbidities), acute COVID-19 characteristics (i.e., presentation, need for hospitalization) and post-COVID-19 complications.

Materials and Methods

Following the initial diagnosis of COVID-19 disease, the outcomes of 321 patients were assessed in this prospective observational cohort study. The hospital database was used to obtain information on the patient's characteristics prior to SARS-CoV-2 infections, including their demographics, comorbidities, primary kidney disease, maintenance immunosuppression regimen, history of graft rejection, vaccinations, and HLA class I and class II mismatches (MM) before transplantation. Body mass index (BMI) was calculated for each patient using the standard formula, and patients were divided into 4 categories: underweight (BMI <18.5 kg/m^2), normal weight (BMI 18.5–24.9 kg/m^2), overweight (BMI 25–29.9 kg/m^2) and obese (BMI ≥ 30 kg/m^2). Data were modified after being taken out of the electronic medical record i.e., shown as binary data, to be used for statistical analysis. For the missing data, complete-case analyses were used, i.e., subjects with missing data were excluded from the individual variable analysis. No input of missing data was performed, and this resulted in different numbers of patients in the analysis of different variables. Patients have been assessed in regular follow-ups, with the first evaluation occurring three months after the acute SARS-CoV-2 infection. To assess clinical complications, patients were interviewed by a standardized survey by trained transplant nephrologists to recount symptoms during the acute illness and whether they persisted or some new occurred to assess clinical complications. They received a thorough physical examination as well. Individually, additional diagnostic techniques from the laboratory and radiology were used. Acute COVID-19 features, changes in immunosuppressive therapy, and treatment during the infection were all documented. Venous blood samples were collected for complete blood count, biochemistry, coagulation examinations (prothrombin time, activated partial thromboplastin time and fibrinogen), D-dimers, C3, C4, total complement, platelet aggregation with ADP (adenosine 5′-diphosphate), serum electrophoresis and virology (molecular diagnostic detection for cytomegalovirus (CMV), Epstein-Barr virus (EBV) and BK virus (BKV)). The HLA class I MMs (HLA-A and -B) were determined at a broad serological level, while HLA class II MM (HLA-DRB1) were determined at split serological level based on low-resolution typing before the transplantation. Anti-HLA antibodies' detection were performed by Luminex bead-based technology (Immucor or One lambda). Results were compared with historical values. No data regarding the SARS-CoV-2 serology was available. Allograft dysfunction was defined as the new onset increase in serum creatinine by 25% or by newly developed proteinuria. The primary outcomes included the development of anti-HLA *de novo* DSA and non-DSA specificities. Categorical data were presented by absolute and relative frequencies. The normality of the distribution of continuous variables was tested by the Shapiro-Wilk test. Continuous data were described by the median and the limits of the interquartile range (IQR). The Mann–Whitney U test was used to compare the median between the two groups, while Fisher's exact test was used to analyze the differences between proportions. Logistic regression analysis was used to analyze the independent factors associated with the development of anti-HLA *de novo* DSA and non-DSA. A stepwise multivariable logistic regression was used to assess the

association between potential risk factors and the development of anti-HLA *de novo* DSA and non-DSA specificities after COVID-19, adjusting for known confounders. Variables assessed included demographic characteristics (i.e., age, gender, primary kidney disease), clinical characteristics (i.e., different comorbidities), acute COVID-19 characteristics (i.e., presentation, need for hospitalization) and post-COVID-19 complications. Parameters with statistical significance in the univariate analysis were incorporated into the multivariate logistic regression model for in-depth analysis. The level of significance was set at an Alpha of 0.05. Considering the relatively small sample size and the possibility of overfitting in the multivariate logistic regression model, we adopted a stepwise forward method (probability for stepwise: entry $P < 0.05$, removal $P > 0.1$) for logistic regression analysis to reduce the number of independent variables entering the model. There was no substitution of the missing data. The statistical analysis was performed using MedCalc® Statistical Software version 19.6 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020) and the IBM SPSS Stat. 23 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.).

Results

Study population

From March 2020 to December 2022, 408 out of the initial cohort of 1432 patients who received renal allograft at Clinical Hospital Centre Zagreb developed COVID-19 disease, proved by positive SARS-CoV-2 real-time reverse transcriptase polymerase chain reaction (RT-PCR) on the nasopharyngeal swab and were potentially eligible for investigation. Twenty-five patients died in the period during or after the infection, and 62 patients have not been assessed in our clinic and were therefore excluded from the study population (Figure 1). Most frequent causes of death reported in COVID-positive patients were sepsis (19 patients), acute respiratory insufficiency (4 patients), and acute myocardial infarction (3 patients). Sepsis and acute respiratory insufficiency were reported together as causes of death in 3 patients, while sepsis and acute myocardial infarction were reported together in 1 patient.

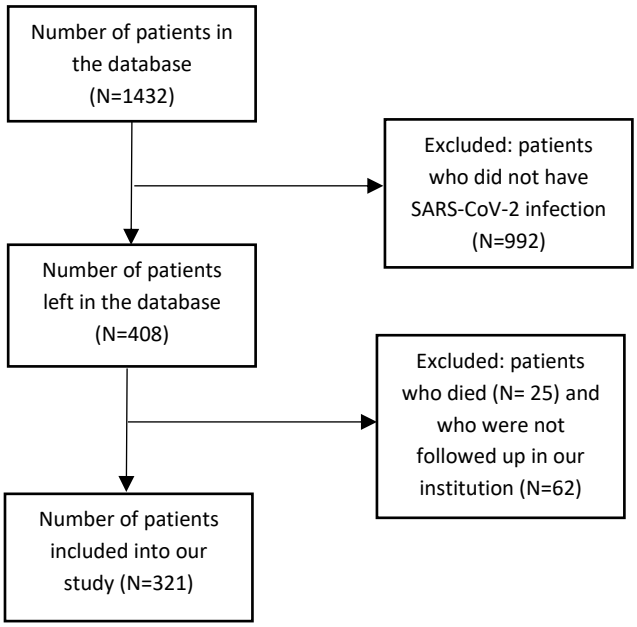

Figure 1. Flowchart of the study population.

There was a slight overrepresentation of male patients in the studied population (57%). According to BMI calculations prior to SARS-CoV-2 infection, there were 105 (32,8%) patients who were of normal weight, compared to 4 (1,3%), 144 (45%), and 67 (20,9%) patients who were underweight, pre-obese, and obese, respectively. Forty-six patients (14,4%) had a history of allograft rejection.

One-hundred-and-fifty patients (46,6%) received at least one dose of the anti-SARS-CoV-2 vaccine before the infection. One hundred twenty-five (39,1%) patients required hospitalization, 141 (44,1%) developed pneumonia and 4 patients (1,3%) required mechanical ventilation. Treatment included immunosuppression modification in 233 patients (77,1%) and remdesivir in 53 patients (16,6%). CMV-

hyperimmune globulin was introduced in 30 patients (9,4%), and 13 (4,4%) patients received intravenous immunoglobulins (IVIg). In the short follow-up period after COVID-19 disease, one or more rehospitalizations were necessary for 70 patients (21,9%). Biopsy was performed in 25 (7,8%) patients due to worsening of allograft function. After SARS-CoV-2 infection CMV, BKV, and EBV reactivations were confirmed in 72 (22,5%), 61 (19,1%), and 115 (35,9%) patients, respectively (positive urine and/or blood PCR test). SARS-CoV-2 reinfection was confirmed in 78 (25,2%) patients.

Analysis of anti-HLA DSA and non-DSA specificities

Donor and recipient pairs were analyzed for HLA class I (HLA-A and –B) and HLA class II (HLA-DRB1) mismatches (MM). The following MM for HLA-A resulted: no MM in 55 (26,2%) patients, one MM in 124 (59%) and two MM in 31 (14,8%) patients. The MM analysis for HLA-B found no MM in 19 patients (9%), one MM in 119 (56,7%) and two MM in 72 (34,3%) patients. The following MM occurred for HLA-DRB1: no MM in 43 (20,5%) patients, one MM in 151 (71,9%) patients and two MM in 16 (7,6%) patients.

Anti-HLA class I antibodies developed *de novo* in 84 (26,3%) patients, while anti-HLA class II antibodies developed *de novo* in 83 (25,9%) patients. We have noticed a higher prevalence of certain anti-HLA class II antibodies among the patients, which we have shown in Table 1. There was an increase in the incidence of HLA-DR antibodies developed in 45 (14,1%) patients, with prevalence of HLA DR53 specificities developed in seventeen (5,3%) patients. An even greater increase was observed for HLA-DQ antibodies developed in 63 (19,7%) patients with increased incidence for specificities HLA-DQ7 (N=26, 8,1%), HLA-DQ8 (N=31, 9,7%) and HLA-DQ9 (N=28, 8,8%). HLA-DP antibodies developed in 4 (1,3%) patients. *De novo* anti-HLA DSA developed in 57 (18,7%) patients (Table 1).

Table 1. Anti-HLA donor-specific antibodies (DSA) and non-DSA developed *de novo* after SARS-CoV-2 infection. HLA, human leukocyte antigen.

HLA	**% of patients**
HLA class I	26,3
HLA class II	25,9
HLA DR	14,1
HLA DP	1,3
HLA DQ	19,7
HLA DQB1*06	1,3
HLA DR53	5,3
HLA DQ8	9,7
HLA DQ9	8,8

HLA DQ7	8,1
DSA	18,7

Analysis of predictors for anti-HLA *de novo* DSA development

Our bivariate analysis identified 10 significant predictors for the development of anti-HLA *de novo* DSA after SARS-CoV-2 infection (Table 2). The strongest predictors for *de novo* DSA development were previous graft rejection and female sex, while higher BMI and vaccination after COVID-19 decreased the probability of *de novo* DSA development. Furthermore, higher initial immunosuppression maintenance doses of prednisolone, adjustment of immunosuppressive therapy (decreasing Tacrolimus/Cyclosporin A dose), and use of CMV immunoglobulins and IVIg during the infection were also proven to be statistically significant predictors for *de novo* DSA development. From the post-COVID-19 period, a performed biopsy was a strong predictor of concurrent *de novo* DSA development. EBV reactivation also appeared to be in a positive correlation with *de novo* formed DSA. Stepwise multivariate regression analysis was used to further examine significant predictors for *de novo* DSA development. Two predictors (female sex (OR = 2,75) and previous graft rejection (OR = 5,97)) had a unique statistically significant contribution to the model. They are significant predictors that increase the probability of DSA development, while obesity decreases the probability (OR = 0,24). The model was completely statistically significant (X2= 26.9; P<0.001) and explained from 13% (according to Cox and Snell (12)) to 22% (according to Negelkerke (13)) variance in the presence of *de novo* DSA (Table 2).

Table 2. Bivariate and multivariate analyses used to examine predictors of *de novo* DSA development. F, female; BMI, body mass index; EBV, Epstein-Barr virus

BIVARIATE ANALYSIS	**ß**	**P**	**OR**	**95% CI**
Sex (F)	0,87	**0,004**	2,38	1,32 - 4,29
Previous graft rejection	1,84	**<0,001**	6,30	3,17 - 12,5
Vaccination after COVID-19 infection	-0,83	**0,03**	0,43	0,20 - 0,93
BMI	-0,09	**0,007**	0,91	0,84 - 0,97
Prednisolone dose	0,10	**0,002**	1,11	1,04 - 1,18
COVID period				
Decreasing Tacrolimus / Cyclosporin A	0,96	**0,03**	2,62	1,09 - 6,29
Hyperimmune anti-CMV globulin	1,02	**0,02**	2,76	1,19 - 6,42
Intravenous Immunoglobulins	1,32	**0,04**	3,73	1,09 - 12,72
Post COVID period				
Biopsy	1,20	**0,009**	3,33	1,35 - 8,24

EBV	0,73	**0,01**	2,08	1,16 - 3,75
MULTIVARIATE ANALYSIS				
Sex (F)	1,16	**0,006**	**3,20**	1,39 - 7,37
Previous graft rejection	1,79	**<0,001**	**5,97**	2,35 - 15,17
Nutritional status (obesity)	-1,41	**0,03**	0,24	0,07 - 0,89
Constant	-2,21	**<0,001**		

ß – coefficient of regression

Analysis of predictors for *de novo* HLA-DQ and HLA-DR antibodies development

The bivariate analysis identified 5 statistically significant predictors for the development of *de novo* HLA-DQ antibodies post-COVID-19 (Table 3). The strongest predictor for *de novo* HLA-DQ antibody development was previous graft rejection. Higher prednisolone maintenance doses and performed biopsies were also positive predictors. Better allograft function estimated by the Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI) during, and post SARS-CoV-2 infection decreased the probability of *de novo* HLA-DQ antibody development. Stepwise multivariate regression analysis showed that patients with BMI within the normal range (OR = 2,24) and those with previous graft rejection (OR = 3,84) had increased probability for *de novo* HLA-DQ development, while better allograft function estimated by CKD EPI values (during COVID-19 infection) decreases the probability (OR = 0,97). The model was completely statistically significant (X2= 31,1; P<0.001) and explained from 10% (according to Cox and Snell (12)) to 16% (according to Negelkerke (13)) variance in the presence of HLA-DQ (*de novo*) (Table 3).

Table 3. Multivariate analysis was used to examine predictors of *de novo* DQ antibody development (multivariate logistic regression – Stepwise method). eGFR, estimated glomerular filtration rate; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration.

BIVARIATE ANALYSIS	**ß**	**P**	**OR**	**95% CI**
Previous graft rejection	1,58	**<0,001**	4,87	2,49 - 9,52
Prednisolone dose	0,08	**0,006**	1,09	1,02 - 1,16
COVID period				
eGFR (CKD EPI)	-0,02	**0,002**	0,98	0,96 - 0,98
Post COVID period				
eGFR (CKD EPI)	-0,02	**0,008**	0,98	0,97 - 0,99
Biopsy	0,93	**0,04**	2,54	1,06 - 6,07
MULTIVARIATE ANALYSIS				

Nutritional status (normal weight)	0,81	**0,01**	2,24	1,19 - 4,21
Previous graft rejection	1,35	**<0,001**	3,84	1,83 - 8,05
eGFR (CKD EPI) – post COVID-19	-0,02	**0,01**	0,97	0,96 - 0,98
Constant	-1,02	**0,02**		

ß – coefficient of regression

The bivariate analysis identified 4 statistically significant predictors for the development of *de novo* HLA-DR antibodies after COVID-19 disease (Table 4). The strongest protective effect was exhibited by better allograft function estimated by CKD-EPI value during SARS-CoV-2 infection and at the post-COVID check-up. Previous graft rejection and IV Ig application increased the probability of *de novo* HLA-DR antibody development. Furthermore, the multivariate statistical analysis confirmed that previous graft rejection (OR = 3,84) is a significant factor that increases the probability of *de novo* HLA-DR antibody development, while better allograft function estimated by CKD EPI values (during COVID-19 infection) decreases the probability (OR = 0,97). The model was completely statistically significant (X2= 17,4; P<0.001) and explained from 7% (according to Cox and Snell (12)) to 13% (according to Negelkerke (13)) variance in the presence of HLA-DR (*de novo*) (Table 4).

Table 4. Bivariate and multivariate analyses used to examine predictors of *de novo* HLA-DR antibody development. CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration.

BIVARIATE ANALYSIS	**ß**	**P**	**OR**	**95% CI**
Previous graft rejection	1,08	**0,004**	2,95	1,41 - 6,19
COVID period				
eGFR (CKD EPI)	-0,02	**0,004**	0,98	0,96 - 0,99
Intravenous Immunoglobulins	1,43	**0,02**	4,19	1,3 - 13,5
Post COVID period				
eGFR (CKD EPI)	-0,03	**0,001**	0,97	0,96 - 0,99
MULTIVARIATE ANALYSIS	ß	P	OR	95% CI
Previous graft rejection	1,35	**0,003**	3,84	1,58 - 9,36
eGFR (CKD EPI) – post COVID-19	-0,02	**0,04**	0,97	0,96 - 0,98
Constant	-1,03	**0,04**		

ß – coefficient of regression

Discussion

Anti-HLA de novo DSA developed in 57 out of 305 (18,7%) patients, which is less than the percentage obtained in the study from Girnita et al. (21), where 14 of 46 (30%) patients developed DSA, and more than in the study from Masset et al. (22) where the global incidence of post-COVID-19 DSA was 4% (7 out of 179 patients). According to our results, patients who developed *de novo* DSA were more likely to be female. The history of graft rejection appeared to be a common predictive factor for the development of DSA, HLA-DQ, and HLA-DR, which could be explained by an increased predisposition to the alloimmune response of those patients. Interestingly, obesity appeared to be a protective factor against the development of *de novo* DSA. The presence of obesity in transplanted patients reduced the likelihood of *de novo* DSA development after SARS-CoV-2 infection, whereas normal body weight was associated with an increased prevalence of newly developed HLA-DQ antibodies. The connection between obesity-related inflammation and transplant rejection is insufficiently understood. Although inflammatory cytokines and cytotoxic cells present in chronic inflammation in obese people boost the inflammatory response, it is unknown if this process affects allosensitivity (23). Obese individuals' functional T regulatory cells' impairment may be a factor in their lack of immunological response and possibly their decreased association with organ rejection (23). A study from Killian et al. (24) found that patients with new or increased DSA levels were less likely to have received at least one dose of a COVID-19 vaccination prior to infection (0% vs. 28%, $p = 0.018$), while our bivariate analysis found vaccination after COVID-19 infection to be a protective factor against *de novo* DSA development. Furthermore, the results of our bivariate analysis showed that a higher pre-admission immunosuppressant dose of prednisolone increased the likelihood of both *de novo* DSA and HLA-DQ development, which is in accordance with the results from Killian et al. (24). Their study found that patients with new or increased DSA levels had higher pre-admission immunosuppressant doses, which is consistent with more recent transplantation and stronger immunosuppression (24). Moreover, treatment with IVIg and CMV-hyperimmune globulin increased the likelihood of *de novo* DSA and HLA-DR development. Application of IVIg and CMV hyperimmune globulin was initiated in patients with a worse clinical presentation of COVID-19 disease, suggesting that those patients were more likely to develop *de novo* DSA and HLA-DR. The severity of COVID-19 disease was proven to be positively correlated with anti-SARS-CoV-2 antibody levels (25). Some authors suggest that kidney transplant recipients after COVID-19 infection exhibit a generalized humoral immune response to both donor-HLA and SARS-CoV-2, even though the relationship between anti-SARS-CoV-2 antibodies and anti-HLA DSA is not entirely understood (26). Our bivariate analysis showed that lowering the doses of maintenance immunosuppression (decreasing the dose of Tacrolimus / Cyclosporin A) during the time of acute SARS-CoV-2 infection positively correlated with the probability of *de novo* DSA development. The idea behind reducing immunosuppression during the acute COVID-19 disease was to help the immune system fight the infection as a functioning immune system is critical for protection against

severe forms of COVID-19 disease which was confirmed by Liang et al. (27) who found that cancer patients with neutropenia have an unfavorable course of COVID-19 disease. Some authors suggest that decreased immunosuppression during viral disease treatment is the reason for the increased graft rejection rates (28,29). However, it should be noted that the patients who required immunosuppressive dose adjustment also had severe forms of COVID-19 disease. Better allograft function during the acute COVID-19 disease (estimated by CKD EPI values), appeared to be a common protective factor against both HLA-DQ and HLA-DR development. Two positive predictors of *de novo* DSA development, namely graft biopsy (performed in patients with worsening renal function) and reactivation of EBV after infection, suggest an immunomodulatory effect of the SARS-CoV-2 virus. The reactivation of dormant viruses such as EBV after COVID-19 infection is attributed to the ability of the virus to trigger immune system dysregulation (30). However, since graft biopsies were indicated only in patients with declining graft function (who may have already developed graft rejection at the time of the procedure), it is questionable whether graft biopsy per se can be a predictive factor for the development of *de novo* DSA. Similar to our study, Killian et al. (24) found an increased DSA response to be associated with impaired allograft function.

In this study *de novo* anti-HLA class I antibodies were slightly more prevalent (84/236 (26,3%)) than anti-HLA class II (83/237 (25,9%)) antibodies developed in post-COVID-19 infection period which is contrary to the results obtained in the study from Girnita et al. (21) where more anti-HLA antibodies were predominantly directed against HLA Class II (20/26, 77%). Girnita et al. (21) also found that most DSAs targeted HLA-DQ (71%), with a dominant IgG isotype and IgG1 subtype prevalence (93%), and/or IgG3 (64%), followed by IgG2 (36%) (21). In our study, HLA-DQ was the most prevalent type of *de novo* class II HLA antibodies while developed in 63 (19,7%) patients, followed by HLA-DR which developed in 45 (14,1%) patients. The HLA-DQ serotype which developed most frequently was HLA-DQ8 (9,7%), while HLA-DQ9 and HLA-DQ7 were reported slightly less frequently; 28 (8,8%) and 26 (8,1%) respectively. HLA-DR53 was the most prevalent HLA-DR serotype developed post-COVID-19 infection in kidney transplant recipients (17 (5,3%)).

Vaccinations are regarded as immunosensitizing events among kidney transplant recipients (31). Several studies showed that in contrast to immunocompetent patients, a decreased proportion of solid organ transplant recipients mount a positive antispike serologic antibody response after SARS-CoV-2 vaccines (32–34). These patients are, therefore, at an increased risk of developing a breakthrough infection which can present as a severe form of COVID-19 disease (35–37). It is still debated whether SARS-CoV-2 vaccination influences immune response against HLA antigens. Vnučak et al. (38) reported on a 25-year-old female kidney transplant recipient who developed new acute humoral and cellular rejection 2 weeks after administration of the adenovirus vectored SARS-CoV-2 vaccine. The example of a 78-year-old kidney transplant patient who had acute T cell-mediated rejection following the second dose of the SARS-CoV-2 mRNA vaccine was also described by Jang et al. (39). Another case report described acute

cellular rejection in a 51-year-old kidney transplant recipient after vaccination with an inactivated SARS-CoV-2 vaccine (40). However, based on the limited series of 17 adult kidney transplant candidates, Kumar et al. (41) concluded that the SARS-CoV-2 mRNA vaccine may not be a significant source of allosensitization. They assessed changes in panel reactive antibodies (PRA) and the flow cross-match among their patients and found that both Class I and Class II PRA remained unchanged pre- and post-vaccination (41). Moreover, Nishida et al. (42) concluded that SARS-CoV-2 mRNA vaccines do not induce anti-HLA antibody development. Among 63 adult kidney transplant recipients who received two doses of the SARS-CoV-2 mRNA vaccine, conversion from negative to positive flow PRA was noted in only one patient. The 8 DSA-positive recipients' mean fluorescence intensity (MFI) did not differ significantly between before and after immunization, and no further DSA was formed in those patients (42). Furthermore, Kilian et al. (24) stated that vaccination against the SARS-CoV-2 virus might be important in the prevention of alloimmunity. Recent reports of immunoglobulin A nephropathy following COVID-19 vaccination have increased (43–45). Application of SARS-CoV-2 vaccine based on adenoviral vector was a reason for the development of immunoglobulin A nephropathy in a 73-year-old kidney transplant 5 weeks after the second adenovirus vectored SARS-CoV-2 vaccine (44). Alonso et al. (45) reported on a 30-year-old kidney transplant recipient who, 34 days after receiving the second dose of mRNA vaccine, developed immunoglobulin A nephropathy.

The proposed mechanism of viral infection as a trigger of rejection is complex and not fully understood. SARS-CoV-2 virus binds its spike protein to the angiotensin converting enzyme 2 receptor to invade the cell (46). Kidney cells are known to express one of the highest levels of angiotensin converting enzyme 2 on their surface, which allows direct invasion by the SARS-CoV-2 virus (47). The presence of viral particles in renal tubule epithelial cells and glomerular capillary endothelial cells was proven by electron microscopy (48). It has been postulated, based on studies of other viral infections, that viruses cause structural changes in infected glomerular and tubular epithelial cells as well as in capillary endothelial cells, where it upregulates the expression of adhesion molecules, which stimulates the infiltration of the graft with immune cells (49). This process leads to the development of inflammation and the release of lymphokines and other inflammatory mediators. Interferons may enhance the expression of renal graft tissue MHC antigens and, together with direct activation of cytotoxic T cells, mediate acute graft rejection (49). Another proposed mechanism points out the potential cross-reactivity between viral antigens and donor histocompatibility molecules, which would then consequently induce an immune reaction against these viral-induced antigens (11). In addition, viral infection-induced activation and proliferation of B lymphocytes are thought to increase the production of alloantigens and trigger complement-mediated injury to the kidney (49,50). Furthermore, the deposition of circulating antibody-viral antigen complexes could lead to worsening of graft function (51). In 80% of renal and heart transplant recipients with a history of CMV infection, increased levels of anti-endothelial cell antibodies can be found (52). These antibodies are implicated in the pathophysiology of antibody-

mediated graft rejection, which has a poorer prognosis than cellular rejection (53–55). However, histological findings of biopsy specimens sometimes fail to show antibody-mediated kidney injury (11,56).

This study has a few limitations. First, the visitation schedule was determined by the recovery from acute COVID-19. The fact that each patient's examination did not take place at the same time could have an impact on the results. Additionally, the overestimation of *de novo*-developed anti-HLA DSA and non-DSA may have resulted from the lack of baseline data on anti-HLA DSA and non-DSA specificities prior to COVID-19 disease. In addition, it's possible that the number of EBV reactivations was overestimated due to the lack of baseline information on EBV DNA expression. As not all of the patients who received medical care during the acute COVID-19 were treated in our facility, we also lacked information on all of their intrahospital laboratory results and treatment. Finally, this investigation was conducted in a tertiary referral facility as a single center. This, together with the small sample size of the study, may limit the generalizability of our findings. However, this research is among the first to address the issue of post-COVID-19 immunogenicity in renal transplant recipients. It included both inpatient and outpatient patients and gave vital insights into clinical problems that could develop even in people who initially show no symptoms.

Conclusions

Since these findings suggest that the SARS-CoV-2 virus has an immunomodulatory effect and may be connected to serious clinical consequences like acute graft rejections and increased mortality in kidney transplant recipients, additional research is urgently required. SARS-CoV-2 immunogenicity may be a problem impacting a larger population, even though no such research has been conducted in the general population. To direct steps to prevent major complications and mortality, a greater emphasis should be placed on transplant patients' post-COVID-19 clinical assessment, particularly considering *de novo* DSA and HLA formation. All COVID-19 survivors should be monitored for a longer amount of time in order to detect and address any newly emerging disorders. It is necessary to conduct additional long-term follow-up studies.

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Biography

I was born on March 22nd, 1999, in Čakovec, Croatia. After finishing elementary school, I attended Gymnasium Josip Slavenski Čakovec. During my high school years, I participated in scientific and research projects and thus developed my passion for science. After graduating from high school, I started my studies at the School of Medicine, University of Zagreb. During my studies I developed a special interest in internal medicine and became a member of the Student Section for Hypertension. During my time at the University, I was awarded the Dean's Award as the best student of my class in the academic year 2019/2020. In the same academic year, I was awarded Certificate of Excellence in Pathophysiology, which is awarded to students with outstanding performance in pathophysiology courses. I was an elected class representative and member of the 2020-2023 eMed Student Council.