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Polyomavirus Nephropathy in ABO Blood Group-Incompatible Kidney Transplantation: Torque Teno Virus and Immunosuppressive Burden as an Approximation to the Problem

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Introduction: Earlier reports suggest that patients after ABO-incompatible kidney transplantation (ABOi) are at enhanced risk of developing BK-virus (BKV, also known as BK polyomavirus [BKPyV]) nephropathy (BKPyVAN). It remains elusive whether this is a result of more intense immunosuppression or an ABOi-associated "intrinsic attribute." To address this question, we measured Torque Teno virus (TTV) loads as a quantitative proxy for immunosuppressive depth in ABOi recipients and compared them to human leukocyte antigen–incompatible (HLAi, i.e. pretransplant donor-specific antibody-positive) and standard-risk transplant recipients.

Methods: Our retrospective study screened 2256 consecutive kidney transplantations performed between 2007 and 2020 at the Medical University of Vienna. Out of 629 in-principle eligible transplantations, we were able to include 465 patients: 42 ABOi, 106 HLAi, and 317 control recipients. Longitudinal TTV- polymerase chain reaction (PCR) and BKV-PCR was carried out at predefined timepoints and ranged from pretransplant until month 24 posttransplantation. TTV loads and immunosuppression were evaluated in the context of BKV-associated complications.

Results: ABOi recipients had a higher TTV load compared to HLAi and controls both at month 3 (median 1.5×10^9 vs. 2.4×10^8 vs. 9.1×10^7; $P = 0.010$) and at month 6 (3.1×10^9 vs. 1.4×10^7 vs. 6.4×10^7; $P = 0.014$) posttransplantation. Tacrolimus exposure was significantly higher in ABOi patients compared to HLAi and control patients (ABOi vs. HLAi: $P = 0.007$; ABOi vs. controls: $P < 0.0001$). Biopsy-proven BKPyVAN was more frequent in ABOi recipients when compared to HLAi and control recipients (11.9% vs. 2.8% vs. 4.1%; $P = 0.046$).

Conclusion: Our data support the assumption that ABOi patients are indeed at higher risk to develop BKPyVAN. A higher TTV load and immunosuppressive burden suggest that intense immunosuppression, rather than an "intrinsic attribute" conferred by ABOi, may contribute to this finding.

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Uncontrolled polyoma BKV replication does frequently start within the first months after kidney transplantation and, if uncontrolled, may lead to BKPyVAN.[1,2] Persistent viral replication may often precede a functional decline of the allograft and can lead to substantial tissue injury or even premature graft loss.[3,4] A major hurdle in the treatment of BKV is the lack of any proven treatment option.[5] Therefore, screening for viral replication via PCR in blood is paramount for the identification of patients at risk for

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BKPyVAN.[5,6] Several risk factors have been identified and the degree of immunosuppression has been shown to play a prominent role.[7-9] Thus, it may be hypothesized that transplant recipients undergoing desensitization protocols associated with enhanced immunosuppression, including ABOi and/or HLAi transplants are considered as high-risk of developing BKV infection. In 2012, Sharif *et al.*[10] compared ABOi and HLAi patients and found a significantly higher rate of biopsy-proven BKPyVANs in ABOi patients (17.7% vs. 5.9%). This finding was also addressed by 2 recent meta-analyses that included >10,000 patients out of >30 studies, both pointing toward an elevated risk of developing BKV infection after ABOi transplantation.[11,12] However, it remains elusive, whether the suggested increased risk of BKV infections in ABOi patients when compared to other high immunologic-risk cohorts such as HLAi patients, is merely a result of the global immunosuppressive burden or as stated by Sharif *et al.*,[10] a consequence of ABOi-associated "intrinsic attributes." In this regard, TTV load in peripheral blood is a promising candidate to quantify immunosuppressive depth after kidney transplantation. This is apathogenic and in nearly all transplant recipients, detectable virus was shown to correlate with the intensity of the individual immunosuppression, and depending on its replicative dynamics, might therefore support clinicians in identifying patients at risk of graft rejection or infectious complications.[13-19] In a previous study, an increase in TTV loads was significantly associated with the overall risk for infectious complications, including presumptive BKPyVAN.[20] Fernández-Ruiz *et al.*[21] reported that higher plasma TTV loads, measured 1 month after transplantation were a significant risk factor for BKPyV-DNAemia. Yet, it is still not understood how TTV loads in renal transplant recipients change under different types of induction treatments. In this retrospective study, we compared TTV and BKPyV-DNAemia loads in 3 historic cohorts, including ABOi, HLAi, and standard immunologic-risk kidney transplant recipients. Different dynamics of TTV loads in patients with intensified immunosuppression and varying induction regimens may help to understand the underlying reasons for the previously-reported higher BKV incidence in ABOi patients.

METHODS

Study Design and End Points

The aim of this retrospective study was to compare TTV loads between patients undergoing ABOi transplantation, patients undergoing HLAi transplantation (HLAi, transplant across preformed donor-specific antihuman leukocyte antigen antibodies), and an ABO-compatible, pretransplant donor-specific antibody-negative control group (representing patients with standard immunologic risk). Then, we correlated these findings with BKV-associated end points. Our primary outcome of interest was the frequency of "definite" BKPyVAN defined as biopsy-proven BKV-nephropathy. Secondary outcomes were "presumptive" BKPyVAN, defined as >10,000 viral copies/ml in plasma (in ≥1 measurement) even in the absence of histologic features of BKPyVAN or a transplant biopsy, and BKPyV-DNAemia, defined as detection of BKV in blood in at least 2 independent samples within our predefined time points. Relevant clinical and laboratory parameters at the time of transplantation were extracted from digitalized patient records. The study protocol was designed in accordance with the Declaration of Helsinki and approved by the local ethics committees of the Medical University of Vienna (EK 2256/2020).

Samples

In our center, routine TTV monitoring was introduced in 2016. To increase the sample size, BKV and TTV loads, if not readily available, were quantified retrospectively from biobanked plasma samples dating back to as far as 2007. All samples were tested at the Center for Virology, Medical University of Vienna, Austria.

Details of quantifying TTV replication in plasma or serum via PCR were described previously.[13,22] Time points for retrospective testing was day 0 (D0, day of transplant) and months 1 (M1), 3 (M3), 6 (M6), 9 (M9), 12 (M12), 18 (M18), and 24 (M24) after transplantation. In ABOi patients, TTV was also assessed 1 month before transplantation (M−1), because a substantial proportion received CD20 antibody rituximab at M−1 and the immunosuppressive regimen was initiated in all ABOi patients 1 to 2 weeks before transplantation, which could both have impacted on TTV loads at D0. Therefore, TTV baseline was defined as TTV load at D0 in the HLAi group and control group and at M−1 in the ABOi group.

BKV-PCR measurements were performed according to the center clinical routine standards.[23] If screening measurements were missing, BKPyV-DNAemia was also tested retrospectively (if available at M3, M6, M9, M12, M18, and M24]. A more detailed description of the methods, including the different sources of the plasma or serum samples are provided in the Supplementary Methods section.

Inclusion Criteria

Criteria for inclusion were defined as follows: (i) all patients with a graft survival of at least 4 months after transplantation (due to the expectation that the typical onset of BKV-replication is after M3), (ii) clinical follow-up of at least 12 months after transplantation,

and (iii) the availability of at least 3 BKPyV-DNAemia measurements within the observation period of 24 months and at least 2 within the first 12 months after transplantation. Pediatric patients and patients undergoing kidney transplantation after any other organ or after combined solid organ transplantation were excluded. Details with respect to the necessity to define different sampling time frames for the 3 cohorts are described in the Supplementary Methods section.

Kidney Transplant Biopsies

Diagnosis of definite BKPyVAN was based on the combination of BKV infection-associated lesions, including intranuclear inclusions, cellular atypia, tubulitis or tubular epithelial cell degeneration, and immunohistochemical staining for SV40 large T-antigen (Mouse IgG_{2A} anti-SV40 monoclonal antibody, clone MRQ-4, Cell Marque, Rocklin, CA).[24] Included cases of definite BKPyVAN were retrospectively reclassified by 1 experienced nephropathologist (NK) and scored according to the polyomavirus nephropathy scoring system by Nickeleit *et al*.[25]

Induction Treatment and Maintenance Immunosuppression

At the Medical University of Vienna, the first ABOi transplantation was performed in 2007. In the earlier phase of our ABOi transplantation program (i.e., before 2016), our protocol included i.v. rituximab (375 mg/m^2 body surface area) and pretransplant antigen-specific immunoadsorption (IA, Glycosorb ABO, Glycorex Transplantation, Lund, Sweden) together with pretransplant immunosuppression in all cases according to the published protocol by Tydén *et al*.[26] As of 2016, after it had been demonstrated that pretransplant rituximab can safely be omitted in patients with lower anti-ABO antibody titers,[27,28] a center-specific algorithm was created. In short, patients with baseline anti-ABO titers ≤1:256 received no rituximab and only underwent antigen-specific IA starting 1 week before the planned transplantation date and also received standard triple immunosuppression (tacrolimus, mycophenolate mofetil, and glucocorticoids) 7 days before transplantation (target tacrolimus trough level at the day of transplantation: 12–15 ng/ml). Patients with baseline anti-ABO titers >1:256 additionally received i.v. rituximab (375 mg/m^2 body surface area) approximately 4 weeks before transplantation and IA as well as triple immunosuppression, both initiated 14 days before transplantation (target tacrolimus trough level at the day of transplantation: 12–15ng/ml). Induction with IL-2R antibody basiliximab (20 mg i.v. on days 0+4) was administered in 74% of patients. Posttransplant target tacrolimus trough levels were defined as 10 to 15 ng/ml within the first 3 months, and thereafter at 7 to 10 ng/ml until month 6. All patients received *Pneumocystis jiroveci* and cytomegalovirus prophylaxis for the first 3 to 6 months after transplantation and protocol biopsies were performed after 7 to 10 days. On-demand IA was performed in case of titer rebound >1:16 within the first weeks after transplantation. Until 2014, patients also received low-dose i.v. immunoglobulins as part of the induction regimen.

Our desensitization protocol for patients with preformed donor-specific antibody was described earlier.[29] In brief, semiselective peritransplant IA was performed with protein A (Immunosorba) or GAM-146-peptide (Globaffin) -coated twin column systems. One IA was performed before transplantation followed by up to 10 treatments thereafter with >10 treatments in selected cases only. Initially, antithymocyte globulin (ATG) was given at a dose of 2.5 mg/kg bodyweight/d. After 2016, the dose was reduced to 1.5 mg/kg/d. Target tacrolimus trough levels were set at 10 to 15 ng/ml within the first 3 months and between 7 and 10 ng/ml thereafter.

Standard immunologic-risk patients received basiliximab for induction (20 mg i.v. on days 0+4). Target tacrolimus trough levels were set to 7 to 10 ng/ml within the first 1 to 3 months and then to 4 to 7 ng/ml according to our center standard and to the treating physicians discretion.

All patients received initial triple immunosuppression, including tacrolimus (99%), mycophenolate mofetil (1 g twice daily) as well as prednisolone with a prolonged taper in ABOi and HLAi patients. Mycophenolate mofetil was kept at a target dose of (1 g twice daily) in all 3 groups. Dose reductions were performed only in case of intolerance, cytopenia, or infectious complications.

Statistics

Categorical variables were expressed as absolute and relative frequencies. Group comparisons of categorical variables were analyzed by Fisher exact or chi-square tests. Continuous parameters are presented as median and interquartile range (IQR) or mean and SD, whichever was appropriate depending on the distribution of the data. Group comparisons of continuous parameters were performed with nonparametric testing (Mann-Whitney *U* test) or (Kruskal-Wallis-test). Tacrolimus trough levels were compared as follows: for each patient and week, 1 median trough level was calculated. For tacrolimus levels below the detection limit (<2.4 ng/ml), the value 1.2 ng/ml was entered. All analyses were performed using IBM SPSS Statistics Version 24 (IBM, Armonk, NY) and illustrated with GraphPad Prism Version 9.5.1 (GraphPad software, San Diego, CA). Tacrolimus trough level area under the curve

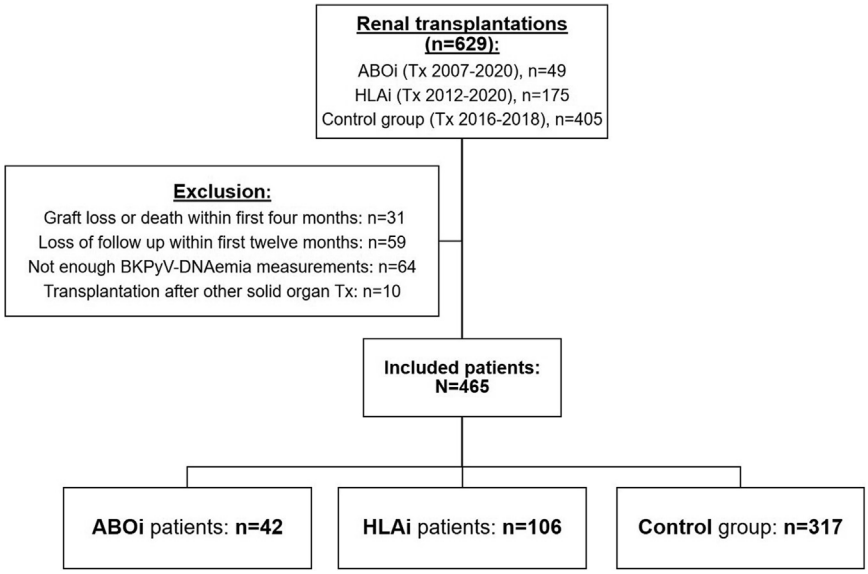

Figure 1. Flowchart illustrating patient inclusion. Flowchart illustrating patient selection based on prespecified inclusion criteria. ABOi, ABO-incompatible; BKPyV, BK polyomavirus; HLAi, human leukocyte antigen–incompatible; Tx, transplantation.

(AUC) was calculated with a median tacrolimus trough level for each patient and each week, with week zero including median tacrolimus levels within the first week after transplantation. Longitudinal logarithmic TTV trajectories were computed with a mixed linear model using group and time as independent variables. Assuming an increase of TTV loads until month 3 and a decrease thereafter, we applied a hockey stick model with a knot at month 3. This was performed with SAS software version 9.4 for Windows (Cary, NC). A 2-sided $P < 0.05$ was considered statistically significant. Time points with missing data were excluded. Extrapolation of missing data was not performed.

RESULTS

Patient Characteristics

Our retrospective study screened 2256 consecutive kidney transplantations within our transplant database that were performed between 2007 and 2020 at the Medical University of Vienna. We identified 629 patients who were transplanted within the prespecified time periods as detailed in the Supplementary Methods section. After review of medical records, 162 patients were excluded, mostly due to insufficient numbers of BKPyV-DNAemia measurements or too short follow-up (Figure 1), resulting in 465 patients available for final analysis: 42 ABOi patients, 106 HLAi patients, and a control group of 317 patients representing standard immunologic-risk patients. The control group included 13.9% live-donor kidney transplants.

Of all 465 patients, 405 (87%) had complete follow-up and a functioning allograft at M24. Of the 60 patients without follow-up until M24, 9 (1.9%) had lost their grafts, 10 (2.2%) had died, and 41 (8.8%) had returned to their respective referral centers.

Baseline characteristics of all included patients are provided in Table 1. In the ABOi group, rituximab at M−1 was used for desensitization in 24 patients (57%), 1 patient (2.4%) with additional preformed low-level antihuman leukocyte antigen donor-specific antibody also received ATG. In this patient, semiselective IA was used. In the HLAi group, 102 patients (96.2%) underwent IA and 99 (93.4%) received ATG. Initial median daily ATG dose was 150 mg (IQR: 100–175).

Overall TTV Trajectories

In total, 4495 TTV load measurements (median 9/patient, IQR: 7–12) were available for final analysis (ABOi: $n = 318$ [median 6/patient, IQR 5-9]; HLAi: $n = 899$ [median 7/patient, IQR: 5–11]; control group: $n = 3278$ (median 9/patient, IQR: 8–13)]. ABOi patients showed the highest baseline TTV loads (Kruskal-Wallis-test for intergroup comparison between the 3 groups, $P = 0.011$). Thereafter, median TTV loads increased constantly within the first 3 months after transplantation in all groups. In ABOi patients, TTV loads remained high until M6 and showed a gradual decline thereafter, whereas HLAi and control patients already descended from their TTV peak at M3 (Figure 2). Intergroup comparison showed that median TTV loads differed significantly at baseline ($P = 0.011$), M3 ($P = 0.010$), and M6 ($P = 0.014$), but not at M9, 12, or 24 after transplantation. As depicted in Figure 2 and shown in Table 2, at M3 and M6, ABOi patients had the highest median TTV loads (M3: ABOi vs. HLAi: 1.5×10^9 vs. 2.4×10^8, $P = 0.062$, ABOi vs. control group: 1.5×10^9 vs. 9.1×10^7, $P = 0.004$; M6: ABOi vs. HLAi:

Table 1. Baseline characteristics

Variables	ABOi (n = 42)	HLAi (n = 106)	Controls (n = 317)
General, n (%)			
Female sex	9 (21.4)	55 (51.9)	93 (29.3)
Underlying kidney disease, n (%)			
Vascular kidney disease	2 (4.8)	4 (3.8)	44 (13.9)
Diabetic kidney disease	1 (2.4)	4 (3.8)	38 (12.0)
Glomerulonephritis	11 (26.2)	39 (36.8)	69 (21.8)
Cystic kidney disease	10 (23.8)	13 (12.3)	37 (11.7)
Other	10 (23.8)	22 (20.8)	64 (20.2)
Unknown	8 (19)	24 (22.6)	65 (20.5)
Variables recorded at the time of transplantation			
Recipient age (yr), median (IQR)	54 (45–63)	55 (45–60)	55 (45–65)
Donor age (yr), median (IQR)	56 (49–62)	52 (44–62)	55 (44–67)
Prior kidney transplant, n (%)	3 (7.1)	71 (67)	41 (12.9)
Preformed anti-HLA DSA, n (%)[a]	3 (7.1)	106 (100)	0 (0)
Deceased donor, n (%)	0 (0)	106 (100)	273 (86.1)
HLA mismatch (A, B, DR), median (IQR)	4 (3–5)	3 (2–4)	3 (2–4)
Induction therapy, n (%)			
Pretransplant rituximab	24 (57.1)	0 (0)	0 (0)
Antithymocyte globulin	1 (2.4)	99 (93.4)	0 (0)
CD25 antibody basiliximab	31 (73.8)	7 (6.6)	317 (100)
Baseline immunosuppressive regimen, n (%)			
Tacrolimus[a]	42 (100)	105 (99.1)	315 (99.4)
Mycophenolate mofetil / mycophenolic acid	41 (97.6)	106 (100)	316 (99.7)
Steroids	42 (100)	106 (100)	317 (100)

ABOi, ABO-incompatible; DSA, donor-specific antibody; eGFR, estimated glomerular filtration rate; HLA, human leukocyte antigen; IQR, interquartile range.
[a]Overall, 3 patients received cyclosporine A as part of their initial triple immunosuppressive regimen.

3.1×10^9 vs. 1.4×10^7, $P = 0.004$, ABOi vs. control group: 3.1×10^9 vs. 6.4×10^7, $P = 0.014$). Only 4 patients had TTV loads $<10^2$ at M3 and M6 after transplantation (HLAi: $n = 2$, control group: $n = 2$). In 3 of those, TTV loads remained $<10^3$ over the further course. Linear mixed model calculation, including all available TTV loads until M3 showed significantly different TTV slopes between the control group ($\beta = 1.27$, reference group, indicating a 10log-increase with every month from M−1 [ABOi] or D0 [HLAi and controls] to M3) and the ABOi group (ABOi: $\beta = 1.08$; $P = 0.04$) and between the control group and the HLAi group (HLAi: $\beta = 1.45$, $P = 0.02$, Supplementary Table S1). HLAi patients showed highest TTV slopes in the first 3 months after transplantation (ABOi vs. HLAi and ABOi vs. controls, $P < 0.05$, Supplementary Figure S1, Supplementary Table S1).

Tacrolimus Trough Levels and Trough Level AUC Between the Groups

Median tacrolimus trough levels differed significantly in intergroup comparison between the 3 groups at M1 (ABOi, median 11.3 ng/mL [IQR: 10.2–12.6], HLAi: 10.1 [IQR: 8.3–11.9], and control group: 8.8 [IQR: 7.2–10.7], Kruskal-Wallis-test, $P < 0.001$) and at M3 (ABOi: 9.6 [IQR: 8.0–11.8], HLAi: 8.3 [IQR: 6.4–9.9] and control group: 7.8 [IQR: 6.3–9.8], $P = 0.002$), but not at M6 (ABOi: 7.6 [IQR: 6.2–9.1], HLAi: 7.0 [IQR: 5.7–8.8], and control group: 6.9 [IQR: 5.5–8.2], $P > 0.05$) or any later time point (tacrolimus trough levels over the course are displayed in Table 2). Between-group comparison is displayed in Figure 3.

Correspondingly, the tacrolimus trough level AUC within the first 12 and 26 weeks (as provided in Figure 4a and b and Table 2, calculated based on multiple single trough levels expressed as ng/ml) was highest in the ABOi group (12-week trough level AUC: ABOi: 135.3 vs. HLAi: 117.8, $P = 0.007$, ABOi 135.3 vs. control group: 106.9, $P < 0.0001$; 26-week trough level AUC: ABOi: 258.3 vs. HLAi: 225.0, $P = 0.009$, ABOi 258.3 vs. controls: 213.8, $P < 0.0001$). Tacrolimus trough levels correlated significantly with TTV loads at M3, M6, M9, and M24 but not at M1 or M24 (Supplementary Figure S2).

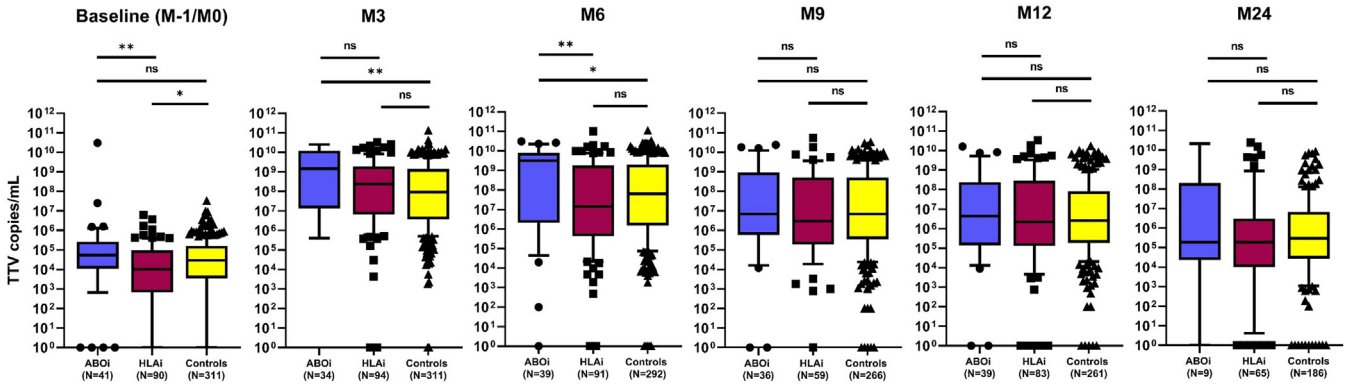

Figure 2. TTV levels over the course of 24 months. Box plots illustrating TTV levels over the course of 24 months. Whiskers represent the 10th to 90th percentile. Values below and above the whiskers are drawn as individual points. At each time point, P-value refers to comparisons between 2 groups. Numbers in brackets represent patients with available parameters at each time point. ABOi, ABO-incompatible; HLAi, human leukocyte antigen–incompatible; M, month; ns, not significant; TTV, Torque Teno virus. *$P < 0.05$; **$P < 0.01$.

Table 2. Laboratory parameters of different groups

Variables	ABOi ($n = 42$)	HLAi ($n = 106$)	Controls ($n = 317$)	ABOi vs. HLAi, *P*-value	ABOi vs. control, *P*-value
TTV load, copies/ml, median (IQR)					
Baseline[a]	5.4×10^4 (1.2×10^4 to 2.5×10^5)	1.0×10^4 (6.7×10^2 to 9.6×10^4)	2.9×10^4 (3.5×10^3 to 1.6×10^5)	0.007	0.104
Mo 3	1.5×10^9 (1.6×10^7 to 1.2×10^{10})	2.4×10^8 (6.6×10^6 to 1.9×10^9)	9.1×10^7 (3.7×10^6 to 1.4×10^9)	0.062	0.004
Mo 6	3.1×10^9 (2.1×10^6 to 7.9×10^9)	1.4×10^7 (4.3×10^5 to 1.8×10^9)	6.4×10^7 (1.5×10^6 to 2.0×10^9)	0.004	0.014
Mo 9	7.0×10^6 (6.6×10^5 to 8.9×10^8)	3.0×10^6 (1.9×10^5 to 5.1×10^8)	7.0×10^6 (3.6×10^5 to 5.0×10^8)	0.401	0.712
Mo 12	4.4×10^6 (1.4×10^5 to 2.4×10^8)	2.2×10^6 (1.3×10^5 to 2.9×10^8)	2.6×10^6 (1.9×10^5 to 7.9×10^7)	0.620	0.519
Mo 18	9.1×10^5 (2.7×10^4 to 1.2×10^8)	3.1×10^5 (1.7×10^4 to 6.9×10^7)	4.4×10^5 (2.7×10^4 to 5.0×10^6)	0.504	0.367
Mo 24	1.9×10^5 (4.6×10^4 to 1.0×10^8)	1.9×10^5 (1.0×10^4 to 2.7×10^6)	2.6×10^5 (2.7×10^4 to 6.8×10^6)	0.693	0.804
Tacrolimus trough levels ng/ml					
Mo 1, median (IQR)	11.3 (10.2–12.6)	10.1 (8.3–11.9)	8.8 (7.2–10.7)	0.159	<0.0001
Mo 3, median (IQR)	9.6 (8.0–11.8)	8.3 (6.4–9.9)	7.8 (6.3–9.8)	0.036	0.021
Mo 6, median (IQR)	7.6 (6.2–9.1)	7.0 (5.7–8.8)	6.9 (5.5–8.2)	0.892	0.324
Mo 9, median (IQR)	6.7 (5.5–8.4)	6.7 (5.2–8.4)	6.5 (5.4–7.8)	0.368	0.818
Mo 12, median (IQR)	6.2 (5.3–8.0)	6.7 (5.1–8.2)	6.3 (5.3–7.8)	0.563	0.902
Mo 18, median (IQR)	6.8 (5.2–8.0)	6.5 (5.3–7.6)	6.5 (5.3–8.2)	0.403	0.767
Mo 24, median (IQR)	5.9 (5.4–6.7)	6.1 (5.0–7.1)	6.4 (5.2–7.6)	0.503	0.270
12-wk trough level AUC	135.3	117.8	106.9	0.007[b]	<0.0001[b]
26-wk trough level AUC	258.3	225.0	213.8	0.009[b]	<0.0001[b]
Serum creatinine mg/dl, median (IQR), *n*[c]					
Mo 12	1.4 (1.3–1.7), $n = 42$	1.4 (1.1–1.7), $n = 99$	1.4 (1.1–1.7), $n = 296$	0.099	0.247
Mo 24	1.5 (1.2–1.7), $n = 38$	1.4 (1.1–1.7), $n = 83$	1.4 (1.1–1.8), $n = 254$	0.348	0.771

ABOi, ABO-incompatible; AUC, areas under the curve; HLAi, human leukocyte antigen–incompatible; IQR, interquartile range; TTV, Torque Teno virus.
[a]Because of rituximab being administered approximately 1 month before transplantation and the upfront start of pretransplant immunosuppressive medication in most ABOi patients, their TTV baseline level was assessed 4 weeks before transplantation (M−1), all other patients (HLAi and controls) had baseline TTV measurement at D0.
[b]*P*-values for comparison of trough level AUCs were based on Mann-Whitney U tests of all median weekly tacrolimus trough levels within the stated time points.
[c]Number of available measurements.

Effect of Rituximab on TTV Loads

Among all analyzed individuals, patients in the ABOi group receiving rituximab had the highest TTV loads at M3. Supplementary Figure S3 shows significantly higher TTV loads at M3 compared to ABOi patients without rituximab induction (median: 4.3×10^9 [IQR: 3.8×10^8 to 1.5×10^{10}] vs. 2.9×10^8 [8.8×10^5 to 4.5×10^9], $P = 0.045$) and versus HLAi and control patients (*P*-values: 0.003 and 0.0002). This difference was not seen in any other month (data not shown). Notably, ABOi patients receiving rituximab also had significantly higher tacrolimus trough levels at M1 (median: 12.1 ng/ml [IQR: 10.4–13.1] vs. 10.6 [8.0–11.7], $P = 0.046$), but not at M3 (10.8 ng/ml [8.0–13.0] vs. 8.9 [8.2–10.6], $P = 0.106$] when compared to ABOi patients without rituximab induction.

BKV End Points

In total, 4405 (median 9/patient, IQR: 7–12) BKPyV-DNAemia measurements at different months were available, consisting of ABOi ($n = 406$, median/patient 9 [IQR: 6–13]), HLAi: ($n = 989$, median 8 [IQR: 6–12]), and control group ($n = 3011$, median 9 [IQR: 7–11]). Our primary end point, definite BKPyVAN, occurred significantly more frequently in the ABOi group (ABOi: $n = 5/42$ [11.9%] vs. HLAi: 3/106 [2.8%] vs. controls 13/317 [4.1%], $P = 0.046$; Figure 5). Presumptive BKPyVAN was similar between the groups (HLAi: $n = 17$ [16.0%] compared to $n = 6$ [14.3%] in ABOi and $n = 33$ [10.4%] in the control group, $P = 0.273$), respectively. Within our predefined observation period of 24 months, overall BKPyV-DNAemia incidence was comparable between HLAi and ABOi patients (HLAi: 30.2% vs. 21.4% in ABOi vs. 24.9% in controls, $P = 0.446$; Table 3). As provided in Figure 5, these findings were confirmed via comparison of cumulative event rates (Log-rank test: $P = 0.045$ for definite BKPyVAN). Peak BKPyV-DNAemia was also not significantly different, but we observed a trend toward higher peak BKV loads in the ABOi group (ABOi: median 1.8×10^5 copies/ml [IQR: 3.3×10^3 to 7.0×10^5], HLAi: 1.3×10^4 [2.2×10^3 to 5.6×10^4], and controls: 6.3×10^3 [1.3×10^3 to 6.0×10^4], $P = 0.06$; Figure 6].

In the ABOi group, BKV-related outcomes did not differ between patients with and without rituximab

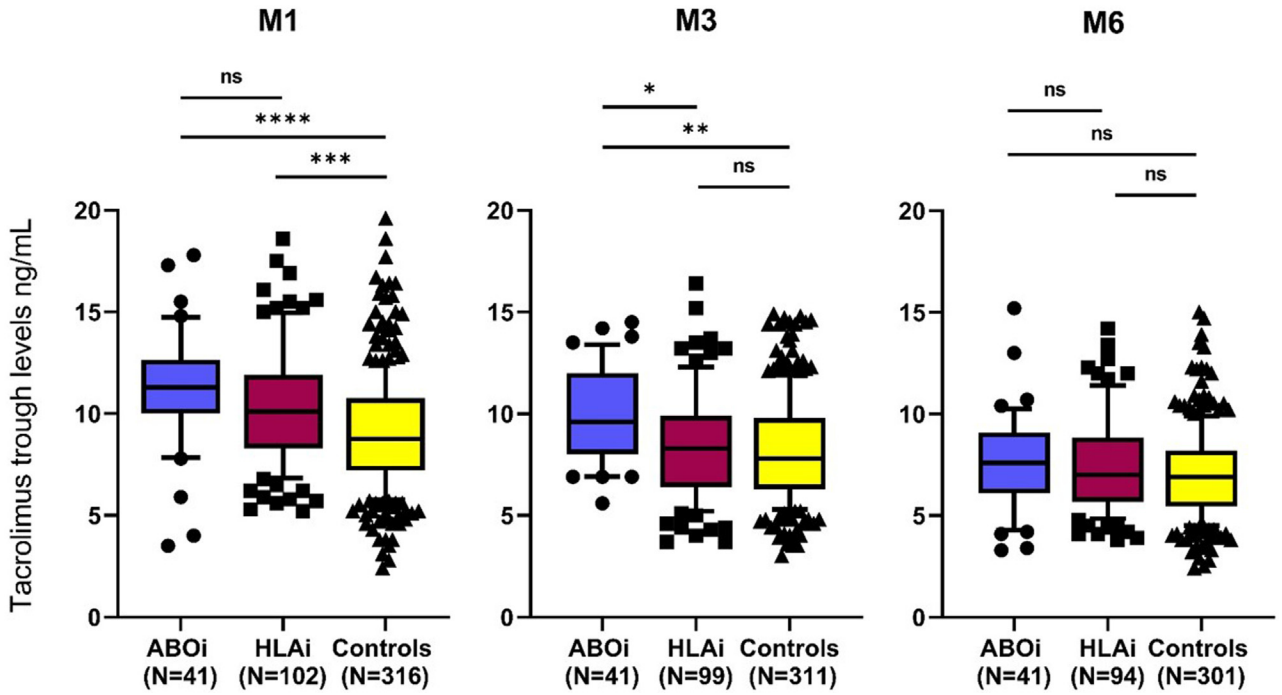

Figure 3. Tacrolimus trough levels at M1, M3, and M6 after transplantation. Box plots illustrating tacrolimus trough levels at M1, M3, and M6 after transplantation. Whiskers represent the 10th to 90th percentile. Values below and above the whiskers are drawn as individual points. At each time point, *P*-value refers to comparisons between 2 groups. Numbers in brackets represent patients with available parameters at each time point. ABOi, ABO-incompatible; HLAi, human leukocyte antigen–incompatible; M, month; ns, not significant; TTV, Torque Teno virus. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

induction (Supplementary Table S2). Graft losses within the first 2 years that were clearly attributable to BKPy-VAN occurred in 3 patients, 1 in each of the 3 cohorts.

DISCUSSION

Our study is the first to compare longitudinal TTV loads between cohorts with different underlying immunologic risk and enhanced immunosuppression. We included ABOi and HLAi recipients and related TTV kinetics to the observed rates of BKV-associated complications. Our main research question was whether TTV loads in ABOi recipients may help to explain their putative predisposition towards development of such complications. As a major finding, we demonstrate that in the first 6 months after transplantation, median TTV loads are highest in patients after ABOi transplantation, although all 3 cohorts showed ascending loads until month 3. Notably, in ABOi patients, TTV loads also remained high for an extended period of time. Afterwards, TTV loads aligned between the 3 groups and gradually decreased. High TTV loads in ABOi transplant recipients were reported in a previous study from our center, but the underlying factors remained speculative.[30] Subgroup analysis of our study suggests that the pretransplant administration of rituximab significantly contributed to the increased TTV loads in a substantial proportion of ABOi patients. Although this finding might not be surprising *per se*, it is still not clear why the effect on B cell depletion exerted by rituximab does lead to more pronounced effects on TTV loads when compared with ATG, which was used as induction therapy in our HLAi cohort. A profound effect of rituximab on TTV loads was also observed by Studenic *et al.*[31] in a study with patients with rheumatoid arthritis. In their study, rituximab was associated with the highest TTV loads compared with TNF-alpha antagonism with infliximab, IL-6 blockade with tocilizumab, or costimulation blockade with abatacept; however, no comparison group with cell-depleting agents such as ATG was included in this analysis.[31] It was observed that TTV loads decreased significantly in patients with leukemia receiving myelosuppressive treatment before bone marrow transplantation.[32] Given that lymphocytes were proposed to constitute one of the primary replicative reservoirs of TTV, our findings may also have implications with respect to the generalizability that TTV loads may represent the overall immunosuppressive burden after lymphocyte depletion, when using different substances such as ATG or rituximab. It may therefore be questioned if cell-depleting agents such as ATG significantly reduce the replicative reservoir of TTV resulting in lower TTV plasma loads, especially when compared to less aggressive induction regimens.

Notably, in our study ABOi patients also had the highest tacrolimus trough levels and tacrolimus trough

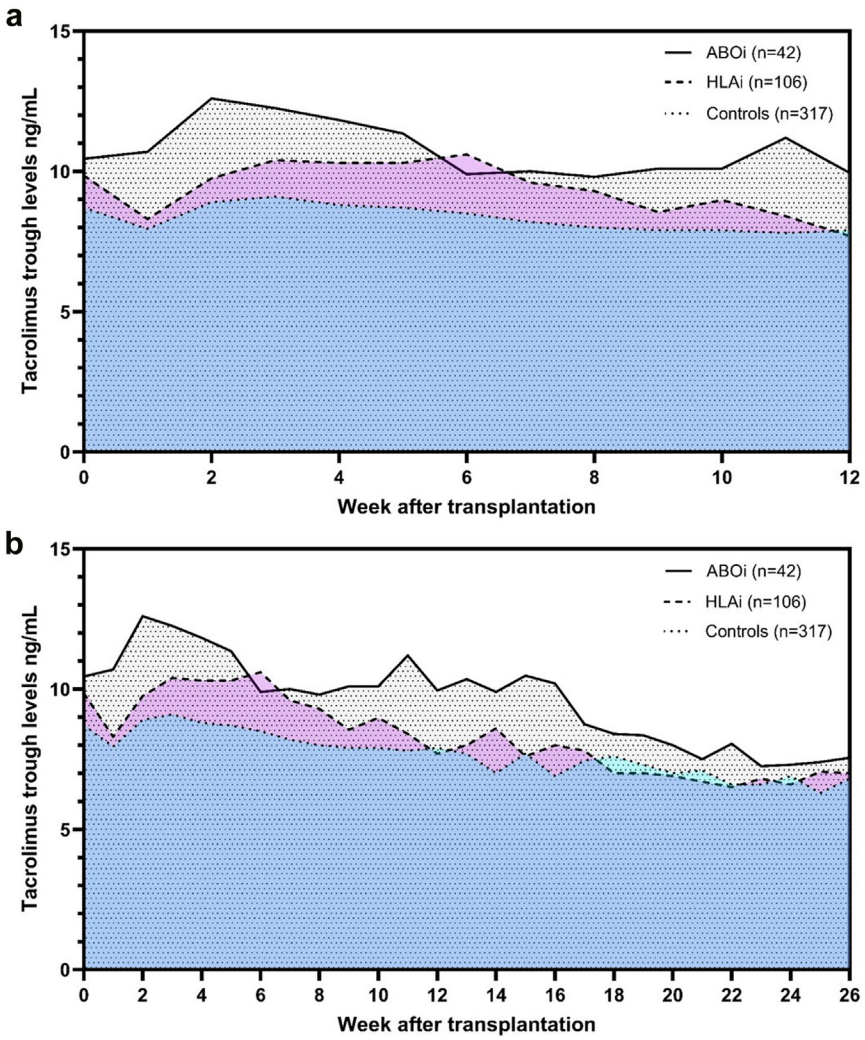

Figure 4. AUC of tacrolimus trough levels within the first 12 and 26 weeks after transplantation. AUCs comparing tacrolimus trough levels between the 3 groups until (a) week 12 and (b) 26 weeks. Trough level AUC after 12 weeks included 5064 tacrolimus trough level medians (derived from 8471 single measurements) and trough level AUC after 26 weeks included 8342 trough level medians (based on 15,533 single measurements). ABOi, ABO-incompatible; AUC, Area under the curve; HLAi, human leukocyte antigen–incompatible.

level AUCs. However, it needs to be mentioned that due to their specific immunosuppressive protocol, ABOi patients have mostly already reached their therapeutic tacrolimus trough level at the day of transplantation, which might help to sustain higher and more stable levels posttransplant.

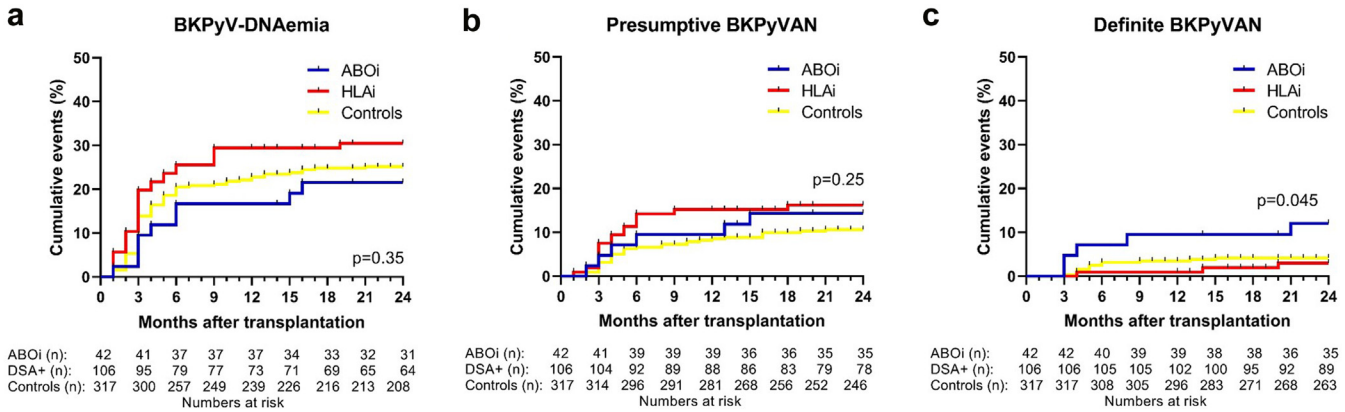

Figure 5. Analysis of cumulative event rates for BKPyV outcomes. Cumulative event rates comparing BKPyV end points between the 3 groups. Log-rank test revealed no significant difference between the groups regarding (a) BKPyV-DNAemia ($P = 0.35$) or (b) presumptive BKPyVAN ($P = 0.25$). (c) Definite BKPyVAN was significantly different with highest events in ABOi patients ($P = 0.045$). ABOi, ABO-incompatible; BKPyV, BK polyomavirus; HLAi, human leukocyte antigen–incompatible.

Table 3. BKV-related outcomes

Variables	ABOi (n = 42)	HLAi (n = 106)	Controls (n = 317)	P-value
Measured BKPyV-DNAemia levels per patient, median (IQR)	9 (6–13)	8 (6–12)	9 (7–11)	0.361
BKPyV-DNAemia, n (%)[a]	9 (21.4)	32 (30.2)	79 (24.9)	0.446
Peak BKPyV-DNAemia, median (IQR)	1.8×10^5 (3.3×10^3-7.0×10^5)	1.3×10^4 (2.2×10^3 to 5.6×10^4)	6.3×10^3 (1.3×10^3 to 6.0×10^4)	0.060
Presumptive BKPyVAN, n (%)	6 (14.3)	17 (16.0)	33 (10.4)	0.273
Definite BKPyVAN, n (%)	5 (11.9)	3 (2.8)	13 (4.1)	0.046
PvI score, n (%)				0.196
PvI 1	2 (40.0)	2 (66.7)	6 (46.2)	
PvI 2	0 (0)	1 (33.3)	5 (38.5)	
PvI 3	3 (60.0)	0 (0)	2 (15.4)	
PVN score, n (%)				0.061
PVN 1	2 (40.0)	1 (33.3)	0 (0)	
PVN 2	1 (20.0)	2 (66.7)	11 (85)	
PVN 3	2 (40.0)	0 (0)	2 (15)	
Biopsies during 'presumptive' BKV-DNAemia level, n (%)[a]	5/6 (83.3%)	10/17 (58.8%)	21/32 (65.6%)	0.555
Graft loss associated with BK infection, n (%)	1 (2.4)	1 (0.9)	1 (0.32)	0.265

ABOi, ABO-incompatible; BKV, BK-virus; BKPyVAN, BK polyomavirus-associated nephropathy; HLAi: human leukocyte antigen–incompatible; IQR, interquartile range; PvI, polyomavirus replication/load level; PVN, polyomavirus nephropathy (score according to Nickeleit *et al.*[25]).
[a]At least 2 BKV detections in plasma.

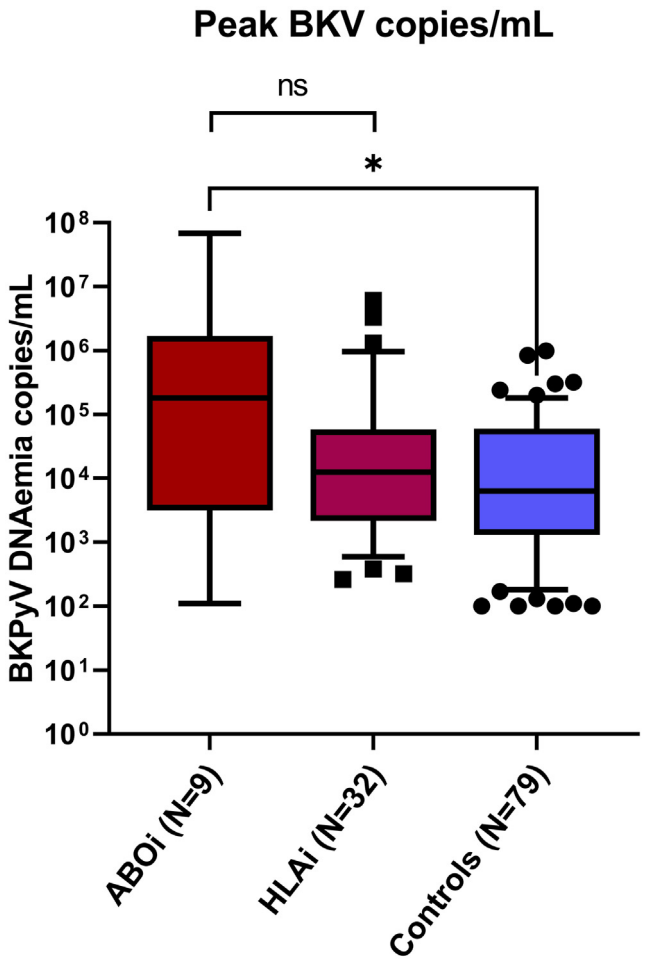

Figure 6. Peak BKPyV-DNAemia. Box plots illustrate peak BKPyV-DNAemia in all 3 groups. Whiskers represent the 10th to 90th percentile. Values below and above the whiskers are drawn as individual points. ABOi, ABO-incompatible; BKPyV, BK polyoma virus; HLAi, human leukocyte antigen–incompatible; TTV, Torque Teno virus. *$P < 0.05$.

Higher TTV loads were associated with an increased risk for infection, including BKV and other viruses.[20] In our study, ABOi patients had the highest incidence of definite BKPyVAN, in line with Sharif *et al.*[10] In contrast to the latter, our analysis also included the end points BKPyV-DNAemia and presumptive BKPyVAN. We observed that those outcomes did not differ significantly between the groups. However, 6 out of 9 ABOi patients with BKPyV-DNAemia had $>10^4$ copies/mL, and 5 out of those had definite BKPyVAN. In contrast, less than half of all control group patients with BKPyV-DNAemia reached presumptive BKPyVAN levels. On the basis of the relation between DNAemia loads and the percentage of SV40 large T-antigen-positive tubules,[33] we hypothesize that higher peak BKPyV-DNAemia loads might explain the increased rate of definite BKPyVAN in the ABOi group, which was also demonstrated in an analysis by Schachtner *et al.*[34] Fortunately, the directly BKV-attributable graft losses were low in all groups, also in line with the study from *Sharif et al.*[10]

Ko *et al.*[35] also reported that overall incidences of BKPyV-DNAemia did not differ between ABOi and ABO-compatible living-related kidney transplant recipients. In their analysis, presumptive BKPyVAN ($>10^4$ copies/mL), on the contrary, was more frequent in ABOi/human leukocyte antigen–compatible compared to ABO-compatible/HLAi recipients (23.5% vs. 7.6%) and compared to ABOi and HLAi patients in our study (ABOi: 14.3% and HLAi 16.0%), respectively. However, there are differences between the 2 study cohorts. In the study from Ko *et al.*,[35] all ABOi patients received rituximab. ATG on the other hand, a suggested risk factor for BKV infections, was not used in their study, but was used in our HLAi group.[35-37]

Higher TTV levels in the ABOi group support the hypothesis that the overall intensity of immunosuppression may increase the risk for severe BKV infections rather than an inherent ABOi-specific risk factor. A study by Kwon *et al*.[38] compared the risk for BKV infections in ABOi patients between 2 time periods with different immunosuppressive regimens. After reduction of rituximab doses and target tacrolimus trough levels, initially higher BKV infection rates decreased and were similar to ABO-compatible transplantations.[38] Several earlier studies also described a tacrolimus dose or trough level dependent risk of BKV infections and most of them used definite BKPyVAN as outcome.[39-42] Nevertheless, future investigations are needed to clarify if tacrolimus dose dependently increases the frequency of overall BKPyV-DNAemia or, as suggested by our study, only exacerbates BKPyV-DNAemia loads in patients with otherwise comparable BKPyV-DNAemia incidences.

Our study has some inherent limitations, including the retrospective study design as well as the use of historic transplant cohorts, which impact on the generalizability of our results. Nevertheless, only by consecutive inclusion of all ABOi transplantations and retrospective BKV/TTV-PCR testing from archived samples, were we able to analyze the so far biggest cohort of ABOi patients in the context of TTV. Furthermore, despite the long time period, almost all patients received tacrolimus and mycophenolate mofetil, excluding TTV level alterations due to different immunosuppressive regimens.

Two[20,21] out of 3[18] previous studies identified an association between TTV loads and the risk for BKV infection. In this current study we did not analyze this research question for several reasons. In a previous publication from our center, Doberer *et al*.[20] showed that every TTV load log level increase led to an 11% increased risk of infections. They observed that this effect was strongly driven by BK infections (presumptive BKPyVAN or definite BKPyVAN; odds ratio: 1.21, $P = 0.005$) and by cytomegalovirus. However, the latter study prospectively measured TTV loads initially weekly until discharge and then at months 3 and 6. Infectious events were correlated with the TTV load that was measured before the event happened. In our study, we retrospectively measured TTV loads from available stored plasma samples. Because some transplantations were performed >10 years before the study, we were not able to obtain sufficient plasma samples to measure TTV loads in comparable close time intervals. This cross-sectional approach is therefore unsuited to analyze intrapatient TTV load dynamics and to predict BKV end points with preceding TTV measurements. Furthermore, it was shown that TTV loads react on changes of immunosuppression with a certain time lag: it takes at least 3 months after transplantation for TTV loads to initially stabilize.[43] As presented in Figure 5, a substantial proportion of BKV events already occurred at month 3; a TTV-based prediction of those events would therefore not be clinically meaningful. Lastly, although we have included a control cohort, our findings were not validated in an external cohort. So far, TTV monitoring was not widely available. Currently, a Real-time PCR assay from bioMérieux (TTV R-GENE, bioMérieux, France) is commercially available. A recent study[44] compared our in-house PCR, which was used in our previous studies,[13,17,20,30] with the commercially available CE-marked PCR from bioMérieux. Both tests showed high correlation and reproducibility (estimate 0.91, 95% confidence interval: 0.89–0.93). We therefore argue that our findings can be translated to other centers and are not restricted to our in-house TTV PCR. An EU-funded prospective trial testing TTV-guided immunosuppression will also include serial BKV monitoring and potentially enable the validation of our findings.[45]

In conclusion, ABOi patients had the highest and most prolonged TTV peaks as well as the highest tacrolimus trough levels and trough level AUCs. We found that TTV loads were significantly higher after ABOi with rituximab, although this was not reflected by a higher number of BKV complications in this group. Our findings therefore argue against an intrinsic predisposition of ABOi towards BKV infection. As indicated by comparable BKPyV-DNAemia incidence but higher DNAemia peaks, we hypothesize that infections with BKV may not be more frequent after ABOi, but owing to the more intense immunosuppression, may follow a more severe course. Therefore, this is in line with the recommendation, that ABOi patients should be regarded as high-risk to develop BKV-associated complications and be subjected to close BKV-surveillance.[5] Whether TTV-based guidance of immunosuppression may help to prevent BKV-associated complications in ABOi or HLAi recipients warrants future prospective studies.

DISCLOSURE

All the authors declared no competing interests.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon reasonable request from the corresponding author, FE. Clinical and patient data are confidential and are subject to General Data Protection Regulation.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Figure S1. Longitudinal TTV levels.

Figure S2. Correlation of tacrolimus through levels and TTV loads

Figure S3. Rituximab effect on TTV levels in ABOi patients.

Table S1. TTV slopes between baseline and M3.

Table S2. BK-related outcomes in ABOi patients with and without rituximab.

Strobe Statement.

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