University of Groningen

Incidence and outcome of BK polyomavirus infection in a multicenter randomized controlled trial with renal transplant patients receiving cyclosporine-, mycophenolate sodium-, or everolimus-based low-dose immunosuppressive therapy

van Doesum, Willem B.; Gard, Lilli; Bemelman, Frederike J.; de Fijter, Johan W.; van der Heide, Jaap J. Homan; Niesters, Hubert G.; van Son, Willem J.; Stegeman, Coen A.; Groen, Henk; Riezebos-Brilman, Annelies

Published in:
Transplant Infectious Disease

DOI:
[10.1111/tid.12687](https://doi.org/10.1111/tid.12687)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Final author's version (accepted by publisher, after peer review)

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van Doesum, W. B., Gard, L., Bemelman, F. J., de Fijter, J. W., van der Heide, J. J. H., Niesters, H. G., van Son, W. J., Stegeman, C. A., Groen, H., Riezebos-Brilman, A., & Sanders, J. S. F. (2017). Incidence and outcome of BK polyomavirus infection in a multicenter randomized controlled trial with renal transplant patients receiving cyclosporine-, mycophenolate sodium-, or everolimus-based low-dose immunosuppressive therapy. *Transplant Infectious Disease*, 19(3), [e12687].
<https://doi.org/10.1111/tid.12687>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Received Date : 26-Oct-2016

Revised Date : 02-Dec-2016

Accepted Date : 04-Dec-2016

Article type : Original Report

**Incidence and outcome of BK polyomavirus infection in a multicenter
randomized controlled trial with renal transplant patients receiving
cyclosporine-, mycophenolate sodium-, or everolimus-based low-dose
immunosuppressive therapy**

Willem B. van Doesum^{1*}, Lilli Gard^{2*}, Frederike J. Bemelman³, Johan W. de Fijter⁴, Jaap J. Homan van
der Heide³, Hubert G.M. Niesters², Willem J. van Son¹, Coen A. Stegeman¹, Henk Groen⁵, Annelies
Riezebos-Brilman², Jan Stephan F. Sanders¹

¹Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen,
University of Groningen, Groningen, The Netherlands

²Department of Clinical Virology, University Medical Center Groningen, University of ³Renal
Transplant Unit, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

⁴Renal Transplant Unit, Department of Nephrology, Leiden University Medical Center, Leiden, The
Netherlands

Groningen, Groningen, The Netherlands

This article has been accepted for publication and undergone full peer review but has not
been through the copyediting, typesetting, pagination and proofreading process, which may
lead to differences between this version and the Version of Record. Please cite this article as
doi: 10.1111/tid.12687

This article is protected by copyright. All rights reserved.

⁵Department of Epidemiology, University Medical Center Groningen, University of Groningen,
Groningen, The Netherlands

*These authors shared co-first authorship.

Running Title: VAN DOESUM ET AL.

Correspondence:

W.B. van Doesum

Department of Internal Medicine,

Division of Nephrology,

University of Groningen,

University Medical Center Groningen

Groningen, The Netherlands.

Email: w.b.van.doesum@umcg.nl

Abbreviations: AUC₁₂, areas-under-the-concentration-over-time curves; BKPyV, BK polyomavirus; BKVAN, BK polyomavirus-associated nephropathy; BPAR, biopsy-proven acute rejection; CsA, cyclosporine; EMM, estimated marginal mean; EVL, everolimus; GEE, generalized estimating equations; HLA, human leukocyte antigen; MDRD, modification of diet in renal disease; MPS, mycophenolate sodium; Pred, prednisolone; mTOR, mammalian target of rapamycin; RT-PCR, real-time polymerase chain reaction; RTR, renal transplant recipients; SV40, simian virus 40; TAC, tacrolimus; VL, viral load.

Abstract

Introduction: It remains unclear whether overall degree of immunosuppression or specific effects of individual immunosuppressive agents are causal for increased occurrence of BK polyomavirus (BKPyV) infection in renal transplant recipients (RTR).

Methods: A prospective, multicenter, open-label randomized controlled trial in 361 *de novo* RTR was performed. A total of 224 RTR were randomized at 6 months into three treatment groups with dual therapy consisting of prednisolone (Pred) plus either cyclosporine (CsA), mycophenolate sodium (MPS), or everolimus (EVL). Primary outcomes were incidence of BK viruria, BK viremia, and BKPyV-associated nephropathy (BKVAN).

Results: From 6 months, incidence of BK viruria in the MPS group (43.6%) was significantly higher than in the other groups (CsA: 16.9%, EVL: 19.8%) ($P=.003$). BKVAN was diagnosed in 3 patients, all treated with MPS (7.8%, $P=.001$). Longitudinal data analysis showed a lower BKPyV load and a significantly faster clearance of BK viruria in the CsA group compared to the MPS group ($P=.03$).

Conclusions: Treatment with MPS was associated with an increased incidence of BK viruria. Dual immunosuppressive therapy with CsA and Pred was associated with the lowest rate of BKPyV replication and the fastest clearance of the virus.

Keywords:

BK virus nephropathy, cyclosporine, everolimus; immunosuppression, mycophenolate sodium, polyomavirus, renal transplantation.

1 INTRODUCTION

Changes in immunosuppressive protocols applied in transplantation medicine have led to decreased allograft rejection rates. Currently triple immunosuppressive regimens are mostly applied, including prednisolone (Pred), a calcineurin inhibitor (tacrolimus [TAC], cyclosporine [CsA]), and an antimetabolite (mycophenolic acid, mycophenolate mofetil [MMF]). These drugs act on different phases of the immune proliferative steps, thereby inhibiting the immune response in a multi-hit model.

With these new, more potent therapeutic strategies, other problems have emerged in the field of transplantation, such as viral infections with BK polyomavirus (BKPyV). BKPyV can cause hemorrhagic cystitis in patients after bone marrow transplantation and BKPyV-associated nephropathy (BKVAN) in renal transplant recipients (RTR).^{1,2} In the last decades, an increase in BKVAN in up to 10% of RTR has been observed, with an associated risk of losing the allograft of up to 50%.² Multiple risk factors have been identified, including: human leukocyte antigen (HLA) mismatching, donor age, deceased donor status, male gender, viral co-infections, and anti-rejection treatment with anti-thymocyte globulin or intravenous immunoglobulin.³⁻⁶ Currently, no effective antiviral therapy is available for treatment of BKPyV replication. Reduction of immunosuppression, e.g., reduction of MMF and/or calcineurin inhibitor, is commonly regarded as the best method to control BKPyV replication, but increases the risk of allograft rejection.^{7-10.}

Because immunosuppressants are regarded as important risk factors in the development of BKPyV-related pathology, the question remains if one immunosuppressive agent or the total immunosuppressive load is responsible for this increased risk of developing BKVAN.

The aim of this study was to investigate the isolated effect of the calcineurin inhibitor CsA, the antimetabolite MMF (as mycophenolate sodium [MPS]), and mammalian target of rapamycin (mTOR) inhibitor everolimus (EVL) on BKPyV replication and on the duration of BKPyV replication, and to study the clinical applicability of each of these individual agents as dual immunosuppressive

therapy (with Pred), as a possible alternative for high-risk transplant patients (patients with a high HLA mismatch, older patients, transplantations with a high cold ischemia time). In this study, the incidence of BK viremia, viremia, and BKVAN was studied in a randomized controlled, prospective multicenter trial with 224 *de novo* RTR receiving dual immunosuppressive therapy consisting of Pred and either CsA, MPS, or EVL.

2 PATIENTS AND METHODS

2.1 Patients

From November 2005 till June 2009 a total of 361 RTR between 18 and 70 years, receiving a first or second renal transplant at the University Medical Center Groningen, Academic Medical Center of Amsterdam, or Leiden University Medical Center, were enrolled in a prospective, multicenter, open-label randomized controlled trial. Exclusion criteria were as follows: HLA-identical sibling donor, a third or fourth transplant, current or historical panel reactive antibodies of >50%, ABO incompatibility, a serum cholesterol >8.5 mmol/L despite adequate HMG co-A reductase inhibition, and female patients unwilling to use adequate contraception.

During the first 6 months post transplantation, patients were treated with a similar standard immunosuppressive regimen. Details about this study protocol are described by Bemelman et al.¹¹ Briefly, induction therapy consisted of 20 mg basiliximab (Simulect®, Novartis Pharma) intravenously prior to transplantation and on day 4 post transplantation, Pred 50 mg once daily from day 1-4, followed by 10 mg once daily from day 4 onwards, MPS (Myfortic®, Novartis Pharma) 720 mg/day from day 1 onwards, and CsA micro-emulsion (Neoral®, Novartis Pharma) from day 1 onwards. Dosage of CsA was calculated with estimated drug exposure, using population-based pharmacokinetic modeling, with serial (full and limited) sampling for calculation of the areas-under-

the-concentration-over-time curves (AUC_{12}). Target values of AUC_{12} for CsA were 5400 mcg*h/L in the first 6 weeks and 3250 mcg*h/L thereafter.¹¹

Follow-up of the study was 24 months after renal transplantation. Patients underwent renal biopsy at 6 months and at 24 months. Biopsy-proven rejection was treated with methylprednisolone pulses. Refractory rejection episodes were treated with rabbit anti-thymocyte globulin (5 doses 2.5 mg/kg; Merieux).¹¹

RTR with no sign of rejection in the protocol biopsy at 6 months after transplantation were randomized into three different treatment arms, consisting of Pred + CsA (target AUC_{12} 3250 mcg*h/L) (CsA group) ; Pred + MPS (target AUC_{12} 40 mg*h/L or a trough level > 2 mg/mL) (MPS group); or Pred + EVL (target AUC_{12} 150 mg*h/L) (EVL group). Patients with signs of (sub)clinical rejection in the 6-month protocol biopsy were excluded from the study and received triple immunosuppressive therapy consisting of Pred, CsA, and MPS. Pred dose was 5-10 mg daily.

After enrolling 39 RTR, inclusion of patients in the MPS arm was prematurely stopped by the Data Safety Monitoring Board owing to an unacceptable high rate of acute rejection. In patients with clinical rejection, CsA was added to the immunosuppressive protocol.

During the total study period, urine and serum samples were collected at baseline, 2 and 6 weeks, and 3, 6, 12, 18, and 24 months and stored at -20°C. Baseline was defined as the day of transplantation, shortly before transplantation. BK viral load (VL) was measured retrospectively. Protocol biopsies and biopsies performed for suspicion of BKVAN were stained for simian virus 40 (SV40) large T antigen. Histologically proven BKVAN was defined as interstitial inflammation and tubulitis, in combination with a positive SV40 nuclear staining in tubular epithelial cells. In this clinical trial, therapy adaptations upon BKPyV infection were not protocolized. RTR with signs of (subclinical) rejection in the 6-month protocol biopsy were excluded from the immunosuppressive study protocol, but were monitored with the same monitoring intervals as RTR who were included,

including serum and urine sampling and a renal biopsy at t=24 months at the time points mentioned above. Results from 0-6 months and 6-24 months were analyzed separately. Patients ID and project number retrospectively provided comparison of the three research groups in data from 0-6 in patients who were to be randomized at t=6 months, which enabled baseline comparison.

The study was conducted according to the Good Clinical Practice guidelines and in accordance to the ethical principles of the Declaration of Helsinki and was approved by the Dutch Medical Ethical Board for medical research. All patients gave written informed consent. The study was registered under the Dutch Medical Ethical Committee Trial ID: NTR1615, Acronym: MECANO).

2.2 BkPyV *real-time polymerase chain reaction (RT-PCR)*

An internally controlled in-house developed quantitative BkPyV RT-PCR, amplifying a 131 bp of the VP2 gene, with a detection limit of $2 \log_{10}$ copies/mL, was used for the detection of BkPyV DNA (Supplementary Table S1). BK viremia and BK viremia were defined as a concentration of BkPyV $> 2 \log_{10}$ copies/mL in urine and serum respectively. The term 'return to latency' was defined as a concentration $< 2 \log_{10}$ copies/mL BkPyV in urine subsequent to a positive urine test.

According to the manufacturer's instructions, DNA was extracted from a 190 μ L sample with the addition of 10 μ L internal control, Phocine herpesvirus.¹² PCR reactions were performed in a total reaction volume of 50 μ L, consisting of 20 μ L DNA, 2x Taqman Universal Mastermix (Life Technologies, USA), 300 nM of primers, 100 nM of probes, 5 mg/mL Bovine serum albumin, and DNase/RNase free water (Sigma). The PCR reactions were run on the ABI PRISM7500 (Life Technologies, USA), with thermal profile: 50°C for 2 minute, 95°C for 10 minute, followed by 42 cycles of 95°C for 15 second, and 60°C for 1 minute.

2.3 Statistics

Statistical analysis was performed using IBM SPSS Statistics 22. Baseline characteristics and incidence of BKPyV infection (viruria, viremia, nephropathy) between the three different treatment groups, primary infections, and reactivations were compared using Chi-square test and ANOVA for categorical variables and continuous variables, respectively. Statistical analyses were performed based on intention-to-treat population. Longitudinal data were analysed using generalized estimating equations (GEE) with an exchangeable correlation matrix. Short-term effect (0-6 months) and long-term effect (6-24 months) were analysed separately. Estimated marginal means (EMM) with 95% confidence intervals from the GEE analyses were plotted in graphs. Biopsy-proven acute rejection (BPAR) during 24 months was compared using survival analysis with log-rank test. Figures were plotted using Graphpad Prism 5.01. Two-sided *P*-values <.05 were considered significant.

3 RESULTS

3.1 Baseline characteristics

In this study 361 RTR were enrolled, of whom 276 RTR underwent a protocol biopsy at 6 months. Reasons for discontinuation of the study are listed in Figure 1. Borderline changes and Banff grade 1A, grade 1B, or higher acute rejection, were found in 50 of 276 RTR, and 2 RTR were excluded because of other complications. In total, 224 RTR were randomized into the three different treatment groups. In patients not randomized at 6 months, the number of deceased donors and the cold ischemia time were significantly higher, compared to patients randomized at 6 months (Supplementary Table S2). In the patients randomized at 6 months, the baseline characteristics between the three different treatment groups did not differ significantly (Table 1).

In all three research groups together, from 6-24 months, 558 of 896 time-point samples were collected and available for analysis (62.3%). In total, 157 of the 224 randomized RTR (70%) were treated per protocol, completed follow-up, and underwent a renal biopsy at 24 months (Figure

1). Of the 89 RTR assigned to treatment with CsA, 74 (83%) were still treated according to protocol 2 years after transplantation, vs 58 of 96 (60%) RTR in the EVL group and 25 of 39 (64%) RTR in the MPS group. Figure 1 summarizes randomization of patients and reasons for discontinuation of the study protocol.

3.2 Drug exposure

From 0 to 6 months, no differences in mean CsA and MPS AUC_{12hrs} in the three treatment groups, randomized at 6 months, were found. No differences in mean CsA and MPS AUC_{12hrs} were found between randomized and not randomized patients at 6 months. In the CsA group, CsA AUC_{12hrs} at 6 and 24 months were 3280 ± 971 mcg*h/L and 3278 ± 907 mcg*h/L, respectively. Mean AUC_{12hrs} in the MPS group were 47 ± 20 mg*h/L at 6 months and 49 ± 23 mg*h/L at 24 months. In the EVL group, the mean AUC_{12hrs} was 203 ± 21 mg*h/L 1 month after conversion and 159 ± 44 mg*h/L at 24 months. Mean AUC_{12hrs} of the CsA, MPS, and EVL group at 6, 7, 12, 18, and 24 months are depicted in Supplementary Figure S1. Pred exposure was not measured via AUC. Pred doses at t=6, 7, 12, 18, and 24 are depicted for the three treatment groups in Supplementary Figure S2 and did not differ among the three groups.

3.3 Primary outcomes BK viruria, viremia, and BKVAN

3.3.1 BK viruria

Of the 224 patients included, 65 tested positive for BKPyV replication (29.0%). From 0 to 6 months, no differences in BKPyV replication are seen between the three treatment groups. In this period, the incidence of BKPyV replication in urine was 12 (13.5%) in the CsA group, 8 (20.5%) in the MPS group,

and 16 (16.7%) in the EVL group ($P=.60$). From 6 to 24 months the incidence of BK viruria was 15 (16.9%) in the CsA group, 17 (43.6%) in the MPS group, and 19 (19.8%) in the EVL group ($P=.003$). The incidence of viruria was not significantly different between patients randomized at 6 months and patients who were not randomized (Supplementary Table S3). Furthermore, the incidence of viruria between patients treated per protocol and patients who switched from immunosuppression for medical reasons, did not differ significantly in the three treatment groups (Supplementary Table S4).

3.3.2 *BK viremia and BKVAN*

In total 31 RTR tested positive for BKPyV replication in serum. Incidence of BK viremia before 6 months was 7 (7.9%), 3 (7.7%), and 6 (6.3%) in the CsA, MPS, and EVL group, respectively ($P=.90$). From 6 to 24 months the incidence of BK viremia in the three groups was 4 (4.5%), 3 (7.7%), and 3 (3.1%) ($P=.51$). Three patients developed BKVAN. All three patients were treated with MPS ($P=.001$) (1.3% of the total cohort, 7.7% of MPS). In Table 2 primary outcome of the study from 6 to 24 months are depicted. The incidence of BK viremia and BKVAN within 24 months was not significantly different between randomized patients ($n=224$) vs patients excluded at 6 months ($n=137$) (Supplementary Table S3) and incidence of BK viremia and BKVAN did not differ between patients treated per protocol and patients who switched from immunosuppression during the study (Supplementary Table S4).

3.4 *Longitudinal analysis*

GEE analysis of long-term effect ($t=6$ - 24 months) was performed on the BKPyV VL in urine (Figure 2). Longitudinal EMM of concentration of BKPyV ($\log_{10}$ copies/mL) in urine from 6 to 24 months are depicted in Figure 2A. A significantly lower mean BKPyV concentration, from 6 to 24 months, was

found in the CsA group compared to the MPS ($P=.002$) and the EVL group ($P=.004$) with an EMM of 1.27, 2.55, and 2.46 $\log_{10}$ copies/mL in the CsA, MPS, and EVL group respectively. This difference remained statistically significant after adjustment for donor age, donor type, CMV status of the donor, and HLA mismatch (A, B, and DR) (CsA vs MPS $P=.004$, CsA vs EVL $P=.03$) (Figure 2A).

In Figure 2B the course of BKPyV VLs EMM in urine over 24 months is displayed. BKPyV VL in urine decreased in the CsA group with an EMM from 6 to 24 months of 2.07 to 0.63 $\log_{10}$ copies/mL, whereas it remained persistently high in the MPS group with an EMM from 6 to 24 months of 2.20 to 2.43 $\log_{10}$ copies/mL ($P=.05$) (Figure 2B). This difference became significant after adjustment for donor type, donor age, CMV status donor, HLA mismatch (A,B, and DR), with an EMM in the CsA group from 6 - 24 months of 2.26 of 0.86 $\log_{10}$ copies/mL, and an EMM in the MPS group of 2.46 to 2.66 $\log_{10}$ copies/mL ($P=.03$).

In serum no significant differences in course of BKPyV infection were found.

3.5 *Death, graft loss, and BPAR*

The combined incidence of death, graft loss, and allograft rejection from 6 to 24 months was 13 (14.6%), 9 (23.1%), and 5 (5.2%) in the CsA, MPS, and EVL group, respectively ($P=.001$). In total, 8 (9.0%), 8 (20.5%), and 1 (1.0%) BPAR episodes were reported in the CsA, MPS, and EVL group between 6 months and 24 months (Figure 3, $P< 0.001$). These were clinical rejections in 'for cause' biopsies – biopsies on indication – in 100% of the cases. No signs of clinical rejection were found in the 24-month protocol biopsies. The majority of these rejections were Banff type I rejections (Table 3). No cases of antibody-mediated rejection occurred.

When the sequence of rejection and BKPyV-related pathology is examined, most BKPyV infection episodes are not related to an episode of allograft rejection. No significant differences in incidence of BK viruria or viremia, after an episode of allograft rejection, were found between the

three treatment groups (Supplementary Tables S5 and S6). BKVAN was exclusively found in the MPS group. These three cases of BKVAN were in patients who did not experience allograft rejection during their 24-month post-transplantation follow-up.

3.6 Renal function

Estimated glomerular filtration rates (eGFR, MDRD formula) were compared between BK viruria-positive and BK viruria-negative patients. At 24 months, the mean eGFR was 38.8 and 39.3 mL/min/1.73 m² in the BKPyV-negative and BKPyV-positive group and were not significantly different ($P=.88$).

4 DISCUSSION

In this study, maintenance treatment with dual immunosuppressive therapy with Pred + MPS was associated with an increased risk of BK viruria. In contrast, patients treated with Pred and either CsA or EVL had a low incidence of BK viruria. Furthermore, while 3 cases of BKVAN occurred in the Pred + MPS group, no BKVAN was observed in patients treated with Pred in combination with either CsA or EVL within 24 months post transplantation. Longitudinal analysis showed a significantly better clearance of BK viruria in patients treated with CsA, with undetectable VLs ($< 2 \log_{10}$ copies/mL) from 12 months onwards, while patients treated with MPS or EVL maintained higher levels of BK viruria up to 24 months.

We therefore draw three main conclusions. First, in this study with relatively low immunosuppression, the incidence of BKVAN and BKPyV-related pathology was considerably lower than the incidence described in the literature. Second, BKVAN only occurred in patients treated with

Pred and MPS. Third, immunosuppressive therapy with Pred + CsA was associated with a shorter return to latency period in case of BKPyV infection, than either Pred + MPS or Pred + EVL.

Many studies have described BKPyV infection and BKPyV-related pathology in RTR patients treated with triple immunosuppressive therapy. Some studies found an increased risk of BKVAN using TAC compared to CsA.¹³⁻¹⁵ Furthermore, several studies indicate a reduced risk of development of BKPyV-related pathology in RTR treated with EVL and either low-dose CsA or low-dose TAC, compared to MPA with CsA, or MPA with TAC.^{6,16-18} In contrast, our study is the first randomized clinical trial to our knowledge comparing long-term Pred-based dual immunosuppressive therapy following 6 months of uniform triple therapy in RTR on incidence of BKPyV, thereby enabling the study of the isolated effect of different immunosuppressive agents on BKPyV replication.

Possible reasons for the effective clearance of BKPyV in patients treated with CsA can be found in *in vitro* studies. Several studies indicate a suppressive effect of CsA on BKPyV-infected Vero E6 cells, which are renal tubular epithelial cells isolated from the African green monkey.¹⁹⁻²² This suppressive effect could be an explanation for the low incidence of BK-related pathology and effective clearance of the virus in patients treated with CsA.

Although we do not see an significant increase in the incidence of BK viremia in patients treated with MPS compared to CsA and EVL, we did observe an increased incidence of viruria and BKVAN. We therefore hypothesized that BKPyV infection is more severe, with prolonged infection episodes and can more easily progress to BKVAN in this group, which was confirmed by our longitudinal analyses.

It is unclear whether MPS directly affects viral replication of BKPyV, but it is thought that MPS is an important factor in maintaining or losing BKPyV-specific T cell immunity. Tapering of antimetabolite is generally accepted as the most effective treatment of BKPyV infection and this can restore BKPyV-specific T cell constitution to levels enabling clearance of the virus.²³⁻²⁶ Loss of

effective BKPyV-specific immunity could be a reason for the increased incidence and prolonged detection of BKPyV in urine and increased occurrence of BKVAN in MPS-treated patients in this study, thereby adding an additional reason to focus on tapering antimetabolite immunosuppressive drugs when encountering BKPyV replication and related pathology. Egli et al.²⁷ reviewed the role of BKPyV-specific T cells and BKPyV-specific immunity in BKPyV infection. They described that an increase of BKPyV-specific T cells measured directly *ex vivo* was only observed in patients with decreasing BKPyV plasma concentrations. Therefore, BKPyV-specific immunity plays a pivotal role in BKPyV replication and progression to BKVAN vs viral clearance, and thereby this indicates a role for BKPyV-specific T cells as a potential marker for regaining control over BKPyV replication.²⁷

Egli et al.²⁷ also concluded that risk stratification prior to transplantation can be achieved, but requires expansion of BKPyV-specific T cells *in vitro* combined with sensitive assays such as EliSpot or intracellular cytokine staining. Recent studies showed a 10- to 100-fold increase of BKPyV-specific T cells after *in vitro* stimulation in patients with BKVAN, indicating that BKPyV-specific T cells were present in these patients, but might be paralyzed by immunosuppression and unable to control BKPyV replication until *in vitro* wash out and re-stimulation.²⁷ This finding strongly indicated an important role for immunosuppressants in reducing BKPyV-specific immunity, and creates new opportunities for future applications of cellular immunotherapy.²⁷

In the current study, it is noticeable that the increased BKPyV loads in the EVL group are detectable up to 24 months, and this contradicts observations associating EVL with reduced BKPyV replication, compared to other immunosuppressants.²⁸⁻³⁰ Data of the pleiotropic effect of EVL and the involved mechanisms are scarce and show multiple modes of action. EVL is involved in suppressing the cellular immune response via suppression of the T helper-1 response. This suppression is established via inhibition of interleukin-2 signaling.³¹ Moreover, reduced expression of viral surface antigens has been observed in hepatitis B-positive patients treated with EVL in the context of hepatocellular carcinoma.³² In addition, *in vitro* data show a viral proliferative effect of

Accepted Article

EVL in hepatitis E infection through inhibition of the PI3K-PKB-mTOR pathway, a new pathway that is involved in a gatekeeping antiviral defense mechanism.³³ In contrast, Hirsch et al.³⁴ found an inhibiting effect of the mTOR inhibitor sirolimus on BKPyV replication in renal epithelial cells. As EVL is the 40-O-(2-hydroxyethyl) derivative of sirolimus, these agents might be expected to have similar properties. However, although both are registered as mTOR inhibitor and both used in transplantation medicine, the two drugs can exert different effects on patients, such as different tissue and subcellular distribution, different affinities to active drug transporters and drug-metabolizing enzymes, as well as differences in drug-target protein interactions.³⁵ These effects are seen both *in vitro* and clinically in transplant recipients.³⁵ Furthermore, sirolimus-related inhibition of BKPyV was principally seen in early infection (the first 24 hours post infection), while in late infection, with late viral gene expression, this effect was not observed, potentially restricting this effect of sirolimus on BKPyV replication to the early infection phase and giving support to the rationale that this early phase is an mTOR-dependent process.³⁴

Translating these data to our study is highly speculative, but one of the above-mentioned mechanisms could be involved in the protracted high BKPyV loads in urine measured in the EVL group. Furthermore, treatment with dual immunosuppressive therapy could elicit these viral promoting effects, whereas these effects are obscured by effects of other immunosuppressants in treatment with triple-drug therapy. However, whether these above-mentioned mechanisms are also operative in BKPyV infection remains unclear, and as we find opposing effects of some agents, further *in vitro* and *in vivo* research is needed.

As mentioned, this prospective multicenter, open-label randomized controlled trial offered the opportunity to study the isolated effects of CsA, MPS, and EVL on BKPyV replication. However, the study has some limitations. First, owing to the clinical status, patients with a rejection episode before 6 months were excluded from the study. This group potentially consists of patients who were at increased risk of BKPyV infection, because of the necessity of increased immunosuppression. Still,

excluded patients did not have increased frequencies of BKPyV-related pathology compared to randomized patients. Second, in the current study, patients were not treated with TAC, which is more frequently used in current immunosuppressive regimens. In multiple studies, the combination of Pred, MPS, and TAC has been associated with a higher risk of BKPyV complications.^{15,18,36,37} In this study, we cannot directly compare the effect of CsA and TAC on BKPyV replication. Third, measuring of drug exposure with the AUC is a method that nowadays is less commonly used in the clinic. Unfortunately, we cannot provide trough levels of the drugs. Still, the reported AUC data give an indication about the actual level of immunosuppression patients received in the three research groups. Lastly, BKPyV monitoring in urine and serum, and possible therapy adaptations upon BKPyV replication, were not protocolized. However, this fact also minimized interventions and enables the study of long-term VL and viral clearance.

A possible confounding effect of the anti-rejection therapy in the MPS group, causing the BKPyV-related pathology, can be ruled out, as the post-rejection incidence of BKPyV infection is very low, and the incidence of this type of BKPyV infection did not differ among the three treatment groups. Furthermore, methylprednisolone as antirejection therapy is not regarded as a risk factor for BKPyV infection or BKVAN, as is demonstrated in several studies and reviews, while maintenance steroid therapy is.³⁷⁻³⁹ However, drug doses of Pred are not higher in the MPS group than in the other treatment groups. A possible confounding effect of maintenance steroid therapy in the development of BKPyV infection and BKVAN is therefore unlikely. Lastly, a possible confounding effect of the switch of patients to other immunosuppressive medication during this study is also unlikely. Rates of BK viruria, viremia, and BKVAN were compared between the patients treated per protocol and the patients who at some point switched immunosuppression, and showed no significant differences between these subgroups (Supplementary Table S4)

Accepted Article

In summary, we can conclude from this study that dual immunosuppressive therapy with MPS + Pred is associated with a prolonged BKPyV infection period and with a higher incidence of BKVAN, while treatment with CsA + Pred can be regarded as an effective treatment to limit BKPyV replication in the first 2 years post transplantation. This dual treatment can be done in an immunological low-risk transplantation cohort, without increasing the risk of graft loss or allograft rejection.

Acknowledgements

We would like to thank all participating patients in this study. Without them this study would not have been possible. In addition, we would like to thank Dr. Jaap van den Born, Marja van Dijk, and Anita Meter for their help during this study.

Author contributions:

W.B.vD., L.G., F.J.B., J.W.dF., J.H.H.vdH., W.J.vS., C.A.S., A.R.-B., and J.S.F.S.: Substantial contribution in research design; W.B.vD., L.G., F.J.B., J.W.dF., J.H.H.vdH., H.G.M.N., W.J.vS., C.A.S., A.R.-B., and J.S.F.S.: Drafting and revising of the paper; W.B.vD., L.G., F.J.B., J.W.dF., J.H.H.vdH., A.R.-B., and J.S.F.S.: Participated in the performance of the research; H.G.M.N., A.R.-B., and J.S.F.S.: Contributed new reagents or analytic tools; W.B.vD., L.G., F.J.B., C.A.S. H.G., A.R.-B., and J.S.F.S.: Analysis and interpretation of data.

Conflicts of Interest:

Authors declare there are no conflicts of interest.

REFERENCES

1. Hirsch HH, Babel N, Comoli P, et al.; ESCMID Study Group of Infection in Compromised Hosts. European perspective on human polyomavirus infection, replication and disease in solid organ transplantation. *Clin Microbiol Infect*. 2014;20(Suppl 7):74-88.
2. Sawinski D, Goral S. BK virus infection: An update on diagnosis and treatment. *Nephrol Dial Transplant*. 2015;30:209-217.
3. Gonzalez S, Escobar-Serna DP, Suarez O, Benavides X, Escobar-Serna JF, Lozano E. BK virus nephropathy in kidney transplantation: An approach proposal and update on risk factors, diagnosis, and treatment. *Transplant Proc*. 2015;47:1777-1785.
4. Prince O, Savic S, Dickenmann M, Steiger J, Bubendorf L, Mihatsch MJ. Risk factors for polyoma virus nephropathy. *Nephrol Dial Transplant*. 2009;24:1024-1033.
5. Pai D, Mann DM, Malik A, Hoover DR, Fyfe B, Mann RA. Risk factors for the development of BK virus nephropathy in renal transplant recipients. *Transplant Proc* 2015;47:2465-2469.
6. Jacobi J, Prignitz A, Buttner M, et al. BK viremia and polyomavirus nephropathy in 352 kidney transplants: Risk factors and potential role of mTOR Inhibition. *BMC Nephrol* 2013;14:207.
7. Schaub S, Hirsch HH, Dickenmann M, et al. Reducing immunosuppression preserves allograft function in presumptive and definitive polyomavirus-associated nephropathy. *Am J Transplant* 2010;10:2615-2623.
8. Sood P, Senanayake S, Sujeet K, et al. Management and outcome of BK viremia in renal transplant recipients: A prospective single-center study. *Transplantation* 2012;94:814-821.
9. Ginevri F, Azzi A, Hirsch HH, et al. Prospective monitoring of polyomavirus BK replication and impact of pre-emptive intervention in pediatric kidney recipients. *Am J Transplant* 2007;7:2727-2735.
10. Johnston O, Jaswal D, Gill JS, Doucette S, Fergusson DA, Knoll GA. Treatment of polyomavirus infection in kidney transplant recipients: A systematic review. *Transplantation* 2010;89:1057-1070.

- Accepted Article
11. Bemelman FJ, de Fijter JW, Kers J, et al. Early conversion to prednisolone/everolimus as an alternative weaning regimen associates with beneficial renal transplant histology and function: The randomized-controlled MECANO Trial. *Am J Transplant*. 2016; Sep 17. doi: 10.1111/ajt.14048. [Epub ahead of print]
 12. van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture. *J Clin Microbiol*. 2003;41:576-580.
 13. Dharnidharka VR, Cherikh WS, Abbott KC. An OPTN analysis of national registry data on treatment of BK virus allograft nephropathy in the United States. *Transplantation* 2009;87:1019-1026.
 14. Acott P, Babel N. BK Virus Replication following kidney transplant: Does the choice of immunosuppressive regimen influence outcomes? *Ann Transplant* 2012;17:86-99.
 15. Brennan DC, Agha I, Bohl DL, et al. Incidence of BK with tacrolimus versus cyclosporine and impact of preemptive immunosuppression reduction. *Am J Transplant*. 2005;5:582-594.
 16. Moscarelli L, Caroti L, Antognoli G, et al. Everolimus leads to a lower risk of BKV viremia than mycophenolic acid in de novo renal transplantation patients: A single-center experience. *Clin Transplant*. 2013;27:546-554.
 17. Peddi VR, Wiseman A, Chavin K, Slakey D. Review of combination therapy with mTOR inhibitors and tacrolimus minimization after transplantation. *Transplant Rev (Orlando)*. 2013;27:97-207.
 18. Suwelack B, Malyar V, Koch M, Sester M, Sommerer C. The influence of immunosuppressive agents on BK virus risk following kidney transplantation, and implications for choice of regimen. *Transplant Rev (Orlando)*. 2012;26:201-211.
 19. Acott P, O'Regan PA, Crocker JF. Suppression of early and chronic BK polyoma virus replication by mycophenolic acid in Vero cells. *Transpl Int*. 2009;22:225-231.
 20. Acott PD, O'Regan PA, Lee SH, Crocker JF. *In vitro* effect of cyclosporin A on primary and chronic BK polyoma virus infection in Vero E6 cells. *Transpl Infect Dis*. 2008;10:385-390.

- Accepted Article
21. Li YJ, Weng CH, Lai WC, et al. A suppressive effect of cyclosporine A on replication and noncoding control region activation of polyomavirus BK virus. *Transplantation*. 2010;89:299-306.
 22. Li YJ, Wu HH, Weng CH, et al. Cyclophilin A and nuclear factor of activated T cells are essential in cyclosporine-mediated suppression of polyomavirus BK replication. *Am J Transplant*. 2012;12:2348-2362.
 23. Dekeyser M, Francois H, Beaudreuil S, Durrbach A. Polyomavirus-specific cellular immunity: From BK-virus-specific cellular immunity to BK-virus-associated nephropathy? *Front Immunol*. 2015;6:307.
 24. Comoli P, Cioni M, Basso S, et al. Immunity to polyomavirus BK infection: Immune monitoring to regulate the balance between risk of BKV Nephropathy and induction of alloimmunity. *Clin Dev Immunol*. 2013;2013:256923.
 25. Babel N, Volk HD, Reinke P. BK polyomavirus infection and nephropathy: The virus-immune system interplay. *Nat Rev Nephrol*. 2011;7:399-406.
 26. Chakera A, Bennett S, Lawrence S, et al. Antigen-specific T cell responses to BK polyomavirus antigens identify functional anti-viral immunity and may help to guide immunosuppression following renal transplantation. *Clin Exp Immunol*. 2011;165:401-409.
 27. Egli A, Dumoulin A, Köhli S, Hirsch H. Polyomavirus BK after kidney transplantation – role of molecular and immunologic markers. *Trends Transplant*. 2009;3:85-102.
 28. Moscarelli L, Caroti L, Antognoli G, et al. Everolimus leads to a lower risk of BKV viremia than mycophenolic acid in *de novo* renal transplantation patients: A single-center experience. *Clin Transplant*. 2013;27:546-554.
 29. Peddi VR, Wiseman A, Chavin K, Slakey D. Review of combination therapy with mTOR inhibitors and tacrolimus minimization after transplantation. *Transplant Rev (Orlando)*. 2013;27:97-107.

- Accepted Article
30. Polanco N, Gonzalez Monte E, Folgueira MD, et al. Everolimus-based immunosuppression therapy for BK virus nephropathy. *Transplant Proc.* 2015;47:57-61.
 31. Mizuno S, Yamagishi Y, Ebinuma H, et al. Progressive liver failure induced by everolimus for renal cell carcinoma in a 58-year-old male hepatitis B virus carrier. *Clin J Gastroenterol.* 2013;6:188-192.
 32. Teng CF, Wu HC, Tsai HW, Shiah HS, Huang W, Su IJ. Novel feedback inhibition of surface antigen synthesis by mammalian target of rapamycin (mTOR) signal and its implication for hepatitis B virus tumorigenesis and therapy. *Hepatology.* 2011;54:1199-1207.
 33. Zhou X, Wang Y, Metselaar HJ, Janssen HL, Peppelenbosch MP, Pan Q. Rapamycin and everolimus facilitate hepatitis E virus replication: Revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. *J Hepatol.* 2014;61:746-754.
 34. Hirsch HH, Yakhontova K, Lu M, Manzetti J. BK Polyomavirus replication in renal tubular epithelial cells is inhibited by sirolimus, but activated by tacrolimus through a pathway involving FKBP-12. *Am J Transplant.* 2016;16:821-832.
 35. Klawitter J, Nashan B, Christians U. Everolimus and sirolimus in transplantation-related but different. *Expert Opin Drug Saf.* 2015;14:1055-1070.
 36. Hirsch HH, Vincenti F, Friman S, et al. Polyomavirus BK replication in de novo kidney transplant patients receiving tacrolimus or cyclosporine: A prospective, randomized, multicenter study. *Am J Transplant* 2013;13:136-145.
 37. Manitpisitkul W, Drachenberg C, Ramos E, et al. Maintenance immunosuppressive agents as risk factors for BK virus nephropathy: A case-control study. *Transplantation.* 2009;88:83-88.
 38. Dadhania D, Snopkowski C, Ding R, et al. Epidemiology of BK virus in renal allograft recipients: Independent risk factors for BK virus replication. *Transplantation.* 2008;86:521-528.

39. Kuypers DR, Bammens B, Claes K, Evenepoel P, Vanrenterghem Y. Maintenance immunosuppressive agents as risk factors for BK virus nephropathy: The need for true drug exposure measurements. *Transplantation* 2010;89:1296-1297.

Legends to figures

FIGURE 1 Patient disposition of eligible *de novo* renal transplant recipients. Intention to treat (ITT) population: all patients randomized after the 6 month biopsy; PP population: all patients who completed study without major protocol deviations; Safety population: all patients who received at least one dose of study drug and had at least one post-baseline safety assessment. CsA group: prednisolone (Pred) + cyclosporine; MPS group: Pred + mycophenolate sodium; EVL group: Pred + everolimus; CNI: calcineurin inhibitor.

FIGURE 2 Longitudinal analysis of BK viruria. (A) Estimated marginal means (EMM) of BK polyomavirus (BKVPyV) load ($\log_{10}$ copies/mL) from t=6 months to t=24 months by treatment group, prednisolone (Pred) + cyclosporine (CsA) (black), Pred + mycophenolate sodium (MPS) (checkered), Pred + everolimus (EVL) (white). (B) Longitudinal course of BKPyV infection in the three treatment groups: Pred + CsA (black circles), Pred + MPS (open squares), and Pred + EVL (black crosses). *P*-values were calculated using GEE with an exchangeable correlation structure. GEE, generalized estimating equations.

FIGURE 3 Kaplan-Meier estimate of time to biopsy-proven acute rejection (BPAR) over 24 months of treatment. Percentages of patients without allograft rejection are plotted against time in the three treatment groups (A). Black line: prednisolone (Pred) + cyclosporine (CsA), long dashed line: Pred + mycophenolate sodium (MPS), short dashed line: Pred + everolimus (EVL). Patients with and without BK polyomavirus (BKPyV) infection in the Pred + MPS group were plotted (B). Log-rank test was used to determine *P*-values.

TABLE 1 Baseline characteristics of renal transplant recipients and donors

	CsA^a (N = 89)	MPS^b (N = 39)	EVL^c (N = 96)	P- value
Male N (%)	56 (62.9)	25 (64.1)	62 (64.6)	.97
Age, years ± SD	49.2±12.8	53.2±11.2	51.0±12.8	.23
Caucasian n (%)	81 (91.0)	32 (82.1)	81 (84.4)	.27
Primary disease leading to end stage renal failure, n (%)				.99
Polycystic kidney disease	21 (23.6)	9 (23.1)	20 (20.8)	
Glomerulonephritis	17 (19.1)	5 (12.8)	17 (17.7)	
Diabetes mellitus	2 (2.2)	2 (5.1)	4 (4.2)	
Pyelonephritis or interstitial nephritis	3 (3.4)	0 (0)	3 (3.1)	
Focal segmental glomerulosclerosis	3 (3.4)	2 (5.1)	4 (4.2)	
Urologic	5 (5.6)	2 (5.1)	10 (10.4)	
Hypertension	13 (14.6)	9 (23.1)	15 (15.6)	
Vascular	4 (4.5)	2 (5.1)	5 (5.2)	
Other	21 (23.6)	8 (20.5)	18 (18.8)	
Total HLA mismatch, mean ± SD	2.81±1.54	2.81±1.80	2.86±1.50	.96
Number of kidney transplantation absolute				.75

numbers n (%)				
1	85 (95.5)	36 (92.3)	90 (93.8)	
2	4 (4.5)	3 (7.7)	6 (6.2)	
Donor characteristics				
Age, years $\pm$ SD	44.3 $\pm$ 19.4	37.7 $\pm$ 21.0	46.1 $\pm$ 17.4	.06
Type of transplantation (%)				.41
Living related	23 (25.8)	6 (15.4)	21 (21.9)	
Living unrelated	30 (33.7)	10 (25.6)	29 (30.5)	
Deceased donor heart beating	23 (25.8)	16 (41)	28 (29.5)	
Donation after cardiac death	13 (14.6)	7 (17.9)	17 (17.9)	
Cold ischemia time, deceased donors only, hours $\pm$ SD	16.7 $\pm$ 5.7	16.1 $\pm$ 5.6	14.9 $\pm$ 6.2	.37

^aCyclosporine (CsA) = prednisolone + CSA.

^bMycophenolate sodium (MPS) = prednisolone + MPS.

^cEverolimus (EVL) = prednisolone + EVL.

SD, standard deviation; HLA, human leukocyte antigen.

TABLE 2 Primary outcome BK polyomavirus (BK) replication and BKVAN from 6 to 24 months

	CsA^a (N = 89)	MPS^b (N = 39)	EVL^c (N = 96)	Total (N= 224)	P- value
BK viruria N (%)	15 (16.9)	17 (43.6)	19 (19.8)	51 (22.8)	.003
BK viremia N (%)	4 (4.5)	3 (7.7)	3 (3.1)	10 (4.5)	.51
BK nephropathy (%)	0 (0.0)	3 (7.7)	0 (0.0)	3 (1.3)	.001

^aCyclosporine (CsA) = prednisolone + CSA.

^bMycophenolate sodium (MPS) = prednisolone + MPS.

^cEverolimus (EVL) = prednisolone + EVL.

BKVAN, BK polyomavirus-associated nephropathy.

.

TABLE 3 Summary of efficacy related result from 6 to 24 months

	CsA^a (N = 89)	MPS^b (N = 39)	EVL^c (N = 96)	Total (N= 224)	P- value
Primary composite endpoint (%)	13 (14.6)	9 (23.1)	5 (5.2)	27 (12.1)	.001
Death	1 (1.1)	0 (0.0)	4 (4.2)	5 (2.2)	
Graft loss	3 (3.4)	1 (2.6)	0 (0.0)	4 (1.8)	
Number of BPAR total	8 (9.0)	8 (20.5)	1 (1.0)	17 (7.6)	
Biopsy proven acute rejection (BPAR) by Banff grade (%)					.002
I	7 (7.9)	8 (20.5)	1 (1.0)	16 (7.1)	
II	1 (1.1)	0 (0.0)	0 (0.0)	1 (0.4)	
III	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	

^aCyclosporine (CsA) = prednisolone + CSA.

^bMycophenolate sodium (MPS) = prednisolone + MPS.

^cEverolimus (EVL) = prednisolone + EVL.

BPAR, biopsy-proven acute rejection.