Importance of molecular diagnostic of viral infections in renal transplant recipients
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CHAPTER 5

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.

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ABSTRACT

Background
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, multi-center, open-label randomized controlled trial in 361 de novo renal transplant recipients was performed. 224 RTR were randomised at 6 months into three treatment groups with duo therapy consisting of prednisolone and either cyclosporine A (CsA), mycophenolate sodium (MPS), or everolimus (EVL). Primary outcomes were incidence of BK viruria, BK viremia and BK nephropathy.

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=0.003). BKPyV nephropathy was diagnosed in 3 patients, all treated with MPS (7.8%, p = 0.001). 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 = 0.03).

Conclusions
Treatment with MPS was associated with and increased incidence of BK viruria. Duo-immunosuppressive therapy with cyclosporine A and prednisolone was associated with the lowest rate of BKPyV replication and the fastest clearance of the virus.
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, a calcineurin inhibitor (tacrolimus, cyclosporine A), and an antimetabolite (mycophenolic acid, mycophenolate mofetil). 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 polyomavirus nephropathy (BKVAN) in renal transplantation recipients [1,2]. In the last decades an increase in BKPyV associated nephropathy up to 10\% of renal transplant recipients (RTR) has been observed, with an associated risk to lose the allograft of up to 50\% [2]. Multiple risk factors have been identified, including: HLA mismatching, donor age, deceased donor status, male gender, viral co-infections, and anti-rejection treatment with ATG or IVIG [3-6]. Currently, no effective anti-viral 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].

Since immunosuppressants are regarded as important risk factors in the development of BK related pathology, the question remains if either 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 cyclosporine A, the antimetabolite mycophenolate mofetil and M-TOR inhibitor everolimus on BKPyV replication and the duration of BK replication, and to study the clinical applicability of one of these agents as duo-immunosuppressive therapy, 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 viruria, viremia and BKVAN was studied in a randomized controlled, prospective multicentre trial with 224 de novo renal transplant recipients receiving duo immunosuppressive therapy consisting of prednisolone and either cyclosporine A, mycophenolate sodium, or everolimus.
Chapter 5

PATIENTS AND METHODS

Patients
From November 2005 till June 2009 a total of 361 renal transplant recipients (RTR) between 18 and 70 years, receiving a first or second renal transplant at the University Medical Center Groningen (UMCG), Academic Medical Center of Amsterdam (AMC) or Leiden University Medical Center (LUMC), were enrolled in a prospective, multi-center, open-label randomized controlled trial. Exclusion criteria were: HLA-identical sibling donor, a third or fourth transplant, current or historical panel reactive antibodies of more than 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, prednisolone 50 mg once daily from day 1-4, followed by 10 mg once daily from day 4 onwards, mycophenolate-sodium (Myfortic®, Novartis Pharma) 720 mg/day from day 1 onwards, and cyclosporine-micro-emulsion (CsA, Neoral®, Novartis Pharma) from day 1 onwards. Dosage of CsA was calculated with estimated drug exposure, using population-based pharmacokinetic modelling, with serial (full and limited) sampling for calculation of the areas-under-the-concentration-over-time curves (AUC12). Target values of AUC12 for CsA were 5400 mcg*h/L in the first six weeks and 3250 mcg*h/L thereafter [11].

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

Renal transplant recipients (RTR) with no sign of rejection in the protocol biopsy at 6 months after transplantation were randomized into three different treatment arms, consisting of prednisolone and cyclosporine A (target AUC12 3250 mcg*h/L) (CsA); prednisolone and MPS (target AUC12 40 mg*h/L or a trough level > 2 mg/mL) (MPS); or prednisolone and everolimus (target AUC12 150 mg*h/L) (EVL). 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 prednisolone, cyclosporine A and mycophenolate sodium. Prednisolone dose was 5-10 mg daily. After enrolling 39 RTR, inclusion of patients in the MPS arm was prematurely
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stopped by the Data Safety Monitoring Board due 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 weeks, 6 weeks, 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 was measured retrospectively. Protocol biopsies and biopsies performed under suspicion of BK nephropathy, were stained for simian virus 40 (SV40) large T antigen. Histological proven BK nephropathy 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 BK infection were not protocolized. Renal transplant recipients with signs of (subclinical) rejection in the 6 months 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 analysed separately. Patients ID and project number retrospectively provided comparison of the three research groups in data from 0-6 in patients that were to be randomized at t = 6 months, which enabled base line 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).

**BKPyV real time polymerase chain reaction (RT PCR)**

An internally controlled in-house developed quantitative BKPyV RT-PCR, amplifying a 131bp of the VP2 gene, with a detection limit of $2 \log_{10}$ copies/ml, was used for the detection of BKPyV DNA (Supplementary table 1). BK viruria 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 190µl sample with the addition of 10µl internal control, Phocine herpesvirus (PhHV) [12]. PCR reactions were performed in a total reaction volume of 50µl, consisting of 20µl DNA, 2x Taqman Universal Mastermix (Life Technologies, USA), 300nM of primers, 100nM of probes, 5mg/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 2min, 95°C for 10min followed by 42 cycles of 95°C for 15sec, 60°C for 1 min.
Statistics
Statistical analysis was performed using IBM SPSS Statistics 22. Baseline characteristics and incidence of BK 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 (ITT). Longitudinal data were analysed using generalised 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 during 24 months was compared using survival analysis with log-rank test. Figures were plotted using Graphpad Prism 5.01. Two-sided P-values <0.05 were considered significant.

RESULTS

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, Banff grade-1A, grade 1B or higher acute rejection were found in 50 of 276 RTR, and two RTR were excluded due to 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 2). In the patients randomized at 6 months baseline characteristics between the three different treatment groups did not differ significantly (Table 1).
Incidence and outcome of BK polyomavirus infection

Table 1: Baseline characteristics of renal transplant recipients and donors

<table>
<thead>
<tr>
<th></th>
<th>CsA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MPS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EVL&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 89)</td>
<td>(N = 39)</td>
<td>(N = 96)</td>
<td></td>
</tr>
<tr>
<td><strong>Male N (%)</strong></td>
<td>56 (62.9)</td>
<td>25 (64.1)</td>
<td>62 (64.6)</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Age, years ± S.D.</strong></td>
<td>49.2 ± 12.8</td>
<td>53.2 ± 11.2</td>
<td>51.0 ± 12.8</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>Caucasian n (%)</strong></td>
<td>81 (91.0)</td>
<td>32 (82.1)</td>
<td>81 (84.4)</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>Primary disease leading to end stage renal failure, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>21 (23.6)</td>
<td>9 (23.1)</td>
<td>20 (20.8)</td>
<td></td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>17 (19.1)</td>
<td>5 (12.8)</td>
<td>17 (17.7)</td>
<td></td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>2 (2.2)</td>
<td>2 (5.1)</td>
<td>4 (4.2)</td>
<td></td>
</tr>
<tr>
<td>Pyelonephritis or interstitial nephritis</td>
<td>3 (3.4)</td>
<td>0 (0)</td>
<td>3 (3.1)</td>
<td></td>
</tr>
<tr>
<td>Focal segmental glomerulosclerosis</td>
<td>3 (3.4)</td>
<td>2 (5.1)</td>
<td>4 (4.2)</td>
<td></td>
</tr>
<tr>
<td>Urologic</td>
<td>5 (5.6)</td>
<td>2 (5.1)</td>
<td>10 (10.4)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>13 (14.6)</td>
<td>9 (23.1)</td>
<td>15 (15.6)</td>
<td></td>
</tr>
<tr>
<td>Vascular</td>
<td>4 (4.5)</td>
<td>2 (5.1)</td>
<td>5 (5.2)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>21 (23.6)</td>
<td>8 (20.5)</td>
<td>18 (18.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Total HLA mismatch, mean ± S.D.</strong></td>
<td>2.81 ± 1.54</td>
<td>2.81 ± 1.80</td>
<td>2.86 ± 1.50</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Number of HLA mismatch, mean ± S.D.</strong></td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of kidney transplantation absolute numbers n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>85 (95.5)</td>
<td>36 (92.3)</td>
<td>90 (93.8)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4 (4.5)</td>
<td>3 (7.7)</td>
<td>6 (6.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Donor characteristics</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Age, years ± S.D.</td>
<td>44.3 ± 19.4</td>
<td>37.7 ± 21.0</td>
<td>46.1 ± 17.4</td>
<td>0.06</td>
</tr>
<tr>
<td>Type of transplantation (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.41</td>
</tr>
<tr>
<td>Living related</td>
<td>23 (25.8)</td>
<td>6 (15.4)</td>
<td>21 (21.9)</td>
<td></td>
</tr>
<tr>
<td>Living unrelated</td>
<td>30 (33.7)</td>
<td>10 (25.6)</td>
<td>29 (30.5)</td>
<td></td>
</tr>
<tr>
<td>Deceased donor heart beating</td>
<td>23 (25.8)</td>
<td>16 (41)</td>
<td>28 (29.5)</td>
<td></td>
</tr>
<tr>
<td>Donation after cardiac death</td>
<td>13 (14.6)</td>
<td>7 (17.9)</td>
<td>17 (17.9)</td>
<td></td>
</tr>
<tr>
<td>Cold ischemia time, deceased donors only, hours ± S.D.</td>
<td>16.7 ± 5.7</td>
<td>16.1 ± 5.6</td>
<td>14.9 ± 6.2</td>
<td>0.37</td>
</tr>
</tbody>
</table>

<sup>a</sup>: CsA: prednisolone + cyclosporine A,  
<sup>b</sup>: MPS: prednisolone + mycophenolate sodium,  
<sup>c</sup>: EVL: prednisolone + everolimus

In all three research groups together, from 6-24 months, 558 of 896 time points 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 two years after transplantation, versus 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.
Figure 1: Patient disposition of eligible de novo renal transplant recipients.

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: prednisolone + cyclosporine A, MPS: prednisolone + mycophenolate sodium, EVL: prednisolone + everolimus, CNI: calcineurin inhibitor.
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Drug exposure
From 0 to 6 months, no differences in mean CsA and MPS AUC\textsubscript{12hrs} in the three treatment groups, randomized at 6 months, were found. No differences in mean CsA and MPS AUC\textsubscript{12hrs} were found between randomized and not randomized patients at 6 months. In the CsA group CsA AUC\textsubscript{12hrs} at 6 and 24 months were 3280 ± 971 mcg*h/L and 3278 ± 907 mcg*h/L, respectively. Mean AUC\textsubscript{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\textsubscript{12hrs} was 203 ± 21 mg*h/L one month following conversion and 159 ± 44 mg*h/L at 24 months. Mean AUC\textsubscript{12hrs} of the CsA, MPS and EVL group at 6, 7, 12, 18 and 24 months are depicted in Supplementary figure 1. Prednisolone exposure was not measured via area under the curve. Prednisolone doses at t = 6, 7, 12, 18 and 24 are depicted for the three treatment groups in Supplementary figure 2 and did not differ between the three groups.

Primary outcomes BK viruria, viremia, and BKVAN

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 = 0.60). From 6 to 24 months the incidence of BK viruria was 15 (16.9%) in the CsA, 17 (43.6%) in the MPS and 19 (19.8%) in the EVL group (p = 0.003). The incidence of viruria, between patients randomized at 6 months and patients who were not randomized, was not significantly different (Supplementary table 3). Furthermore, the incidence of viruria between patients treated per protocol and patients who switched from immunosuppression due to medical reasons, did not differ significantly in the three treatment groups (Supplementary table 4).

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 = 0.90). 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 = 0.51). Three patients developed BK nephropathy. All three patients were treated with MPS (p = 0.001) (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) versus patients excluded at 6 months (n = 137) (Supplementary table 3) and incidence of BK viremia and BKVAN did not differ between patient treated per protocol and patients who switched from immunosuppression during the study (Supplementary table 4).
Table 2: Primary outcome BKPyV replication and BKVAN from 6 to 24 months

<table>
<thead>
<tr>
<th></th>
<th>CsA (N = 89)</th>
<th>MPS (N = 39)</th>
<th>EVL (N = 96)</th>
<th>Total (N= 224)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK viruria N (%)</td>
<td>15 (16.9)</td>
<td>17 (43.6)</td>
<td>19 (19.8)</td>
<td>51 (22.8)</td>
<td>0.003</td>
</tr>
<tr>
<td>BK viremia N (%)</td>
<td>4 (4.5)</td>
<td>3 (7.7)</td>
<td>3 (3.1)</td>
<td>10 (4.5)</td>
<td>0.51</td>
</tr>
<tr>
<td>BK nephropathy (%)</td>
<td>0 (0.0)</td>
<td>3 (7.7)</td>
<td>0 (0.0)</td>
<td>3 (1.3)</td>
<td>0.001</td>
</tr>
</tbody>
</table>


Longitudinal analysis

GEE analysis of long term effect (t = 6 - 24 months) was performed on the BKPyV viral load in urine (Figure 2). Longitudinal estimated marginal means (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 = 0.002) and the EVL group (p = 0.004) 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 versus MPS p = 0.004, CsA versus EVL p = 0.03) (Figure 2A).

In Figure 2B the course of BKPyV viral loads EMM in urine over 24 months is displayed. BKPyV viral load 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 = 0.05) (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 = 0.03).

In serum no significant differences in course of BKPyV infection were found.
Incidence and outcome of BK polyomavirus infection

**Figure 2:** Longitudinal analysis of BK viruria.
Estimated marginal means (EMM) of BKPyV load ($\log_{10}$ copies/ml) from $t = 6$ months to $t = 24$ months by treatment group, prednisolone + cyclosporine A (black), prednisolone + MPS (checkered), prednisolone + everolimus (white) (Figure 2A).

Longitudinal course of BKPyV infection in the three treatment groups, prednisolone + cyclosporine A (black circles), prednisolone + MPS (open squares) and prednisolone + everolimus (black crosses) (Figure 2B). $P$ values were calculated using GEE with an exchangeable correlation structure.

**Death, graft loss and biopsy proven acute rejection (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 ($p = 0.001$). In total 8 (9.0%), 8 (20.5%) and 1 (1.0%) biopsy proven acute rejection (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 months protocol biopsies. The majority of these rejections were Banff type I rejections (Table 3). There were no cases of antibody mediated rejection.
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 + cyclosporine A, long dashed line: prednisolone + MPS, short dashed line: prednisolone + everolimus. Patients with and without BKPyV infection in the prednisolone + MPS group were plotted (B). Log-rank test was used to determine P values.

Table 3: Summary of efficacy related result from 6 to 24 months

<table>
<thead>
<tr>
<th></th>
<th>CsA(^a) (N = 89)</th>
<th>MPS(^b) (N = 39)</th>
<th>EVL(^c) (N = 96)</th>
<th>Total (N = 224)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary composite endpoint (%)</td>
<td>13 (14.6)</td>
<td>9 (23.1)</td>
<td>5 (5.2)</td>
<td>27 (12.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Death</td>
<td>1 (1.1)</td>
<td>0 (0.0)</td>
<td>4 (4.2)</td>
<td>5 (2.2)</td>
<td></td>
</tr>
<tr>
<td>Graft loss</td>
<td>3 (3.4)</td>
<td>1 (2.6)</td>
<td>0 (0.0)</td>
<td>4 (1.8)</td>
<td></td>
</tr>
<tr>
<td>Number of BPAR total</td>
<td>8 (9.0)</td>
<td>8 (20.5)</td>
<td>1 (1.0)</td>
<td>17 (7.6)</td>
<td></td>
</tr>
</tbody>
</table>

Biopsy proven acute rejection (BPAR) by Banff grade (%)

<table>
<thead>
<tr>
<th></th>
<th>CsA(^a) (N = 89)</th>
<th>MPS(^b) (N = 39)</th>
<th>EVL(^c) (N = 96)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7 (7.9)</td>
<td>8 (20.5)</td>
<td>1 (1.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>II</td>
<td>1 (1.1)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>0 (0.0)</td>
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When the sequence of rejection and BKPyV related pathology is examined, most BKPyV infection episodes are not related with 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 table 5 and 6). 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 months post transplantation follow up.

Renal function
Estimated glomerular filtration rates (eGFR, MDRD formula) were compared between BK viruria positive and BK viruria negative patients. At 24 months 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 = 0.88).

DISCUSSION
In this study, maintenance treatment with duo immunosuppressive therapy with prednisolone and mycophenolate sodium was associated with an increased risk of BK viruria. In contrast, patients treated with prednisolone and either cyclosporine A or everolimus had a low incidence of BK viruria. Furthermore, while 3 cases of BKVAN occurred in the prednisolone and mycophenolate sodium group, no BKVAN was observed in patients treated with prednisolone in combination with either cyclosporine A or everolimus within 24 months post-transplantation. Longitudinal analysis showed a significantly better clearance of BK viruria in patients treated with cyclosporine A, with undetectable viral loads (< 2 log₁₀ copies/ml) from 12 months onwards, while patients treated with mycophenolate sodium or everolimus maintained higher levels of BK viruria up to 24 months. We therefore draw three main conclusions. First, in this study with relative low immunosuppression, the incidence of BKVAN and BKPyV related pathology was considerably lower than the incidence described in the literature. Second, BKPyV associated nephropathy only occurred in patients treated with prednisolone and mycophenolate sodium. Third, immunosuppressive therapy with prednisolone and cyclosporine A was associated with a shorter return to latency period in case of BKPyV infection, than either prednisolone and MPS, or prednisolone and everolimus.

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 tacrolimus compared to cyclosporine A [13-15]. Furthermore, there are several studies that indicate a reduced risk of development of BKPyV related pathology in RTR treated with EVL and, either low dose CsA, or low dose tacrolimus, compared to MPA with CsA, or MPA with tacrolimus [6,16-18]. In contrast, our study is the first randomized clinical trial comparing long term prednisolone based duo immunosuppressive therapy following six 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 cyclosporine A can be found in in vitro studies. Several studies indicate a suppressive effect of cyclosporine A on BKPyV infected Vero E6 cells, which are renal tubular epithelial cells isolated from the African green monkey [19-22]. This could be an explanation for the low incidence of BK related pathology and effective clearance of the virus in patients treated with cyclosporine A.

Although we do not see a 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 BKPV infection is more severe, with prolonged infection episodes and can easier progress to BKVAN in this group, which was confirmed by our longitudinal analyses.

It is unclear whether MPS directly affects viral replication of BKPV. But it is thought that MPS is important factor in maintaining and loss of BKPV specific T cell immunity. Tapering of antimetabolite is generally accepted as the most effective treatment of BKPV infection and this can restore BKPV specific T cell constitution to levels enabling clearance of the virus [23-26]. Loss of effective BKPV specific immunity could be a reason for the increased incidence and prolonged detection of BKPV 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 BKPV replication and related pathology. Egli et al. reviewed the role of BKPV specific T-cells and BKPV specific immunity in BKPV infection. They described that an increase of BKPV specific T-cells measured directly ex vivo was only observed in patients with decreasing BKPV plasma concentrations. Therefore, BKPV specific immunity plays a pivotal role in BKPV replication and progression to BKVAN versus viral clearance, and thereby this indicates a role for BKPV specific T-cells as potential marker for regaining control over BKPV replication[27].

Egli et al also concluded that risk stratification prior to transplantation can be achieved, but requires expansion of BKPV 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 BKPV specific T-cells after in vitro stimulation in patients with BKVAN, indicating that BKPV specific T-cells were present in these patients, but might be paralysed by immunosuppression and unable to control BKPV replication until in vitro wash out and re-stimulation[27]. This strongly indicated an important role for immunosuppressants in suppressing BKPV specific immunity and creates new opportunities for future applications of cellular immunotherapy [27].
Incidence and outcome of BK polyomavirus infection

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 everolimus with reduced BKPyV replication, compared to other immunosuppressants [28-30]. Data of the pleiotropic effect of everolimus and the involved mechanisms are scarce and show multiple modes of action. Everolimus is involved in suppressing the cellular immune response via suppression of the Th1-response. This is established via inhibition of IL-2 signaling [31]. Moreover, reduced expression of viral surface antigens has been observed in hepatitis B positive patients treated with everolimus in the context of hepatocellular carcinoma [32]. In addition, in vitro data show a viral proliferative effect of everolimus in hepatitis E infection through inhibition of the PI3K-PKB-mTOR pathway, a new pathway that is involved in a gate keeping antiviral defense mechanism [33]. In contrast, Hirsch et al. found an inhibiting effect of the mTOR inhibitor sirolimus on BKPyV replication in renal epithelial cells [34]. Since everolimus is the 40-O-(2-hydroxyethyl) derivative of sirolimus one would expect similar properties of these agents. However, although both registered as mTOR inhibitor and both used is 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 effect 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), whether 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 supporting 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 duo 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 since we find opposing effects of some agents, further in vitro and in vivo research is needed.

As mentioned, this prospective multi-center, open-label randomized controlled trial offered the opportunity to study the isolated effects of cyclosporine A, MPS and everolimus on BKPyV replication. However, the study has some limitations. First, due 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, due to 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 tacrolimus, which is more frequently used in
current immunosuppressive regimens. In multiple studies, the combination of prednisolone, MPS and tacrolimus has been associated with a higher risk of BKPyV complications [15,18,36,37]. In this study we cannot directly compare the effect of cyclosporine A and tacrolimus on BKPyV replication. Third, the AUC measuring of drug exposure is a method that nowadays is less commonly used in the clinic. Unfortunately we cannot provide through 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 also minimized interventions and enables the study of long term viral load and viral clearance.

A possible confounding effect of the antirejection therapy in the MPS group, causing the BKPyV related pathology can be ruled out, since the post rejection incidence of BKPyV infection is very low and the incidence of this type of BKPyV infection did not differ between 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 prednisolone 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 thereby 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 of immunosuppression, which showed no significant differences between these subgroups (Supplementary table 4)

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

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REFERENCES


