Supplementary data

Extended Version of Materials and Methods

Study Design and Patients

This was an open-label, single-center, randomized study (Registered at Australian New Zealand Clinical Trials Registry: ACTRN12600000016033ANZCTR). Patients were recruited from a transplant center at the Charles University Teaching Hospital, Pilsen, Czech Republic. From November 2007 through April 2012, eligible for inclusion were all adult renal transplant recipients with recipient (R) and/or donor (D) positive CMV serology. Exclusion criteria included D-/R- serostatus, allergy to (val)ganciclovir or (val)acyclovir, severe leukopenia or thrombocytopenia, participation in another clinical trial and inability to provide informed consent. The study was approved by the local ethics committee and conducted in compliance with the Declaration of Helsinki. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'. Written informed consent was obtained before enrollment. Patients were randomized by the transplant physician using a random-number table, at a 1:1 ratio with the use of block sizes of 4, to valganciclovir or valacyclovir prophylaxis. The order of interventions varied randomly within each block. Randomization was stratified by D/R CMV serostatus. Sequentially numbered sealed envelopes were used for allocation concealment.

Interventions

Patients received valganciclovir (Valcyte, Hoffman-La Roche, Grenzach-Wyhlen, Germany) at a dose of 900mg daily or valacyclovir (Valtrex, Glaxo Wellcome, Dartford, UK) at a dose of 2g four times daily for 3 months beginning day 7 post-transplant at the latest. Antiviral drugs doses were tapered based on renal function according to manufacturers' instructions. PCR for CMV DNA from whole blood was performed at 2-week intervals for the first 3 months and at 4, 5, 6, 9, and 12 months thereafter. PCR was likewise performed if clinically required. Asymptomatic CMV DNAemia occurring during or after prophylaxis was not treated regardless of the viral load. Patients developing severe CMV disease were treated initially by intravenous ganciclovir (Cymevene, Hoffman-La Roche, Basel, Switzerland) at a dose of 5 mg/kg every 12 hours. Other patients were given valganciclovir (900 mg twice daily). Duration of therapy was 21 days or longer in cases with persistent CMV viremia.

The standard immunosuppressive protocol included cyclosporine, mycophenolate mofetil and corticosteroids. Immunological high-risk patients received induction by antithymocyte globulin (Thymoglobuline, Genzyme, Marcy I'Eoile, France) and tacrolimus. Recipients of grafts from highly marginal donors (donation after cardiac death, ≥70 years old, donors with hypertension and significant nephrosclerosis on biopsy, and/or dual kidney transplantation) were treated with anti-IL2R monoclonal antibody (basiliximab) and low-dose tacrolimus. Patients received prophylaxis with trimethoprim-sulfamethoxazole for 4 months and oral amphotericin solution for 1 month. Plasma was tested for polyoma virus DNAemia every month for the first 6 months and at 9 and 12 months with preemptive immunosuppression reduction at a significant viral load (≥10,000 copies/mL).

Study Outcomes and Follow-up

The primary end points were the incidences of CMV DNAemia and biopsy-proven acute rejection (BPAR) at 12 months post-transplant. Secondary end points included CMV disease, patient and graft survival (not censored for death), subclinical rejection and IF/TA assessed by protocol biopsy at month 3, renal function, other infections, and safety evaluated by recording adverse events and routine laboratory parameters. In addition, other potential indirect effects of CMV such as cardiovascular events or new-onset diabetes mellitus, malignancy, and economic data were recorded prospectively. All patients remained on follow-up for a minimum of 12 months post-transplant or until death.

CMV DNAemia was defined by detection of CMV DNA. CMV disease was defined as symptomatic CMV viremia and embraced both CMV syndrome and tissue-invasive disease (1). Suspected acute rejection was confirmed by core biopsy using the up-dated Banff classification (2). BPAR was defined as grade ≥IA or antibody-mediated rejection.

Quantitative PCR for CMV DNA

Quantitative real-time PCR was performed using a commercially available kit (RealStar® CMV PCR kit 1.0, Altona Diagnostics, Germany) according to manufacturers' instructions on a Rotor-Gene™ 2000/3000 system (Corbett Research, Australia). DNA was isolated from 200 µL of whole blood using a commercially available kit

(QuickGene DNA whole blood kit S (DB-S), Kurabo, Japan). Sensitivity of the investigation was 50 copies/mL of whole blood. Physicians assessing the PCR results were blinded to the study group of patients.

Quantitative PCR for Polyomavirus DNA

Quantification of BK virus (BKV) was performed using commercial diagnostic kit (BK Virus R-gene™-Quantification kit, Argene/Biomérieux, Verniolle, France) according to manufacturer's protocol. Amplification reaction targets small T Antigen and detects BKV with sensitivity of 65 copies/mL. PCR was run on Rotor Gene Q (Qiagen, Gaithersburg, USA). DNA was isolated from 200 μL of plasma by QIAamp DNA Blood Mini kit (Qiagen, Gaithersburg, USA). The efficiency of DNA extraction and PCR inhibition was checked in each sample using Real Time PCR amplification of control artificial DNA. Physicians assessing the PCR results were blinded to the study group of patients.

Biopsy Sample Processing

Indications for biopsy included an increase in serum creatinine by more than 20% that could not be explained otherwise, and delayed graft function. Biopsy was also undertaken in cases of suboptimal development of graft function (serum creatinine >160 μmol/L). In patients with functioning grafts, protocol biopsy was performed at 3 months. Using a 16- or 18-gauge needle (biopsy gun) a minimum of two cores were obtained. Tissues for light microscopy were fixed in 4% formaldehyde, embedded in paraffin using routine procedure. Sections 3μm thick were cut from tissue blocks and stained with hematoxylin and eosin, blue trichrome, silver staining, and Congo red staining. Immunohistochemical analysis was performed in the Ventana automated slide stainer without manual antigen retrieval and was detected using Ventana ultraView universal DAB detection kit (Ventana-Roche, Tucson, AZ) as recommended by manufacturer. The primary antibodies against C4d (Venatana-Roche) and SV40 (Ventana-Roche), p53 (Ventana-Roche), and CMV (Dako, Glostrup, Denmark) were used. Appropriate positive controls were employed. In all biopsies, fresh tissue samples were examined using immunoflorescence staining for C4d (Biomedica Grouppe, Vienna, Austria) and for C3 (Dako) depositions in peritubular capillaries paralelly. All biopsies were evaluated according to the the up-dated Banff classification (2). Pathologists evaluating biopsy samples were blinded to the study group of the patients.

Sample Size and Statistical Analysis

The anticipated CMV DNAemia and BPAR rates in the valacyclovir group were 60% and 12%, respectively (3, 4). To detect a reduction in the incidence of CMV DNAemia to 35% and an increase in the incidence of BPAR to 30% in the valganciclovir group it was necessary to enroll at least 60 and 72 patients to ensure 80% power for detection of a treatment difference with type 1 error of 0.05. Given the anticipated number of patients to be lost to follow-up, a total of 80 patients were planned to enroll. Because of a clinically important trend toward a lower BPAR rate in the valganciclovir group (12.5% vs. 25%; P=0.14 by not adjusted log-rank test) after analysis of a planned study population, it was decided to increase sample size. With the assumption of the same difference in BPAR, at least 114 patients were required. Finally, 124 patients were planned to enroll anticipating patients to be lost to follow-up.

Quantitative parametric data were compared using Student's t-test and the Mann-Whitney U-test in non-parametric distribution. Qualitative data were analyzed using χ^2 with Yates correction or Fisher's exact test. Incidence of CMV DNAemia and disease, BPAR, patient and graft survival, polyoma BKV viremia and polyomavirus-associated nephropathy were calculated using Kaplan-Meier curves, with the log-rank test used for comparison. The Cox proportional hazard model adjusting for age, previous transplantation, peak panel reactive antibodies, HLA mismatches, calcineurin inhibitor, induction therapy, donor age, donor type, expanded criteria donor, and delayed graft function was used to calculate aHR and 95% CI for selected variables. Data were analyzed according to the intention-to-treat principle. Statistical calculations were made using SigmaStat 3.1 and Statistica 9.0 software. Values of $P<0.05$ were considered statistically significant.

References

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Supplementary Table 1. Immunosuppressive therapy during the study

Variables	Valganciclovir (n = 60)	Valacyclovir (n = 59)	P value
Switch to tacrolimus during 1st year[a]	16 (64)	24 (69)	0.93
Switch to cyclosporine during 1st year[b]	4 (11)	1 (4)	0.39
Early (< 3 months) mycophenolate mofetil withdrawal[c]	15 (25)	16 (27)	0.96
Maintenance immunosuppression[d]			
Month 1			
Tacrolimus	47 (78)	41 (71)	0.46
Cyclosporine	13 (22)	17 (29)	
Mycophenolate mofetil	59 (98)	57 (98)	0.49
Prednisone	60 (100)	57 (98)	0.99
Month 3			
Tacrolimus	45 (76)	40 (71)	0.71
Cyclosporine	14 (24)	16 (29)	
Mycophenolate mofetil	56 (95)	52 (93)	0.94
Prednisone	59 (100)	55 (98)	0.98
Month 12[e]			
Tacrolimus	44 (77)	43 (80)	0.94
Cyclosporine	13 (23)	11 (20)	
Mycophenolate mofetil	54 (95)	50 (93)	0.94
Prednisone	55 (96)	51 (94)	0.95
Cyclosporine trough level (ng/mL; mean ± SD)[d]			
Week 1	241 ± 53	233 ± 53	0.56
Month 1	250 ± 94	249 ± 76	0.96
Month 3	198 ± 56	213 ± 52	0.45
Month 6	121 ± 47	145 ± 60	0.24
Month 12	117 ± 21	118 ± 49	0.87
Tacrolimus trough level (ng/mL; mean ± SD)[d]			
Week 1	10.6 ± 4.6	10.5 ± 6.6	0.99
Month 1	11.9 ± 6.3	11.8 ± 4.7	0.70
Month 3	9.1 ± 3.4	9.1 ± 3.6	1.0
Month 6	6.5 ± 2.0	6.5 ± 2.2	0.13
Month 12	6.3 ± 2.3	6.5 ± 2.1	0.64
Mycophenolate mofetil dose (g per day; mean ± SD)[d]			
Initial	1.92 ± 0.25	1.90 ± 0.22	0.63
Month 1	1.69 ± 0.45	1.73 ± 0.49	0.56
Month 3	1.45 ± 0.49	1.53 ± 0.57	0.49
Month 6	1.34 ± 0.55	1.39 ± 0.58	0.49
Month 12	1.25 ± 0.56	1.34 ± 0.58	0.51
Mycophenolic acid AUC (mg*h/L; mean ± SD)			
Day 5	44 ± 18	39 ± 26	0.11
Month 3	47 ± 24	37 ± 11	0.06
Prednisone dose (mg per day; mean ± SD)[d]			
Week 1	20 ± 0	20 ± 3	0.75
Month 1	13 ± 2	13 ± 3	0.60
Month 3	10 ± 1	10 ± 2	0.85
Month 6	5 ± 0	5 ± 1	0.75
Month 12	5 ± 1	5 ± 1	0.93

AUC, area under concentration curve.

Data are n (%) unless otherwise indicated.

[a]Assessed in patients initially treated with cyclosporine. Reasons for switch in valganciclovir prophylaxis: 13 acute rejection episode (including borderline changes), 1 subclinical rejection, 1 gingival hyperplasia, 1 acute tubular necrosis; in valacyclovir prophylaxis: 16 acute rejection episode (including borderline changes), 2 gingival hyperplasia, 1 donor-related nephrosclerosis, 1 acute tubular necrosis, 1 cyclosporine nephrotoxicity, 1 acute pancreatitis.

[b]Assessed in patients initially treated with tacrolimus. Reasons for switch in valganciclovir prophylaxis: 2 diarrhea, 2 polyomavirus-associated nephropathy; in valacyclovir prophylaxis: 1 diarrhea.

[c]Reasons for withdrawal in valganciclovir prophylaxis: 7 myelotoxicity, 4 gastrointestinal side effect, 2 severe bacterial infection, 1 acute pancreatitis; in valacyclovir prophylaxis: 7 gastrointestinal side effect, 5 myelotoxicity, 3 severe bacterial infection, 1 acute pancreatitis.

[d]Assessed in patients with functioning graft.

[e]In 2 patients no calcineurin inhibitor was given.

Supplementary Table 2. Variables associated with biopsy-proven acute rejection and polyoma BKV viremia by multivariate Cox proportional hazard model

Variables[a]	Hazard ratio	95% CI	P value
Biopsy-proven acute rejection			
Valacyclovir prophylaxis[b]	2.49	1.09 – 5.65	0.03
Basiliximab induction[c]	0.20	0.05 – 0.77	0.02
HLA mismatches (per 1 increase)	1.58	1.09 – 2.28	0.02
Polyoma BKV viremia			
Valacyclovir prophylaxis[b]	0.43	0.19 – 0.96	0.04
Tacrolimus[d]	2.27	1.09 – 4.73	0.03

BKV, BK virus, CI, confidence interval

Data are n (%) unless otherwise indicated.

[a]Following variables were included to analysis: age, previous transplantation, peak panel reactive antibodies, HLA mismatches, calcineurin inhibitor, type of induction therapy, donor age, donor type, expanded criteria donor, and delayed graft function.

[b]Valganciclovir prophylaxis is the reference group.

[c]No induction therapy is the reference group.

[d]Cyclosporine-based immunosuppression is the reference group.

Supplementary Table 3. Characteristics of patients with CMV disease

Patient/No. of Episodes	Study Group	D/R Status	Time to CMV Disease (days)	Peak CMV DNAemia (copies/mL)	CMV Disease Type
1/1	Valganciclovir	D+/R-	135	6550	Syndrome
2/1	Valganciclovir	D+/R+	135	2800	Syndrome
3/1	Valganciclovir	D+/R-	144	164500	Colitis
4/1	Valacyclovir	D+/R+	106	13350	Syndrome

CMV, cytomegalovirus; D, donor; R, recipient.

Supplementary Table 4. Patient and graft survival at 12 months and renal function parameters

Variables	Valganciclovir (n = 60)	Valacyclovir (n = 59)	P value
Patient survival[a]	60 (100)	56 (95)	0.08
Graft survival[b]	57 (95)	55 (93)	0.68
Delayed graft function	13 (22)	11 (19)	0.86
Serum creatinine (μmol/L; mean ± SD)			
Week 1	288 ± 220	292 ± 200	0.65
Month 1	156 ± 59	148 ± 57	0.17
Month 3	141 ± 36	134 ± 34	0.31
Month 12	136 ± 39	137 ± 44	0.92
eGFR (mL/min; mean ± SD)[c]			
Month 1	48 ± 16	48 ± 16	0.65
Month 3	58 ± 18	57 ± 19	0.57
Month 12	58 ± 23	60 ± 20	0.75

eGFR, estimated glomerular filtration rate.

Data are n (%) unless otherwise indicated.

[a]Reasons for death in valacyclovir prophylaxis: 1 hemorrhagic shock, 1 sepsis, 1 epileptic status.

[b]Reasons for graft loss in valganciclovir prophylaxis: 2 polyomavirus-associated nephropathy, 1 primary nonfunctioning graft; in valacyclovir prophylaxis: 3 death with functioning graft, 1 antibody-mediated rejection.

[c]According to the MDRD7 formula.