Sex-associated differences in cytomegalovirus prevention: Prophylactic strategy is associated with a strong kidney function impairment in female renal transplant patients

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Abbreviations

ATG, Antithymocyte globulin; CMV, Cytomegalovirus; 95% CI, 95% Confidence interval; D^-R^-, Seronegative donor and seronegative recipient; D^+R^-, Seropositive donor and seronegative recipient; D^+R^+, Seropositive donor and seropositive recipient; EBV, Epstein-Barr virus; eGFR, Estimated glomerular filtration rate; eGFR-1y, Estimated glomerular filtration rate one year after transplantation; IQR, Interquartile range; MMF, mycophenolate mofetil; OR, Odds ratio; qPCR, Quantitative polymerase chain reaction; R^+, Seropositive Recipient.

Conflict of interest statement

The authors have no conflicts of interest to disclose

Abstract

Cytomegalovirus (CMV) syndrome or disease, a serious health hazard after renal transplantation, can be prevented using the antiviral drug (val)ganciclovir. (Val)ganciclovir is typically administered following a prophylactic or a pre-emptive strategy. The prophylactic strategy entails early universal administration, the pre-emptive strategy early treatment in case of infection. However, it is not clear which strategy is superior with respect to transplantation outcome and viral clearance. We have retrospectively analysed 540 patients from the multicentre Harmony study: 308 were treated according to a prophylactic, 232 according to a pre-emptive strategy. As expected, we observed an association of prophylactic strategy with lower incidence of CMV syndrome, delayed onset and lower viral loads compared to the pre-emptive strategy. However, the prophylactic strategy was associated with higher incidence of acute rejection (P=0.002) and – for female patients – a strong impairment of glomerular filtration rate (eGFR) one year post-transplant (P<0.001, median difference: 18.5 $mL \cdot min^{-1} \cdot 1.73m^{-2}$). Additionally, the prophylactic strategy was associated with increased incidence of severe BK virus reactivation. Our results suggest for the first time that the prophylactic strategy might lead to inferior transplantation outcomes, providing evidence for a strong association with sex.

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1. Introduction

Cytomegalovirus (CMV) is a herpes virus often reported as the most important viral pathogen after kidney transplantation.[1–3] It is a major cause of morbidity and mortality, being associated with retinitis, pneumonitis, colitis, encephalitis, allograft damage and allograft loss, among others.[1–4] CMV syndrome or disease may occur as a consequence of reactivation of latent infections or through primary infection, acquired from the donor or from the environment.[2] The major risk factor for CMV syndrome or disease is the pre-transplantation serostatus: CMV seronegative transplant recipients with a seropositive donor (D^+R^-) have the highest risk, while seropositive recipients (R^+) have an intermediate risk and seronegative recipients with seronegative donors (D^-R^-) have the lowest risk.[2] Moreover, the use of immunosuppressive drugs like rabbit antithymocyte globulin (ATG) can additionally increase the incidence of CMV (re)activations.[5]

The standards in prevention and treatment of CMV (re)activation are based on ganciclovir or its oral prodrug valganciclovir.[6,7] Two prevention strategies are routinely employed in the clinic: prophylactic and pre-emptive.[2,6,7] The prophylactic strategy is based on the universal administration of (val)ganciclovir in case of patients with a CMV risk constellation, usually during 3-6 months after transplantation.[6,7] In the pre-emptive strategy, patients are regularly monitored for CMV through quantitative polymerase chain reaction or pp65 antigenemia test; (val)ganciclovir is only administered after a positive test, ideally before any symptoms of CMV syndrome or disease manifest.[6,7] The pre-emptive strategy thus leads to a reduction of unnecessary treatments, which is advantageous with respect to the appearance of side effects and resistances against antiviral drugs.[6,7]

While the KDIGO guideline of 2009 preferred prophylaxis as the standard of prevention, the more recent reference CMV management guideline recommends both strategies for the prevention of CMV disease in patients with both high or intermediate CMV mismatch-based risk constellation.[6,7] However, the differences in outcome with regard to other criteria, including renal function and other viral (re)activationsis largely unclear. In addition to antiviral

therapy, CMV-specific T cell immunity has been shown to control CMV viral reactivations, determining the outcome of disease.[8–10] Interestingly, there is evidence of sex differences in both ganciclovir pharmacokinetics and the anti-CMV immune response.[11–14] Thus, female patients have been shown to have a faster ganciclovir clearance, and distinct anti-CMV immunological profiles e.g. higher number of secreting anti-CMV T cells.[11–15] In spite of this, there are to our knowledge still no studies on the influence of sex on the clinical outcomes of CMV prevention strategies. In this work, we provide evidence that prophylaxis might be associated with inferior transplantation outcomes – including a strong reduction of renal function in female patients – at the end of the first year post-transplant.

2. Materials and Methods

2.1. Patient population

As part of the systems medicine project e:KID, we conducted a sub-study within the randomized, multi-centre, investigator-initiated Harmony trial (NCT 00724022)[16,17] to determine the impact of CMV prevention strategy on transplant outcome. For this, CMV, Epstein-Barr virus (EBV) and BK virus (BKV) viral loads, white blood cell count and creatinine were measured at predetermined eight study visits.[17] This viral monitoring was non-interventional and centrally performed and was independent from the internal, interventional viral monitoring (see section 2.3). The study was carried out in compliance with the Declaration of Helsinki and Good Clinical Practice.

2.2. Patient medication

According to study design, patients were treated with a quadruple (arm A) or triple (arms B and C) immunosuppressive therapy.[16] Patients in arm A received an induction therapy with basiliximab and maintenance therapy consisting of tacrolimus (Advagraf®, Astellas), mycophenolate mofetil (MMF) and corticosteroids. Patients in arm B received the same treatment as in arm A, but corticosteroids were withdrawn at day 8. Patients in arm C received the same treatment as in arm B, except induction was achieved with ATG, instead

of basiliximab.

2.3. Patient monitoring

Patients were monitored for graft function along eight visits, scheduled at day 0 (pre-transplantation), 2nd week, 1st month, 2nd month, 3rd month, 6th month, 9th month, and 12th month. To assess graft function, glomerular filtration rate was calculated using the CKD-EPI formula, measured in $mL \cdot min^{-1} \cdot 1.73\ m^{-2}$.[18] Serious adverse events were defined following the Good Clinical Practice guidelines. Suspected episodes of acute rejection had to be confirmed through biopsy; histologic characteristics were described according to the Banff criteria of 2005.[19] Regarding the outcome assessment, acute rejection was analysed including and excluding borderline rejections. Routine surveillance biopsies were allowed but not mandatory.

2.4. Clinical monitoring and management of clinical complications

Viral (re)activations were monitored and managed at local centres as described previously.[17] CMV in particular was monitored for all patients, independently of the prevention strategy. Monitoring was performed independently from the above described CMV viral load measurements and was based on three different methods: serum PCR viral load measurements; test for pp65 antigenemia and symptom monitoring according to the internal centre standards. Diagnosis of CMV syndrome was likewise based on the methods, where a qPCR over 1000 $copies \cdot mL^{-1}$ was defined as positive. Patients with CMV syndrome were treated based on internal centre standards. Suggested treatment was (val)ganciclovir treatment according to local standards with/or without reduction of tacrolimus and MMF dose. No data on the time point of CMV syndrome diagnostic were available for this study; no data on CMV disease were available.

2.5. Screening of CMV, EBV and BKV viraemia

In parallel to the clinical monitoring performed at each centre, peripheral blood samples from

the eight visits were centrally monitored for CMV, EBV and BKV by TaqMan qPCR, as described previously.[17] The centralized viral load assessment was non-interventional.

2.6. Definition of CMV prevention strategy groups and characterization of antiviral treatments

Patients were stratified into two prevention strategy groups based on the (val)ganciclovir treatments during the first 14 days. All patients that started a (val)ganciclovir treatment during the first 14 days were assigned into the prophylactic strategy group; the rest of the sub-cohort was classified in the pre-emptive strategy group. The 14 day threshold was chosen to allow comparability with our previous prospective study on the topic (VIPP), in which recruiting took place during the first two post-transplant weeks.[20,21] CMV syndrome was treated equally for both strategy groups, as explained above. Antiviral treatments with no data on the end time point were disregarded for the calculation of the treatment duration but considered for the calculation of median dose. Accordingly, reported MMF dose and tacrolimus trough correspond to the 14 day threshold.

2.7. Viraemia-based patient classification

To assess the efficacy of prevention strategies regarding viral (re)activations, patients were classified based on their peak viral load values for CMV, EBV and BKV, as previously published.[17] Briefly, the classifications are defined as follows: “detectable viral load” corresponds to patients with at least one viral load measurement over detection limit (250 copies·mL^{-1})[17], “elevated viral load” to patients with at least one viral load measurement over 2000 copies·mL^{-1}, “high viral load” to patients with at least one viral load measurement over 10000 copies·mL^{-1}. These groups can overlap with each other.

2.8. Analytical approach

The association between transplantation outcomes and prevention strategy was analyzed using the following approach: First, crude single-parameter associations between prevention strategy and the outcome were assessed; whenever possible, the associations were also

calculated stratified for the risk constellations of the outcome at hand. Second, to assess independence from confounders of potential associations, multi-parameter backwards elimination regression analysis was performed. Third, the difference of single-parameter strategy-outcome associations between sexes were assessed. Lastly, if there was a significant difference between the sexes and a significant association with the outcome was observed for only one sex, the association was tested in that sub-group employing multi-parameter backwards elimination regression analysis.

2.9. Statistical analysis

For descripticve statistics, categorical variables are summarised as numbers and frequencies; quantitative variables are reported as median and interquartile range (IQR). Differences between the groups were calculated using Pearson's chi-square test with continuity correction (or two-tailed Fisher's exact test, when stated); odds ratios (OR) and 95% confidence intervals (95% CI) are provided. In all cases, odds ratio over 1 denote a higher prevalence of the adverse event in the pre-emptive strategy group. Differences in quantitative variables between groups are analysed using the two-tailed Mann-Whitney test. Kaplan-Meier curves for time to occurrence of the first CMV (re)activation were calculated using the R survival package (version 2.43-3); strategy groups were compared using the log-rank test. Correlations are reported employing Spearman's rho and P value).

To control for the influence of confounders in the analysis of prevention strategies, multi-parameter regression was employed. Regression models incorporated as independent variables the prevention strategies and all potential confounding factors, and as dependent variable the outcome variable of interest. For categorical binary outcomes, logistic regression was employed, for continuous outcomes linear regression was used. For the peak viral load outcomes, the decimal logarithm was used. After logarithmisation, viral loads below detection level were set to zero. We considered as potential confounders all measured demographic factors (see Table 1 and Table S1) and centre effects. For the analysis of the eGFR one year after transplantation (eGFR-1y), CMV, BKV and EBV peak viral loads and acute rejection

were additionally included as potential confounders, as these events precede or are simultaneous to eGFR-1y and might hence have an influence on it. To exclude unimportant factors, backward elimination was performed, employing Akaike's information criterion. Factors in the final regression model were therefore considered to be independently associated with the dependent variable, regardless of the corresponding P value. Resulting linear regression models were tested for homoscedasticity of their residuals employing the studentized Breusch-Pagan test; multicollinearity was assessed for all models calculating the generalized variance-inflation factors. If homoscedasticity cannot be assumed ($P<0.050$), robust standard errors are reported. For multicollinearity, a threshold of 5 for the generalized variance-inflation factors was employed to exclude a factor.

The analysis of the interactions of sex effects with the effect of strategy group on outcomes was assessed with a single-parameter ANOVA type 3 for continuous variables and a single-parameter analysis of deviance type 3 of a logistic model for categorical variables. In all cases, a P value below 0.050 was considered significant. P values were not corrected for multiple testing, as this study was of exploratory nature and we prioritized the minimization of type II errors.[22–24] Analyses were performed using R (Version 3.3.2).

3. Results

3.1. Definition of study sub-cohorts

To assess the effects of CMV prevention strategy on transplantation outcome, we retrospectively analysed the cohort of an existent study (N=540 patients) with a female ratio of 35.9% (N=194).[16,17] Patients were grouped into two sub-cohorts, based on whether they started an antiviral therapy during the first two post-transplant weeks (prophylactic strategy group, N=308) or not (pre-emptive strategy group, N=232) (see 2.6). As described previously, viral load (CMV, EBV and BKV), graft function and other clinical markers were collected along eight visits during the first post-transplant year; a total of 3715 blood samples were analysed.[17]

In this work, we have evaluated the effects of prevention strategy on eGFR, incidence of acute rejection, incidence of CMV complications, and incidence of BKV and EBV (re)activations following the analytical approach detailed in section 2.8. After an assessment of the baseline characteristics of the sub-cohorts, we describe in detail the most important findings in the next sections.

3.2. Study sub-cohorts characteristics

To assess differences between the two prevention strategy sub-cohorts regarding demographics or treatment procedures, we performed comparative statistics (see Table 1, for cause of end-stage kidney disease see Table S1). As shown in Table 1, significant (P<0.050) differences were found for MMF daily dose, CMV mismatch-based risk and therapy arm; the difference was highly significant (P<0.001) for the latter two factors.

Variable		**Prophylactic strategy group (N=308)**	**Pre-emptive strategy group (N=232)**	**P value**
Female sex		104 (33.8%)	90 (38.8%)	0.265
Caucasian race		304 (98.7%)	231 (99.6%)	0.397[a]
Recipient age (years)		55 [46-64]	57 [44-64]	0.988
Body mass index ($kg \cdot m^{-2}$)		26.3 [23.5-29.7]	25.4 [22.8-28.4]	0.059
CMV mismatch-based risk	High (D^+R^-)	119 (39.1%)	27 (12.1%)	<0.001
	Medium (R^+)	137 (45.1%)	129 (57.8%)	
	Low (D^-R^-)	48 (15.8%)	67 (30.0%)	
EBV mismatch-based risk	High (D^+R^-)	13 (5.1%)	11 (6.2%)	0.583[a]
	Medium (R^+)	239 (93.4%)	161 (91.0%)	
	Low (D^-R^-)	4 (1.6%)	5 (2.8%)	

Donor age (years)		55 [48-65]	55 [46-65]	0.931
No previous transplantations		298 (96.8%)	216 (94.7%)	0.346
Living donor		31 (10.1%)	35 (15.4%)	0.088
Donors with expanded criteria		136 (44.2%)	99 (42.7%)	0.798
Cold ischaemia time (min)		626 [427-844]	600 [414-840]	0.505
Number of HLA A, B and DR mismatches		3 [2-4]	3 [1-4]	0.457
No panel-reactive antibodies before transplantation		23 (7.6%)	17 (7.7%)	1.000
White blood cell count (cells·L^{-1})		7.2 [5.7-8.9]	7.1 [6.0-8.5]	0.676
Therapy arm	A (basiliximab+steroids)	93 (30.2%)	96 (41.4%)	<0.001
	B (basiliximab)	92 (29.9%)	83 (35.8%)	
	C (ATG)	123 (39.9%)	53 (22.8%)	
Low MMF daily dose (< 2000 mg·day^{-1})		37 (12%)	45 (19.4%)	0.025
Tacrolimus trough level (ng·mL^{-1})		9.9 [7.4-12.5]	9.2 [7-12]	0.145

Table 1 – Differences in patient baseline characteristics between strategy groups. Data are given as number (percentage) or median [interquartile range]. Donors with expanded criteria are defined as follows: age over 60 years or age over 50 years and at least two of the following factors: cerebrovascular accident as the cause of death, hypertension or a serum creatinine level over 1.5 mg·dL^{-1}. P value is calculated based on Pearson's chi-square test or Fisher's exact test for binary variables (marked with [a]) and based on Mann-Whitney test for continuous variables. Data on the cause of end-stage kidney disease are summarized in Table S1.

59 patients (25.4%) of the pre-emptive strategy group were treated with (val)ganciclovir after the second post-transplantation week. In total, 367 patients (68.0%) received (val)ganciclovir during the first post-transplant year, independently of their prevention strategy group; use of antivirals in both groups is shown in Table 2.

Variable	**Prophylactic strategy group (N=308)**	**Pre-emptive strategy group treated with (val)ganciclovir**

		(N=59)
Median time under (val)ganciclovir (days)	118 [87-182]	92 [46-155]
Valganciclovir average daily dose ($mg \cdot day^{-1}$)	277 [165-450]	450 [205-454]
Ganciclovir usage	34 (11.0%)	8 (13.6%)
Intravenous ganciclovir usage	31 (10.1%)	7 (11.9%)

Table 2 – Antiviral treatment details for the two strategy groups. Data are given as number (percentage) or median [interquartile range].

3.3. Prophylactic strategy group was associated with a serious impairment of graft function in female patients

Patients in the prophylactic strategy group had, in general, a poorer transplantation course than those in the pre-emptive strategy group, as confirmed by their significantly higher incidence of total serious adverse events (64.6% vs. 54.3%, P=0.020, OR: 0.65 [0.45-0.94]). For the main outcome, renal function, single-parameter analysis likewise revealed a significant difference between the prevention groups. Thus, eGFR-1y was lower in the prophylactic strategy group compared to the pre-emptive group (45.6 [33.5-58.3] vs. 50.3 [38.1-64.5] $mL \cdot min^{-1} \cdot 1.73m^{-2}$, P=0.011). Of note, a significant difference in eGFR was detected for all visits from the third post-transplant month on (Figure S4).

Multi-parameter analysis could not confirm an independent association of prophylactic strategy with decreased eGFR-1y (Table S2 A). However, we observed that this association is subject to a strong interaction with sex (P=0.003, see Table 3): The significant impairment of eGFR-1y in the prophylactic group was only observed for female patients, with a difference of 18.5 $mL \cdot min^{-1} \cdot 1.73m^{-2}$ (38.4 [28.8-53.6] vs. 56.8 [41.3-67.9] $mL \cdot min^{-1} \cdot 1.73m^{-2}$, P<0.001). Among male patients, the prophylactic strategy group had a slightly higher median eGFR-1y (48.5 [36.3-61.5] vs. 47.2 [37.2-59.6] $mL \cdot min^{-1} \cdot 1.73m^{-2}$, P=1.000). This significant

difference in eGFR for females can be observed already one month after transplantation (Figure 1). Importantly, the multi-parameter analysis confirmed that prevention strategy was independently associated with eGFR-1y in female patients (Table S2 B).

Outcomes		**Effect size of prevention strategies in male patients (N=346)**	**Effect size of prevention strategies in female patients (N=194)**	**P value**
Serious adverse event		0.76 [0.48-1.19]	0.49 [0.26-0.91]	0.237
eGFR-1y		-1.3 [-4.8-4.7]	18.5 [8.2-20.7]	0.003
Acute rejection		0.35 [0.13-0.81]	0.39 [0.12-1.12]	0.847
CMV	Detectable viral load	1.48 [0.80-2.73]	1.28 [0.57-2.85]	0.756
	Elevated viral load	1.77 [0.71-4.5]	1.01 [0.30-3.35]	0.416
	High viral load	1.24 [0.34-4.42]	0.77 [0.06-6.85]	0.654
	Syndrome	0.57 [0.31-0.99]	0.47 [0.19-1.07]	0.683
BKV	Detectable viral load	0.75 [0.48-1.18]	0.82 [0.45-1.51]	0.807
	Elevated viral load	0.64 [0.36-1.13]	0.71 [0.34-1.48]	0.809
	High viral load	0.49 [0.21-1.05]	0.71 [0.22-2.13]	0.545
EBV	Detectable viral load	0.68 [0.38-1.19]	2.26 [1.00-5.25]	0.009
	Elevated viral load	1.27 [0.54-2.97]	4.27 [0.79-43.19]	0.154
	High viral load	1.15 [0.22-5.46]	1.16 [0.01-91.65]	0.998

Table 3 – Differences in effect size of prevention strategy between sexes with respect to outcomes. The P value refers to the P value of the interaction term, as assessed by a single-parameter ANOVA type 3 for continuous variables or a single-parameter analysis of deviance type 3 of a logistic model for categorical variables. The effect sizes are given with 95% confidence intervals as OR (categorical variables) or as difference of medians (continuous variables). An OR over 1 indicates a higher incidence of the outcome in the prophylactic strategy group, compared to the pre-emptive; a positive difference of medians indicates a lower value of the median outcome in the prophylactic strategy group compared to the pre-emptive. For the definition of (re)activation severity degrees see Methods (2.7).

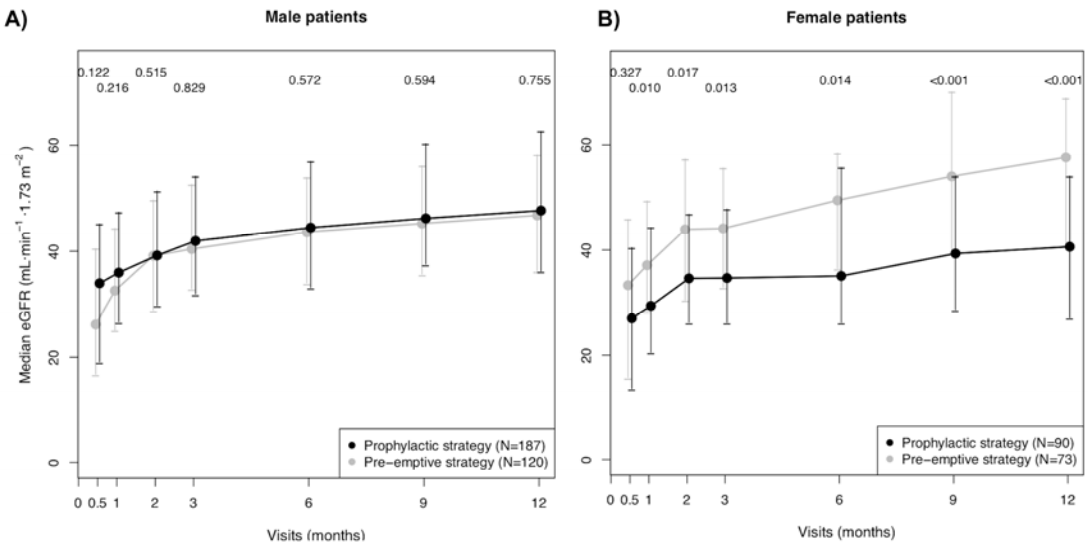

Figure 1. Graft function dynamics of the prevention strategy groups stratified for sex. To exclude the role of donor type in eGFR dynamics, only patients with a deceased donor are shown (for the eGFR dynamics of patients with living donors, see Figure S5). Median eGFR ($mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$) of the prevention strategy groups is plotted for each protocol visit. The bars indicate the interquartile range. The numbers indicate the p value of the difference in eGFR between the prevention strategy groups, as calculated using the Mann-Whitney test. Day 0 is not shown, as it is pre-transplantation.

We further investigated the nature of the difference in eGFR between prevention strategies in female patients, examining the associations of dose and beginning of therapy with eGFR-1y. We did not observe any negative effect of high doses: We compared the female patients in the pre-emptive strategy group that received a valganciclovir treatment, with those in the prophylactic strategy group, as the first group had a significantly higher daily dose than the second (P=0.041). Thus, we observed that these patients had a significantly higher eGFR-1y than those in the prophylactic group (38.4 [28.8-53.6] vs. 57.7 [40.1-66.6] $mL{\cdot}min^{-1}{\cdot}1.73m^{-2}$, P=0.005), in spite of the higher valganciclovir dose. On the other hand, we observed a significant effect of therapy timing in eGFR-1y, with a positive correlation between day of treatment beginning and eGFR-1y (ρ=0.27, P=0.015) among female patients who received (val)ganciclovir.

Based on the encountered strong interaction of sex and prevention strategy for renal function, we decided to evaluate systematically the sex effects for all transplantation outcomes. While no differences between sexes with respect to transplantation outcomes

were observed (Table 4), further interactions of sex with prevention strategy were observed with respect to (Table 3), as described in detail below.

Outcomes		**Male patients (N=346)**	**Female patients (N=194)**	**P value**
Serious adverse event		204 (59.0%)	121 (62.4%)	0.493
eGFR-1y		48.2 [36-5-61.4]	46.8 [33.3-58.8]	0.341
Acute rejection		38 (11.0%)	22 (11.3%)	1.000
CMV	Detectable viral load	57 (16.5%)	35 (18.0%)	0.730
	Elevated viral load	24 (6.9%)	15 (7.7%)	0.866
	High viral load	13 (3.8%)	5 (2.6%)	0.629
	Syndrome	78 (22.5%)	35 (18.0%)	0.261
BKV	Detectable viral load	173 (50.0%)	87 (44.8%)	0.289
	Elevated viral load	76 (22.0%)	45 (23.2%)	0.825
	High viral load	41 (11.8%)	18 (9.3%)	0.438
EBV	Detectable viral load	74 (21.4%)	35 (18.0%)	0.414
	Elevated viral load	28 (8.1%)	9 (4.6%)	0.178
	High viral load	9 (2.6%)	2 (1.0%)	0.343[a]

Table 4 – Differences in outcomes between sexes. Data are given as number (percentage) or median [interquartile range]. P value is calculated based on Pearson's chi-square test or Fisher's exact test for binary variables (marked with [a]) and on Mann-Whitney test for continuous variables. For the definition of (re)activation severity degrees see Methods (2.7). As it can be observed, there were no significant differences between sexes with respect to the measured outcomes.

3.4. Prophylactic strategy was associated with higher incidence of acute rejection independently of sex

Regarding the important complication acute rejection, a significantly higher incidence was found in the prophylactic strategy group (14.9% vs. 6.0%, P=0.002, OR: 0.37 [0.18-0.70]). The observed association between prevention strategy and incidence of acute rejection was confirmed to be independent from potential confounders by multi-parameter analysis (Table

S2 C). As shown in Table 3, there were no significant differences between sexes for this association.

3.5. The prophylactic strategy was associated with lower incidence of CMV (re)activation and syndrome than the pre-emptive strategy

We further tested the effectivity of the strategies in the prevention of CMV complications. The single-parameter analysis showed a higher incidence of CMV viral load in the pre-emptive strategy group (19.8% vs. 14.9%, P=0.167); for CMV syndrome a significantly higher incidence was found in the prophylactic strategy group (see Table S3 A). The latter was not unexpected, as most patients with high CMV risk were in the prophylactic strategy group. Stratifying for CMV risk, a clear trend for lower incidence of CMV (re)activation was observed in the prophylactic strategy group; but not for CMV syndrome (Table S3 B). However, the results of the multi-parameter regression show that prophylactic strategy was independently associated with both lower peak CMV viral load and CMV syndrome incidence (Table S2 D-E). No significant sex association were observed for these outcomes (Table 3 and Table S3 D-E).

Interestingly, CMV incidence showed significantly different temporal patterns in the two strategy groups (Figure 2): While in the pre-emptive strategy group 86.7% of all CMV load events occurred in the first 100 days post-transplant, in the prophylactic strategy group it was only 56.1% (Figure 2A). Moreover, a higher prevalence of detectable CMV viral load was observed in the prophylactic strategy group for all study visits after the third month (Figure

2B).

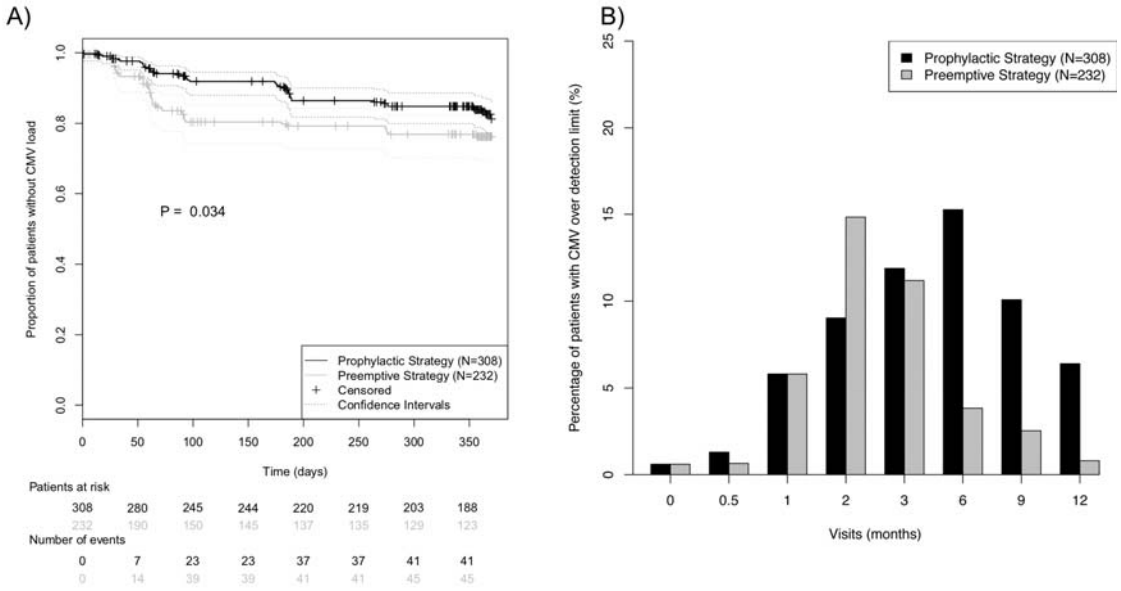

Figure 2. Incidence of CMV (re)activation in the prevention strategy groups during the first post-transplant year. (A) Kaplan-Meier curves for absence of CMV (re)activation during the first post-transplant year. CMV (re)activation was defined as viral load over detection limit. Prevention strategy groups were compared using the log-rank test. (B) Prevalence of CMV viral load over detection limit for each of the eight protocol visits.

3.6. The prophylactic strategy was associated with lower incidence of EBV (re)activation among women and higher incidence of BKV (re)activation

Regarding the effects of prevention strategy for other viruses, no effect of prevention strategy was found for EBV when considering both sexes, neither through (stratified) single-parameter analysis (Table S3 A and S3 C), nor through multi-parameter analysis (Table S2F). However, there was a significant interaction of sex and prevention strategy for EBV (re)activation (P=0.009, Table 3). Thus, there was an association of prophylactic strategy with reduced incidence of EBV (re)activation only for female patients (P=0.049, OR: 2.26 [1.00-5.25], see Table S3 D). An analysis stratified for viral risk constellations (Table S3 F) showed an even stronger association for female patients treated with ATG (P=0.005, OR: 5.65 [1.56-22.86]). Similarly, the multi-parameter analysis showed that prophylactic strategy was independently associated with decreased peak EBV load in female patients (Table S2 G).

On the other hand, we found a higher incidence of severe BKV (re)activation in patients of the prophylactic strategy group (P=0.056, OR: 0.55 [0.29-1.01]), see Table S3 A. The multi-parameter analysis confirmed that prophylactic strategy was independently associated with increased peak BKV load (Table S2 H). No significant sex-associated effect was observed for BKV (re)activation (Table 3 and Table S3 D-E), even though male sex was independently associated with increased peak BKV load in the multi-parameter analysis (Table S2 H).

4. Discussion

The goal of our study was to evaluate the clinical efficacy and sex-associated differences of two common CMV prevention strategies in a large cohort of kidney transplant patients from the multi-centre Harmony study.

The main finding of the study is the first evidence of superiority of the pre-emptive strategy with respect to acute rejection incidence and – for female patients – graft function.[20,21,25–31] While we have already reported a negative effect of prophylaxis on rejection within the VIPP study – albeit only for the D^-R^+ subgroup – this study is the first to report a significant association in the entire cohort.[20,21] The observed association of prevention strategy with eGFR in female patients is especially relevant, as eGFR-1y is an accepted marker for long term transplantation outcomes.[32]

Our results highlight the importance of sex-associated effects in transplantation. In recent years, sex differences have emerged as an essential factor in clinical studies.[33] In transplantation, several complications are associated with sex, including acute rejection, graft loss and viral (re)activations.[11,13,34,35] However, the underlying reasons for these sex differences are not well understood; possible causes include the hormonal regulation of the immune system, the effects of pregnancy, and differences in the metabolism of drugs routinely employed in transplantation.[11] For example, there is tentative evidence of sex-related differences in the pharmacokinetics of (val)ganciclovir.[11,12] Thus, ganciclovir clearance has been observed to be 24% faster in female transplantation patients, suggesting higher activity of the organic anion transporter 1.[11,12] Furthermore, it has been shown

repeatedly that women and men have different anti-CMV immunological profiles, with an impact in the graft function and even the phenotype of the immune system as a whole.[13–15] Notably, in a recent publication, Lindemann *et al.* have observed an association of high number of IL-21 secreting anti-CMV T cells with female sex and lower eGFR in a clinical transplantation context.[13]

Our analyses may provide some evidence on the nature of the observed association of eGFR-1y with prevention strategies in female patients. Although the impaired graft function in the female prophylactic strategy group can be partly explained through the higher incidence of BKV severe (re)activation and rejection, the results of the multi-parameter analysis showed an independent association of prevention strategy with graft function, regardless these adverse events.[17,36,37] Therefore, our results do not support the hypothesis that these adverse events are the main cause for the difference in eGFR-1y between sub-cohorts. Regarding possible nephrotoxic effects of the antiviral drug, we did not find any association of higher valganciclovir doses with lower eGFR – rather, the opposite association was observed – in contrast to Heldenbrand *et al*.[38] The absence of a negative dose-dependent effect suggests that the observed difference was not a consequence of nephrotoxicity of valganciclovir. On the other hand, the time of beginning with the (val)ganciclovir therapy was determinant for the eGFR-1y: the later patients began the therapy, the higher the renal function.

Albeit being highly speculative, we hypothesize that the observed results may be (at least in part) caused by an immunological mechanism. As we previously demonstrated, an increased number of CMV-specific T-cells upon CMV (re)activation is associated with reduced alloreactivity and improved graft function in renal transplantation patients.[39] Similarly, in liver transplantation, primary CMV infection has been found to be associated with donor-specific $CD8^+$ T-cell hyporesponsiveness and increased $V\delta1/V\delta2$ $\gamma\delta$ T-cell ratio – a surrogate marker for operational tolerance.[40] Accordingly, the higher rate of asymptomatic CMV (re)activation found in the pre-emptive strategy group could potentially lead to regulatory $\gamma\delta$ T-cell-based graft protection and explain the better graft function and lower incidence of

acute rejection; an early administration of (val)ganciclovir would therefore hinder the development of this protective immune response. Our hypothesis is compatible with the observed differences between male and female recipients, as sex-associated differences in the anti-CMV immunity have been shown by Lindemann *et al.* to correlate with graft function.[13] Even though this effect cannot explain our observations, it demonstrates how sex and anti-CMV immunity can potentially interact and affect eGFR.[13] Therefore, further research, including systems medicine approaches, is needed to better understand the effects of CMV prevention strategies from an immune, virological and pharmacokinetic point of view – with emphasis on sex-associated differences – and their effects on transplantation outcome.[41,42]

Of interest, the prophylactic strategy group showed a higher incidence of late-onset CMV (from month six on); such increases of viral (re)activation incidence after the end of prophylaxis have been observed before.[27,28] Regarding BKV, the observed association of prophylaxis with increased incidence of severe (re)activation is in line with two recent studies.[43,44] On the other hand, an association of EBV with prevention strategy was observed only for female patients.[45] This is relevant, as there is currently no consensus in the literature on this topic. While a number of publications have observed an effect against EBV or post-transplant lymphoproliferative disease (the main EBV-associated complication), a meta-study with 2366 participants saw no effect of prophylaxis for this disease.[46–50]

This study is based on the prospective Harmony study, a trial designed with the goal of identifying which immunosuppressive drug combination is superior with respect to acute rejections and secondary to a number of other outcome variables, including graft function and viral (re)activations.[16] A potential shortcoming of the present study consists therefore in the fact that prevention strategy groups were not randomized, and no power calculation was performed with respect to this question. Therefore, even though we have controlled for all measured demographic factors in the analyses, we cannot completely exclude bias in unmeasured factors as the cause of the observed differences. A further limitation is related to the criteria employed for choosing prevention strategy for each patient: As the decision to

adopt a prophylactic or a pre-emptive strategy was taken by each individual physician or centre, it is difficult to ascertain the causes for the individual decisions, potentially introducing bias in the use of prevention strategies. On the other hand, our study does have some advantages: We have analysed a larger (N=540) and more heterogeneous cohort (patients with all CMV mismatch-based risk constellations) than most studies on the matter, thereby achieving higher statistical power.[20,21,25–29] Moreover, our study design is closer to clinical reality, with similar valganciclovir doses and prophylaxis duration to those routinely employed in the clinic.[51] Based on the limitations and advantages of the study, we deem our results as evidence that further research is needed to determine the effects of prevention strategies on transplantation outcome, especially the hypothetical interactions with sex.

In summary, our study provides the first evidence in the literature suggesting that the pre-emptive approach might be associated with improved graft function – especially in female patients. Even though the prophylactic strategy was associated with reduced prevalence of CMV (re)activation and syndrome, it was associated with higher incidence of acute rejection and, for female patients, with a strong impairment of the renal function. The effects of prevention strategy on graft function and acute rejection were shown in the multi-parameter analysis as independent from potential bias in the cohort. Further randomized controlled studies are needed to confirm these observations.

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Description of Supporting Information

Table S1. Differences in cause of end-stage kidney disease between strategy groups.

Table S2. Detailed results of the multi-parameter analyses.

Table S3. Results of the single-parameter analyses for virus-related complications. The detailed results of single-parameter associations of prevention strategy, including stratified analysis for risk constellation and sex, are reported. Following complications were analysed: CMV (re)activation and syndrome, EBV (re)activation and BKV (re)activation

Figure S4. Graft function dynamics of the prevention strategy groups. Median eGFR ($mL \cdot min^{-1} \cdot 1.73\ m^{-2}$) of the prevention strategy groups is plotted for each protocol visit. The bars indicate the interquartile range. The numbers indicate the P value of the difference in eGFR between the prevention strategy groups, as calculated using the Mann-Whitney test. Day 0 is not shown, as it is pre-transplantation.

Figure S5. Graft function dynamics of the prevention strategy groups stratified according to sex, for patients with living donor. Median eGFR ($mL \cdot min^{-1} \cdot 1.73\ m^{-2}$) of the prevention strategy groups is plotted for each protocol visit. The bars indicate the interquartile range. The numbers indicate the p value of the difference in eGFR between the prevention strategy groups, as calculated using the Mann-Whitney test. Day 0 is not shown, as it is pre-transplantation. For the eGFR dynamics of patients with deceased donors, see Figure 1.