Viral reactivations and associated outcomes in the context of immune reconstitution after pediatric hematopoietic cell transplantation

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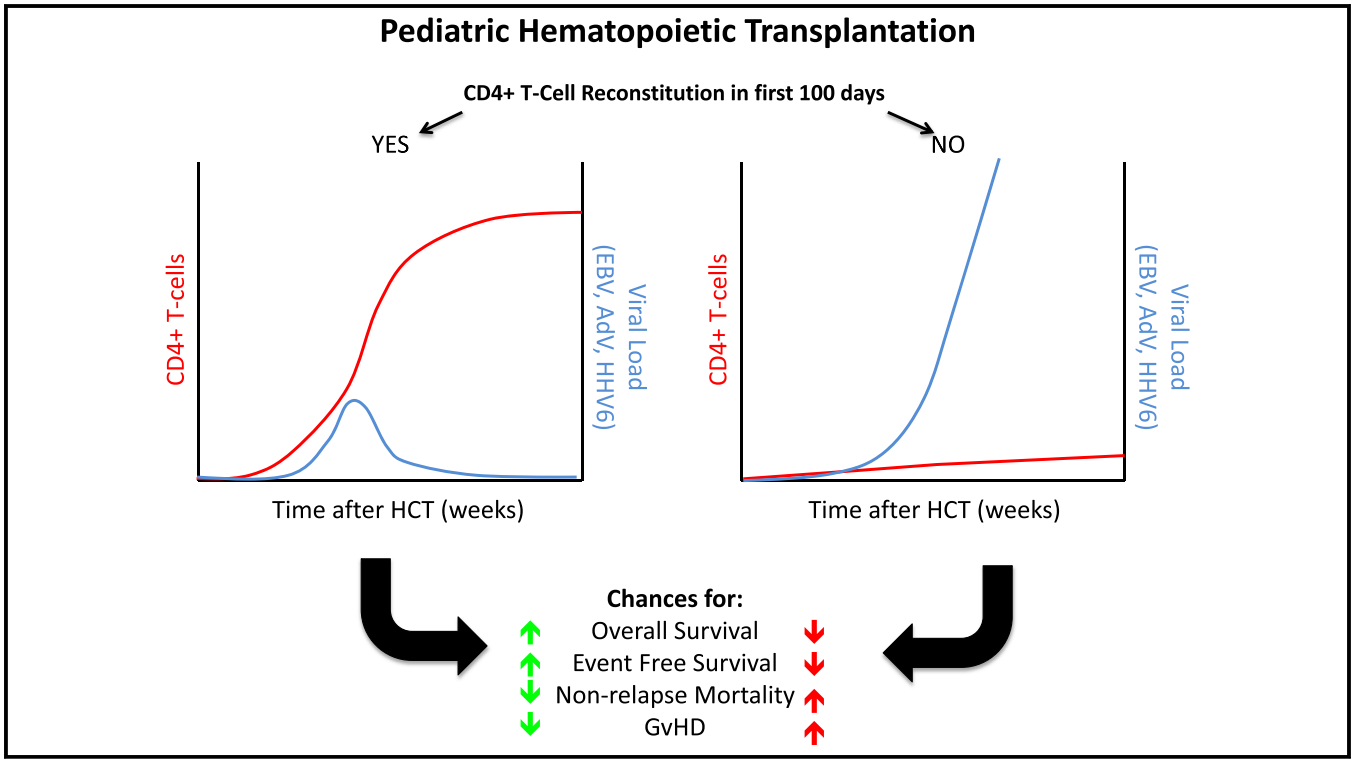
GRAPHICAL ABSTRACT



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Background: Viral reactivations (VRs) after hematopoietic cell transplantation (HCT) contribute to significant morbidity and mortality. Timely immune reconstitution (IR) is suggested to prevent VR.
Objectives: We studied the relation between IR (as a continuous predictor over time) and VR (as a time-varying predictor) and the relation between VR and other clinical outcomes.
Methods: In this retrospective analysis all patients receiving a first HCT between January 2004 and September 2014 were included. IR (CD3/CD4/CD8 T, natural killer, and B cells) was measured biweekly until 12 weeks and monthly thereafter. Main outcomes of interest were VR of adenovirus, EBV, human herpesvirus 6 (HHV6), cytomegalovirus (CMV), and BK virus screened weekly. Clinical outcomes included overall survival (OS), event-free-survival, nonrelapse mortality (NRM), and graft-versus-host disease. Cox proportional hazard and Fine and Gray competing risk models were used.
Results: Two hundred seventy-three patients (age, 0.1-22.7 years; median follow-up, 58 months) were included. Delayed CD4 reconstitution predicted reactivation of adenovirus (hazard ratio [HR], 0.995; $P = .022$), EBV (HR, 0.994; $P = .029$), and HHV6 (HR, 0.991; $P = .012$) but not CMV ($P = .31$) and BK virus ($P = .27$). Duration of adenovirus reactivation was shorter with timely CD4 reconstitution, which was defined as 50×10^6 cells/L or greater within 100 days. Adenovirus reactivation predicted lower OS (HR, 2.17; $P = .0039$) and higher NRM (HR, 2.96; $P = .0008$). Concomitant CD4 reconstitution abolished this negative effect of adenovirus reactivation (OS, $P = .67$; NRM, $P = .64$). EBV and HHV6 reactivations were predictors for the occurrence of graft-versus-host disease, whereas CMV and BK virus reactivation did not predict clinical outcomes.
Conclusion: These results stress the importance of timely CD4 reconstitution. Strategies to improve CD4 reconstitution can improve HCT outcomes, including survival, and reduce the need for toxic antiviral therapies. (J Allergy Clin Immunol 2017;140:1643-50.)

Key words: *Viral reactivations, hematopoietic cell transplantation, immune reconstitution, clinical outcomes*

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative therapy for various hematologic malignancies and benign disorders in children. The success of HCT is hampered by relapse of malignancy and transplantation-related mortality caused by HCT-associated complications, which include opportunistic infections (eg, viral reactivations [VRs]) and graft-versus-host disease (GvHD). Infectious complications and relapse are in part due to absent or delayed T-cell reconstitution.[1,2]

Early $CD4^+$ T-cell reconstitution in particular is suggested to be important for better survival after HCT.[2-7] Delayed immune reconstitution (IR) after HCT was previously found to be a predictor for VR,[7] which might contribute subsequently to acute graft-versus-host disease (aGvHD), graft failure (GF), and increased mortality.[8] Early and improved prediction of HCT-related complications provides the opportunity for earlier initiation of preemptive treatment, which subsequently could improve survival.

Although VR and IR are related and are both associated with clinical outcome,[7-10] their joint time-dependent relation as predictors for clinical outcome remains to be studied. Most studies investigating the role of IR markers as a predictor for VR only include very limited measurements at relatively late time points after HCT. Because most VRs occur early after HCT, this is not optimal. Early IR markers assessed as a continuous value over time might provide better predictors, which can also be used in clinical practice for decision making. The identification of early IR-related predictors might guide us in the initiation of antiviral therapies or targeted antiviral cellular therapies.

We aimed to assess the relation between IR and VR and other clinical outcomes. To achieve this, we performed a large retrospective cohort analysis in which clinical outcomes, such as survival, GvHD, and GF, were related to VR. In this analysis VR was uniquely evaluated as a time-varying predictor. Additionally, this is the first study that related various IR markers as variables that were continuous over time to VRs. This enabled us to determine the effect of IR on VR-associated clinical outcomes.

Abbreviations used
aGvHD: Acute graft-versus-host disease
ATG: Antithymocyte globulin
cGvHD: Chronic graft-versus-host disease
CMV: Cytomegalovirus
EFS: Event-free survival
GF: Graft failure
GvHD: Graft-versus-host disease
HCT: Hematopoietic cell transplantation
HHV6: Human herpesvirus 6
HR: Hazard ratio
IR: Immune reconstitution
NK: Natural killer
NRM: Nonrelapse mortality
OS: Overall survival
RRM: Relapse-related mortality
VR: Viral reactivation

METHODS

Study design and patients

In this analysis we included pediatric patients receiving an allogeneic HCT between January 2004 and September 2014 at the University Medical Centre in Utrecht, The Netherlands. All consecutive patients undergoing their first transplantation were included. The minimum follow-up for surviving patients was 6 months, and data were collected and registered prospectively. Patients were enrolled and data were collected only after obtaining written informed consent in accordance with the Helsinki Declaration. The study was approved by the local ethics committee (trial nos. 05-143 and 11-063k).

Procedures

Conditioning regimens were applied according to (inter-)national protocols. For myeloablative busulfan-containing protocols (administered intravenously), therapeutic drug monitoring was used to aim for an area under the curve of 75 to 95 mg·h/L. Antithymocyte globulin (ATG; thymoglobulin) was administered at a dose of 10 mg/kg starting 5 days before HCT from 2004 to 2010. From 2010 onward, patients weighing more than 40 kg received a lower dose of ATG (7.5 mg/kg). A 50% dose reduction was given when preconditioning lymphocyte counts were less than 300×10^6 T cells/L. Those receiving a cord blood transplant from 2009 onward received ATG starting 9 days before HCT. Starting in 2013, patients receiving a cord blood transplant for malignant indications did not receive ATG. Patients received gut decontamination and infection prophylaxis according to local protocols. All patients received acyclovir prophylaxis at a dose of 500 mg/m^2/d in 3 doses; on discharge, this was switched to valacyclovir. Patients with a viral load exceeding 1000 copies (cp)/mL were treated pre-emptively. GvHD prophylaxis consisted of cyclosporin A (targeted at trough levels of 150-250 μg/L

for all patients) combined with either 1 mg/kg prednisolone (cord blood) or 10 mg/m^2 methotrexate (on days +1, +3, and +6; unrelated donor). Cyclosporin A was continued for at least 3 or 6 months after HCT in patients with benign and malignant indications, respectively. Prednisolone was tapered after 28 days in patients with benign disorders and after engraftment in patients with malignancies. Cord blood recipients were treated with filgrastim from day +7 after HCT until neutrophil counts were greater than 2000 cells/μL. Patients were treated in high-efficiency, particle-free, air-filtered, positive-pressure isolation rooms.

Cytomegalovirus (CMV) and EBV serostatus was assessed in all patients and donors before HCT. After transplantation, all patients underwent weekly PCR viral screening in plasma for human herpesvirus 6 (HHV6), EBV, adenovirus, CMV, and BK virus, irrespective of serostatus and clinical conditions. Viral screening was continued until 3 months after HCT and longer after complications, most notably those requiring additional immune suppression, including GvHD and autoimmune cytopenia. VR in which no distinction was made between reactivation or primo infection was defined as having a viral load of greater than 1000 copies/mL. From 2012 onward, EBV and CMV were reported in international units per milliliter, for which the threshold was set at 5000 and 500 IU/mL, respectively, according to the conversion factor in the same viral laboratory.

Patients with EBV, CMV, or adenovirus reactivation received pre-emptive antiviral treatment. Patients with HHV6 reactivation were treated only in cases of high clinical suspicion of HHV6-associated disease (eg, encephalitis or bone marrow suppression). Rituximab was used for treating EBV reactivation, CMV was treated with either ganciclovir or foscarnet, HHV6 was treated with foscarnet, and adenovirus reactivation was treated with cidofovir. Patients with BK virus–associated hemorrhagic cystitis received hyperhydration (3 L/m^2/d) as supportive care. VR treatment was stopped when viral load was undetectable in 2 subsequent samples or in case of severe toxicity after acquiring low viral loads (<1000 copies/mL).

After reaching a leukocyte count of at least 0.3×10^9/L, absolute numbers of lymphocyte subsets, including overall T cells ($CD3^+$), T helper cells ($CD3^+CD4^+CD8^-$), cytotoxic T cells ($CD3^+CD8^+CD4^-$), B cells ($CD19^+$), and natural killer (NK) cells ($CD3^-CD16^+CD56^+$), were measured by means of flow cytometry at least every other week up to 12 weeks after HCT and monthly thereafter up to 6 months after HCT on EDTA-treated whole blood by using Trucount technology (BD Biosciences, Erembodegem, Belgium).

Outcomes

Main outcome of interest. VRs of adenovirus, EBV, HHV6, CMV, and BK virus were defined as viremia with greater than 1000 copies/mL.

Other outcomes of interest. Overall survival (OS) was defined as time from transplantation to death or last follow-up. Event-free survival (EFS) was defined as time from HCT to last contact whereby GF, relapse of disease, or death were regarded as events. All surviving patients were censored at the date of last contact. Nonrelapse mortality (NRM) was defined as death of a cause other than relapse of a malignancy. Relapse-related mortality (RRM) was defined as death caused by relapse of a malignancy. aGvHD and chronic graft-versus-host disease (cGvHD) were classified according to the criteria of Glucksberg et al[11] and Shulman et al,[12] respectively. GF was defined as nonengraftment (autologous reconstitution) or graft rejection (secondary loss of donor chimerism). In case of nonengraftment, the assessment date was +60 days after HCT. Interstitial pulmonary syndrome and bronchitis obliterans were defined according to internationally accepted criteria, as previously described.[13]

Statistical analysis

Duration of the follow-up was defined as the time from HCT to the last assessment for surviving patients or death. Actual cell counts for $CD3^+$, $CD4^+$, and $CD8^+$ T cells, as well as NK and B cells, considered as continuous values over time were evaluated as predictors for VR. For clinical end points, VRs of adenovirus, EBV, HHV6, CMV, and BK virus were evaluated as predictors. Additionally, reconstitution of $CD4^+$ T cells was assessed as a copredictor for clinical outcome. This was chosen in line with previous findings.[2,10,14,15] $CD4^+$ T-cell reconstitution ($CD4^+$ IR) was defined as reaching 50×10^6 $CD3^+CD4^+$ cells/L or greater in 2 consecutive measurements within 100 days after HCT and considered as a time-varying covariate in accordance with previous publications.[14] In multivariate analysis predictors considered for outcome were patient-related variables (age at transplantation, sex, CMV, and EBV serostatus), disease (malignancies/primary immune deficiencies/bone marrow failure/inborn errors of metabolism), donor factors (stem cell source, HLA disparity, CMV, and EBV serostatus), and conditioning regimen (myeloablative or reduced-intensity conditioning). Because immune suppression was similar for all patients during the first 3 months after HCT, this was not considered a multivariate predictor.

Variables associated with a *P* value of less than .05 by using univariate analysis were selected for testing in a multivariate analysis. Probabilities of events were calculated by using the Kaplan-Meier estimate; the 2-sided log-rank test was used for univariate comparisons. Time-dependent outcomes were analyzed by using Cox proportional hazard models. For the end points of NRM, RRM, aGvHD, cGvHD, and GF, Fine and Gray competing risk regressions were used. Here competing events were RRM, NRM, death not caused by aGvHD or cGvHD, and death not caused by GF, respectively. Statistical analyses were performed with R 3.0.1 software using the packages *survival* and *cmprsk*.

TABLE I. Patients' characteristics

	All patients
No. of patients	273
Male sex (%)	59
Age at transplantation (y)	8.4 (0.1-22.7)
Recipient/donor EBV serostatus	
−/−	19
+/−	60
−/+	3
+/+	18
Recipient/donor CMV serostatus	
−/−	37
+/−	48
−/+	3
+/+	12
Diagnosis (%)	
Malignancy	53
Primary immune deficiency	12
Bone marrow failure	16
Benign non-PID	18
Stem cell source (%)	
Bone marrow	45
Cord blood	52
Peripheral blood stem cells	3
Conditioning regimen (%)	
Busulfan based	83
TBI based	17
Patients receiving serotherapy (%)	71
Follow-up (mo)	58 (0.2-130)

PID, Primary immunodeficiency; *TBI*, total body irradiation.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

RESULTS

A total of 273 patients were included with a median age of 8.4 years (range, 0.1-22.7 years). Patients' characteristics are summarized in Table I. Busulfan combined with fludarabine and ATG (thymoglobulin) was the most frequently used conditioning

TABLE II. Multivariate analysis of $CD4^+$ IR versus VR

End point	HR	95% CI	*P* value	Significance level
CMV reactivation				
$CD4^+$ T-cell counts (continuous over time)	0.998	0.993-1.002	.312	
HHV6 reactivation				
$CD4^+$ T-cell counts (continuous over time)	0.991	0.985-0.998	.012	*
BK virus reactivation				
$CD4^+$ T-cell counts (continuous over time)	0.996	0.99-1.003	.271	
Adenovirus reactivation				
$CD4^+$ T-cell counts (continuous over time)	0.995	0.99-0.999	.022	*
EBV reactivation				
$CD4^+$ T-cell counts (continuous over time)	0.994	0.988-0.999	.029	*

IR was considered a continuous time-varying predictor. VR was defined as having a viral load of greater than 1000 copies/mL in plasma.
HR, Hazard ratio for each point increase in $CD4^+$ T-cell counts.
*$P < .05$.

regimen. Cord blood (52%, n = 142) and bone marrow (45%, n = 123) were the most frequently used cell sources. Median time to follow-up was 58 months (range, 0.2-130 months). Cumulative incidence of VRs was 27%, 18%, 15%, 13%, and 11% for HHV6, CMV, adenovirus, BK virus, and EBV, respectively (see Fig E1 in this article's Online Repository at www.jacionline.org).

We identified only $CD4^+$ IR as a predictor for VR. No associations between $CD3^+CD8^+$ T-cell, NK cell, or B-cell IR and VR were found (see Table E1 in this article's Online Repository at www.jacionline.org). In patients with adenovirus, the chance of reactivation is reduced 5% with every increase of 10/μL $CD4^+$ T cells (hazard ratio [HR], 0.995; 95% CI, 0.99-0.999; $P = .022$; Table II). $CD4^+$ IR similarly predicted EBV (HR, 0.994; 95% CI, 0.988-0.999; $P = .029$) and HHV6 (HR, 0.991; 95% CI, 0.985-0.998; $P = .012$). No relation with CMV reactivation ($P = .31$) and BK virus reactivation ($P = .27$) was identified.

Interestingly, patients with successful early $CD4^+$ IR had shorter duration of adenovirus viremia (Fig 1). The median duration of viremia was only 14 days for patients with $CD4^+$ IR compared with 47.5 days for patients without recovery of $CD4^+$ IR ($P = .011$). Early $CD4^+$ IR did not influence the duration of CMV, EBV, and HHV6 viremia (see Fig E2 in this article's Online Repository at www.jacionline.org).

We next investigated the effects of VRs on clinical outcome parameters. In this analysis patients were considered as having a VR only from the day of reaching greater than 1000 viral copies/mL in plasma, producing a robust estimation of the hazard associated with the different VRs (see Fig E3 in this article's Online Repository at www.jacionline.org). Here we identified adenovirus, EBV, and HHV6 as VR predictors for various clinical outcomes (Table III). Reactivation of adenovirus was a predictor for lower chances of OS (HR, 2.17; 95% CI, 1.28-3.68; $P = .0039$; Fig 2, *A*, and Table III) and EFS (HR, 1.77; 95% CI, 1.05-2.99; $P = .032$; Fig 2, *C*, and Table III) compared with those in patients not experiencing an adenovirus reactivation (HR, 2.17; 95% CI, 1.28-3.68; $P = .0039$; Fig 2, *A*, and Table III). We did not find any VRs associated with RRM (see Table E2 in this article's Online Repository at www.jacionline.org). NRM was associated with both adenovirus (HR, 2.95; 95% CI, 1.57-5.57; $P = .0008$; Fig 2, *E*, and Table III) and EBV (HR, 2.03; 95% CI, 1.01-4.11; $P = .049$) reactivation (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org).

Because $CD4^+$ IR predicted both the incidence and duration of VRs, we subsequently investigated whether $CD4^+$ IR influenced the negative effect of VRs on clinical outcome. Here we found that OS was similar in patients with adenovirus reactivation and concurrent $CD4^+$ IR compared with those patients without an adenovirus reactivation (68% ± 3% vs 66% ± 12%, $P = .67$; Fig 2, *B*). Patients with adenovirus reactivation without $CD4^+$ IR performed considerably worse (OS, 32 ± 10; $P = .0045$ in multivariate analysis for $CD4^+$ IR; Table III). For EFS and NRM, we found comparable results: patients with adenovirus reactivation without $CD4^+$ IR had lower EFS (63% and 62% vs 32% for adenovirus$^-$, adenovirus$^+$/IR$^+$, and adenovirus$^+$/IR$^-$, respectively; $P = .0021$ in multivariate analysis for $CD4^+$ IR; Table III) and higher NRM (18% and 28% vs 54% for adenovirus$^-$, adenovirus$^+$/IR$^+$, and adenovirus$^+$/IR$^-$, respectively; $P = .0005$ in multivariate analysis for $CD4^+$ IR; Table III and see Fig E4, *B*) compared with those with $CD4^+$ IR in the presence or absence of adenovirus.

Although adenovirus was associated with all survival-related parameters, HHV6 and EBV were predictors for the occurrence of GvHD. HHV6 was found to be a predictor for a higher chance on grade 2 to 4 aGvHD (HR, 3.47; 95% CI, 2.11-5.7; $P < .0001$; Table III and see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). Diagnosis was an additional multivariate predictor, with bone marrow failure and primary immune deficiencies having the lowest chance on grade 2 to 4 aGvHD (see Table E3 in this article's Online Repository at www.jacionline.org). For grade 3 to 4 aGvHD, both HHV6 (HR, 2.74; 95% CI, 1.22-6.15; $P = .015$; Table III and see Fig E5, *B*) and EBV (HR, 4.8; 95% CI, 1.31-17.62; $P = .018$; Table III and see Fig E5, *C*) were predictors, with age being an additional predictor (see Table E3). Extensive cGvHD probability was predicted by EBV reactivation (HR, 3.61; 95% CI, 1.13-11.53; $P = .03$; Table III and see Fig E5, *D*). Furthermore, in patients with HHV6 reactivation, $CD4^+$ reconstitution was associated with a protective effect for grade 2 to 4 GvHD ($P = .0074$, see Fig E6 in this article's Online Repository at www.jacionline.org). The same was found for the association between EBV and aGvHD and extensive cGvHD ($P = .04$ and $P < .0001$, respectively; see Fig E6). Receiving cord blood as a cell source was an additional predictor for a lower chance on cGvHD (see Table E3). For the outcomes of GF, lung injury, interstitial pulmonary syndrome, and bronchitis obliterans, we did not identify any VR predictors.

DISCUSSION

To our knowledge, this is the first study to investigate the relation between various IR markers, occurrence and duration of VR, and clinical outcome in a large pediatric cohort. Here, for the

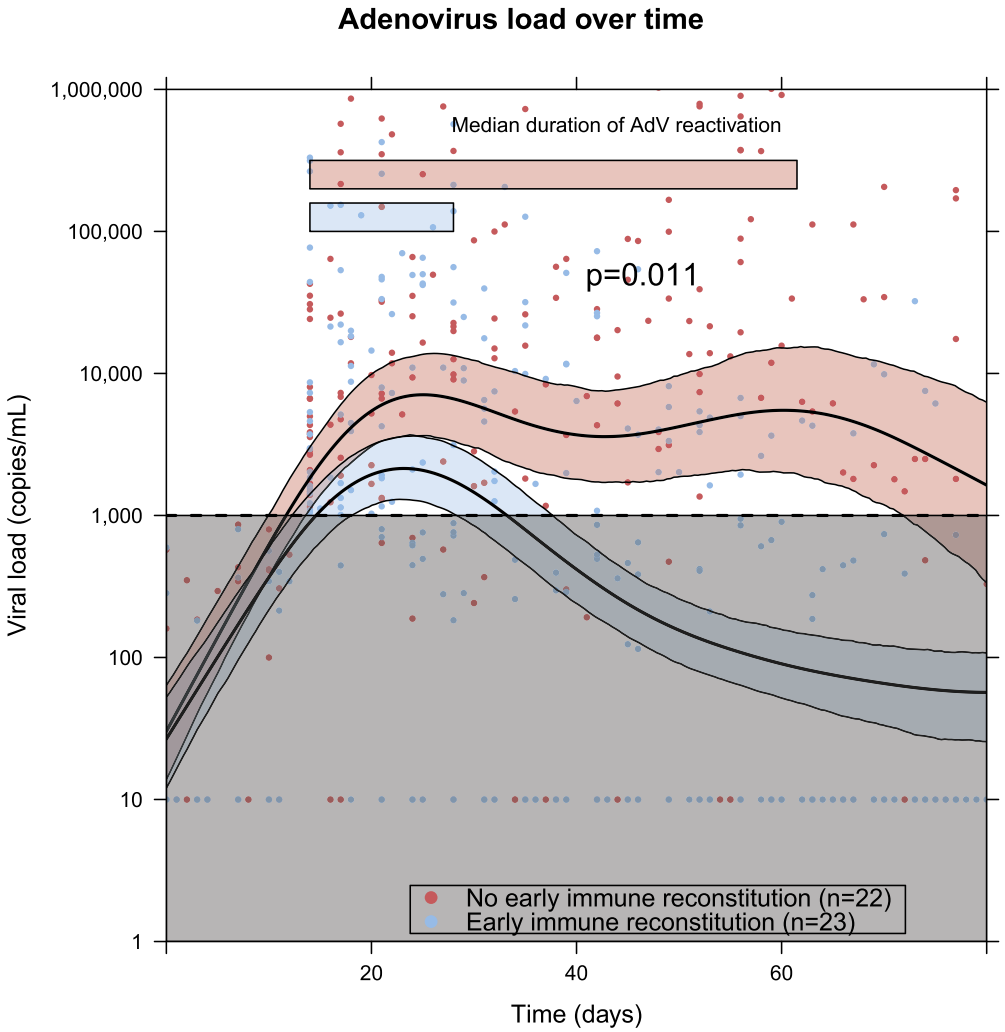

FIG 1. Viral load in patients with adenovirus *(AdV)* reactivation, according to early reconstitution of $CD4^+$ T cells. Early reconstitution was defined as reaching 50×10^6 $CD4^+$ T cells/L or greater in 2 consecutive measurements within 100 days after HCT. Spline regression analysis (5 *df*) of adenovirus load (*bold solid lines* with 95% CIs as *shaded area*) over time (normalized to 14 days before reaching a viral load of 1000) in patients with adenovirus reactivation, with *(blue area/dot/bar)* or without *(red area/dot/bar)* early $CD4^+$ T-cell reconstitution, showing longer adenovirus reactivation and higher adenovirus loads in those without early reconstitution is indicated. Median duration of adenovirus treatment was 14 days for patients with early IR and 47.5 days for the group without early IR. *Dots*, Individual raw adenovirus load. *Bars*, Median duration of adenovirus treatment. *Gray background*, Clinically insignificant viral load.

first time, we considered all predictors in a time-dependent manner, making the findings more robust. With the limitations of a retrospective cohort study taken into account, our data show that from all evaluated immunologic markers, absence of $CD4^+$ T-cell reconstitution best predicted reactivation of adenovirus, EBV, and HHV6. Furthermore, adenovirus predicted lower OS and EFS, adenovirus and EBV predicted higher NRM, and EBV and HHV6 reactivation predicted GvHD. Importantly, $CD4^+$ IR completely abolished the adverse effect of VR on survival parameters. Moreover, $CD4^+$ IR did not influence reactivation of CMV and BK virus, nor were these VRs predictive for clinical outcomes.

Our finding that the absence of $CD4^+$ IR predicts VR is in line with previous findings.[2-7,14] A previously suggested relation between $CD8^+$ T-cell recovery and VR could not be identified,[16] nor could a correlation between T-cell recovery and CMV reactivation be confirmed.[7,15] These discrepancies might be due to the fact that we considered IR to be a continuous and time-dependent variable measured as early as 2 weeks after HCT rather than a binary and time-independent variable at certain time points, as done in most previous analyses. By taking time dependency into account, a more precise insight into the predictive value of the various immune markers will be obtained. Nonetheless, in the implemented models there was no accounting for a virus-induced stimulatory effect on $CD4^+$ T-cell proliferation. However, most patients did not show a steep increase in $CD4^+$ counts after VR.

Considering VRs as predictors of clinical outcome, our findings are partly in line with those of other studies showing that adenovirus reactivation is associated with lower survival,[17] EBV reactivation is associated with lower survival and GvHD,[18] and HHV6 reactivation is associated with a higher risk for aGvHD in myeloablative HCT recipients.[19,20] Some conflicting data exist as well because others did find a relation between HHV6 and higher mortality rates[20,21] or between HHV6

TABLE III. Multivariate analysis of VR and $CD4^+$ IR versus outcome

Variable	Univariate, *P* value	Multivariate, HR	95% CI	*P* value	Significance level
OS					
Adenovirus reactivation, >1000 copies/mL	.00038	2.173	1.282-3.684	.0039	†
Early $CD4^+$ T-cell reconstitution	.002	0.52	0.332-0.816	.0045	†
EFS					
Adenovirus reactivation, >1000 copies/mL	.0101	1.771	1.05-2.988	.032	*
Early $CD4^+$ T-cell reconstitution	.00079	0.515	0.337-0.786	.0021	†
NRM					
Adenovirus reactivation, >1000 copies/mL	<.0001	2.955	1.567-5.571	.0008	‡
EBV reactivation, >1000 copies/mL	.0013	2.028	1.001-4.109	.049	*
Early $CD4^+$ T-cell reconstitution	.00017	0.344	0.189-0.629	.0005	‡
Grade 2-4 aGvHD					
HHV6 reactivation, >1000 copies/mL	<.0001	3.472	2.113-5.704	<.0001	§
Grade 3-4 aGvHD					
EBV reactivation, >1000 copies/mL	.0085	4.799	1.307-17.616	.018	*
HHV6 reactivation, >1000 copies/mL	.014	2.74	1.22-6.155	.015	*
Extensive cGvHD					
EBV reactivation, >1000 copies/mL	.0298	3.611	1.131-11.534	.03	*

VRs and IRs were considered time-varying predictors. A VR was defined as having a viral load of greater than 1000 copies/mL in plasma.
*$P < .05$.
†$P < .01$.
‡$P < .001$.
§$P < .0001$.

or EBV reactivation and aGvHD.[22,23] These discrepancies can most likely be explained by differences in treatment protocols, age, or VR definition. Furthermore, we found a protective effect of $CD4^+$ reconstitution for HHV6- and EBV-induced probability on aGvHD and cGvHD. This might be due to the regulatory properties of subpopulations of $CD4^+$ T cells, again considering that the time dependency of the variables might also have had a significant influence on the found associations.

With respect to CMV reactivation in relation to clinical outcome, we did not find any association, whereas CMV has previously been associated with the occurrence of aGvHD, relapse, and lower survival.[8,15,24] This might be due to the preemptive treatment in our cohort controlling the negative effects associated with CMV. However, most available data on the effect of CMV reactivation are acquired through retrospective multicenter registry studies in which CMV monitoring can differ between the centers, such as when CMV load is only measured when there is a suspicion of CMV-related disease; there might be an underreporting of actual CMV reactivations. In the literature early CMV reactivation is associated with a lower relapse incidence in patients with acute myeloid leukemia, which we could not find. In addition to the reasons discussed above, the relatively low number of patients in this study with acute myeloid leukemia (n = 53) might also have contributed. Significant effects of CMV on survival was shown in large registry studies in which the absolute effect size and thereby the clinical relevance was relatively small (difference of 1.8% in OS and approximately 5% in NRM in studies by the European Society for Blood and Marrow Transplantation[25] and Center for International Blood and Marrow Transplant Research,[26] respectively). Also, BK virus reactivation was found not to predict any of the clinical outcome parameters assessed in this study; however, we did not consider hemorrhagic cystitis to be an outcome measure. Regardless of the fact that BK virus–associated cystitis can be a very painful and long-lasting complication requiring prolonged hospitalization,[27] according to our findings, it does not affect survival or any other clinical outcome analyzed after HCT.

Data from this study imply that antiviral therapies to increase survival in cases of VR might not be needed in patients with $CD4^+$ IR, especially taking toxicities of antiviral drugs (eg, nephrotoxicity and bone marrow suppression) into account. Thus antiviral treatment can be delayed and possibly omitted in cases of $CD4^+$ T-cell recovery at the time of reactivation. Early monitoring of $CD4^+$ IR can be an important tool to identify patients at risk. However, the effect and safety of omitting antiviral therapy in patients with $CD4^+$ IR should be carefully evaluated first.

Although this association between the absence of $CD4^+$ IR and lower survival was described previously,[1,3,5,6] it has never been shown that $CD4^+$ IR affects VR-associated mortality. Therefore an important strategy to prevent mortality associated with VR would be to target for a predictable and certain $CD4^+$ IR after HCT. Because recent findings by our group suggest that high exposure of ATG after HCT significantly delays $CD4^+$ IR,[14] individualized ATG dosing regimens aiming at optimal exposure might quicken $CD4^+$ IR and subsequently enhance $CD4^+$ T-cell predictability. This appears to be more crucial after cord blood transplantation than after bone marrow transplantation.[28] Other strategies that might stimulate $CD4^+$ IR are currently being tested in clinical settings,[29] such as sex hormone inhibitors,[30] low-dose IL-2 treatment,[31] keratinocyte growth factor,[32] and thymosin $\alpha 1$ treatment.[33] However, the effects of these treatment options on $CD4^+$ T-cell recovery remain to be explored.

In conclusion, the results obtained by this study stress the importance of $CD4^+$ IR and provide insight into morbidities and mortality associated with developing a VR. Strategies to better predict $CD4^+$ IR are of the utmost importance to improve survival chances after HCT. In future studies, it would be of great interest to further study a variety of T-cell subsets and (antigen-specific) T-cell functions, which would help qualify the $CD4^+$ T-cell IR in relation to VR.[29] Preferably, these novel strategies should be tested in the context of harmonized clinical trial design and standardized immune monitoring to better compare different strategies, as recently reviewed.[34] Moreover, identifying patients with $CD4^+$ IR and thus at lower risk for VR-related morbidity

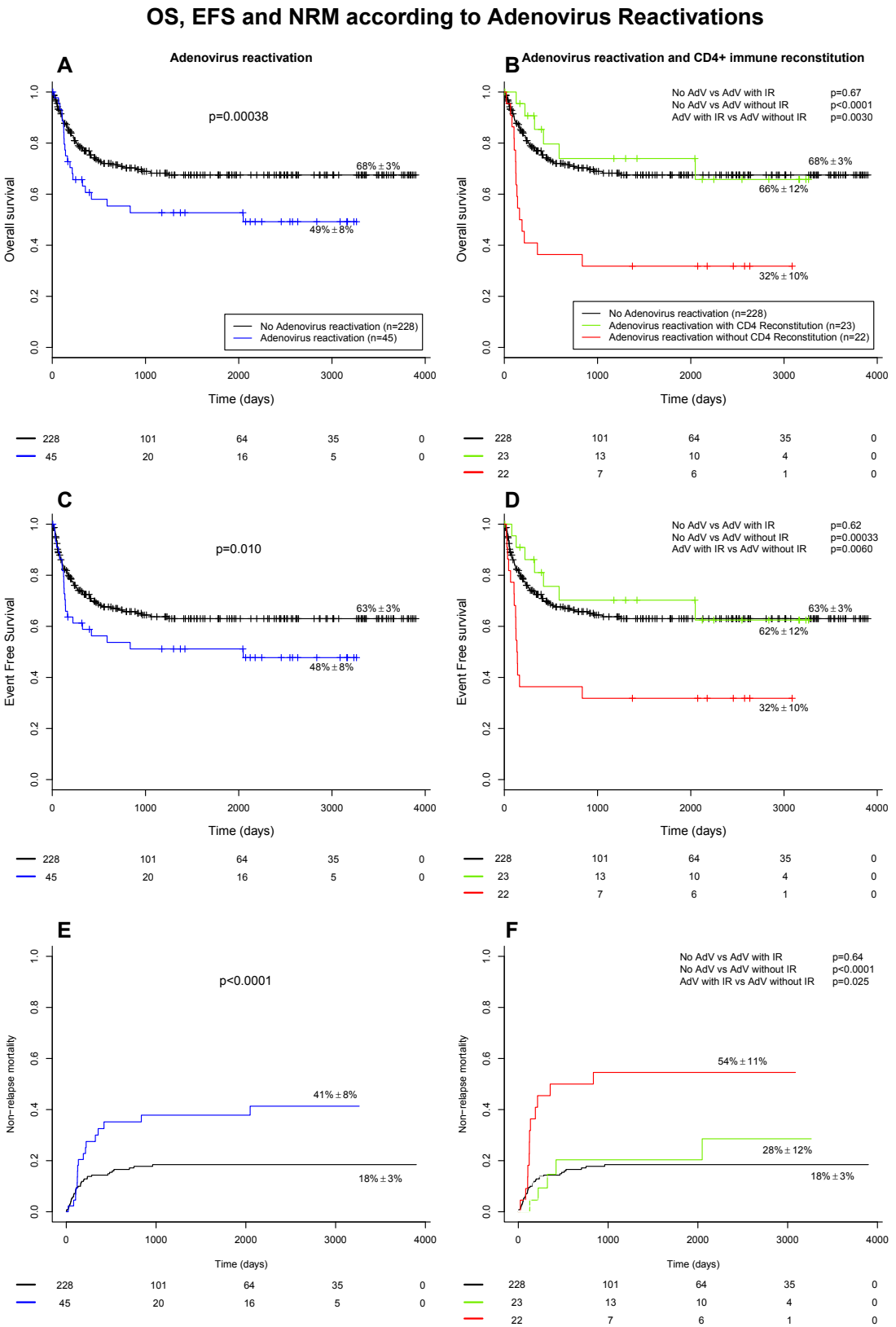

FIG 2. Clinical outcome according to VR and IR. **A, C,** and **E,** Plots on the left show the incidence of OS (Fig 2, *A*), EFS (Fig 2, *C*), and NRM (Fig 2, *E*) in patients without adenovirus *(AdV)* reactivation *(black lines)* versus those with adenovirus reactivation *(blue lines)*. VR was defined as having a viral load of greater than 1000 copies/mL. **B, D,** and **F,** Plots on the right show the effect of adenovirus and $CD4^+$ T-cell reconstitution on the incidence of OS (Fig 2, *B*), EFS (Fig 2, *D*), and NRM (Fig 2, *F*) in patients without adenovirus reactivation *(black lines)* versus those with adenovirus reactivation and subdivided into patients having $CD4^+$ T-cell reconstitution *(green lines)* and patients not having $CD4^+$ T-cell reconstitution *(red lines)*. IR was defined as having 50×10^6 $CD4^+$ T cells/L or greater in 2 consecutive measurements within 100 days after HCT.

and mortality might limit the need for toxic antiviral drugs. On the other hand, the identification of at-risk patients with a delayed $CD4^+$ IR provides the opportunity to pre-emptively intervene with antiviral (cell) therapies. Altogether, finding strategies that lead to a better predictable $CD4^+$ T-cell reconstitution and subsequent prevention of VR might lead to lower morbidity and better survival chances for patients undergoing HCT.

We thank the data managers from the University Medical Centre Utrecht (UMCU; C. E. Gerhardt and A. de Wildt) for collecting patients' characteristic and outcome data and the nursing staff of the UMCU for implementing the study protocol.

Clinical implications: Although VRs have major implications on clinical outcomes after HCT, early T-cell reconstitution decreases the chances of VRs and reduces the associated mortality.

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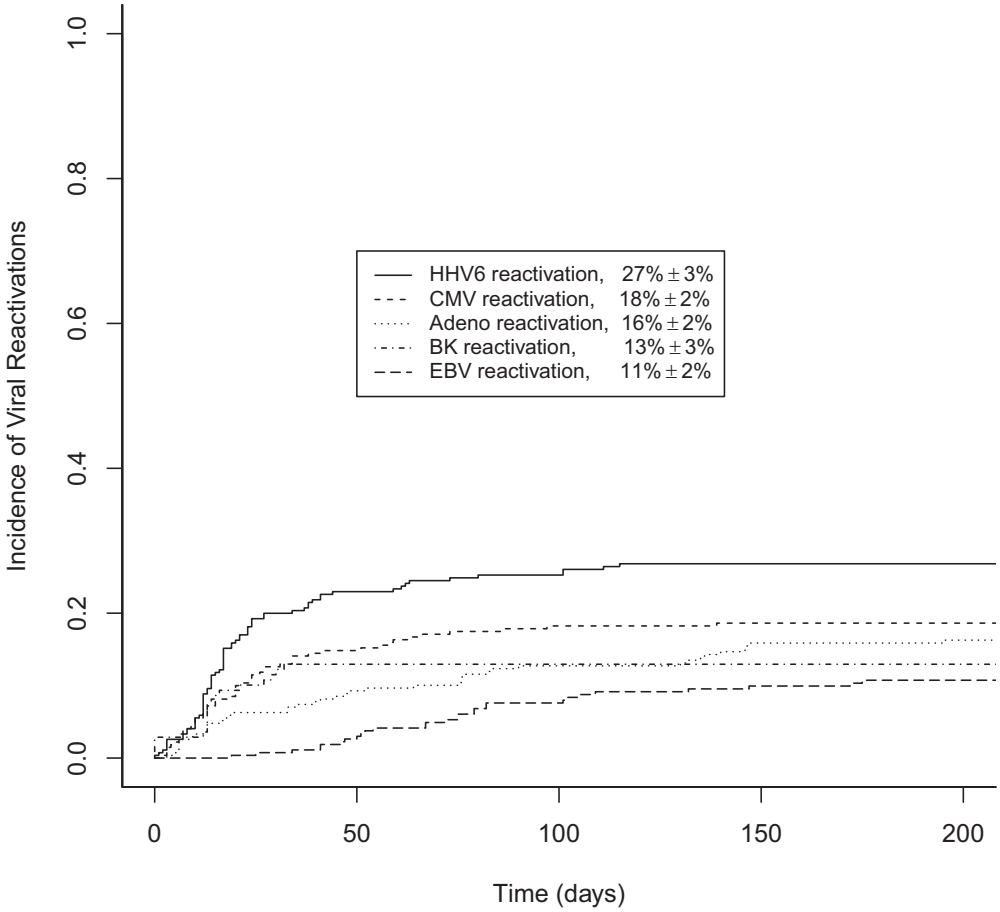

FIG E1. Incidence of VRs of CMV, HHV6, adenovirus *(Adeno)*, EBV, and BK virus. Death not caused by VR was considered a competing event.

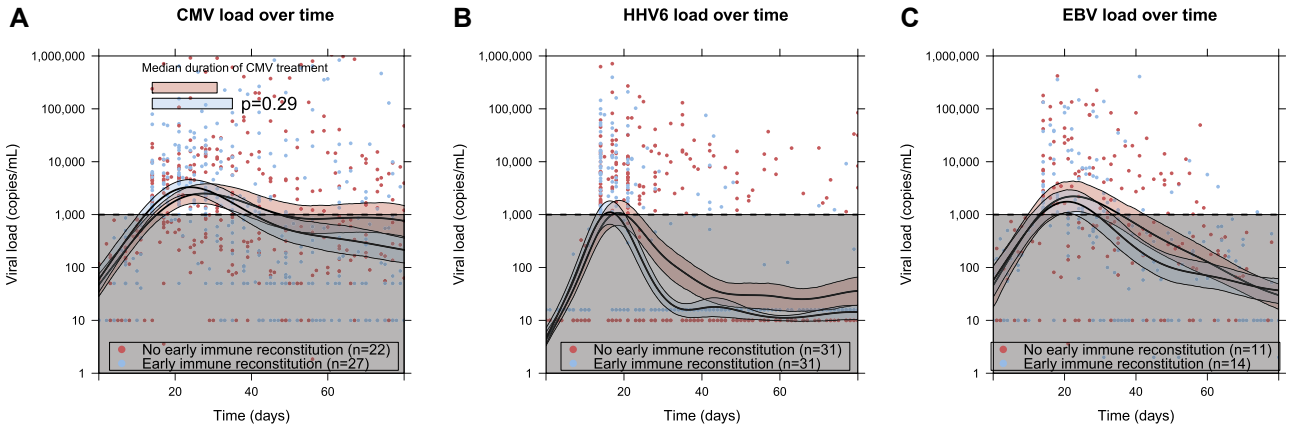

FIG E2. Viral load in patients with early and no early reconstitution of $CD4^+$ T cells: **A,** CMV; **B,** HHV6; and **C,** EBV. Early reconstitution was defined as reaching 50×10^6 $CD4^+$ T cells/L or greater in 2 consecutive measurements within 100 days after HCT. Spline regression analysis of viral load (*bold solid lines* with 95% CIs as *shaded area*) over time (normalized to start of reactivation) in patients with VR with *(blue area/dot/bar)* or without *(red area/dot/bar)* early $CD4^+$ T-cell reconstitution is indicated. *Dots,* Individual raw viral load. *Bars,* Median duration of viral load greater than 1000 copies/mL. *Gray background,* Clinically insignificant viral load.

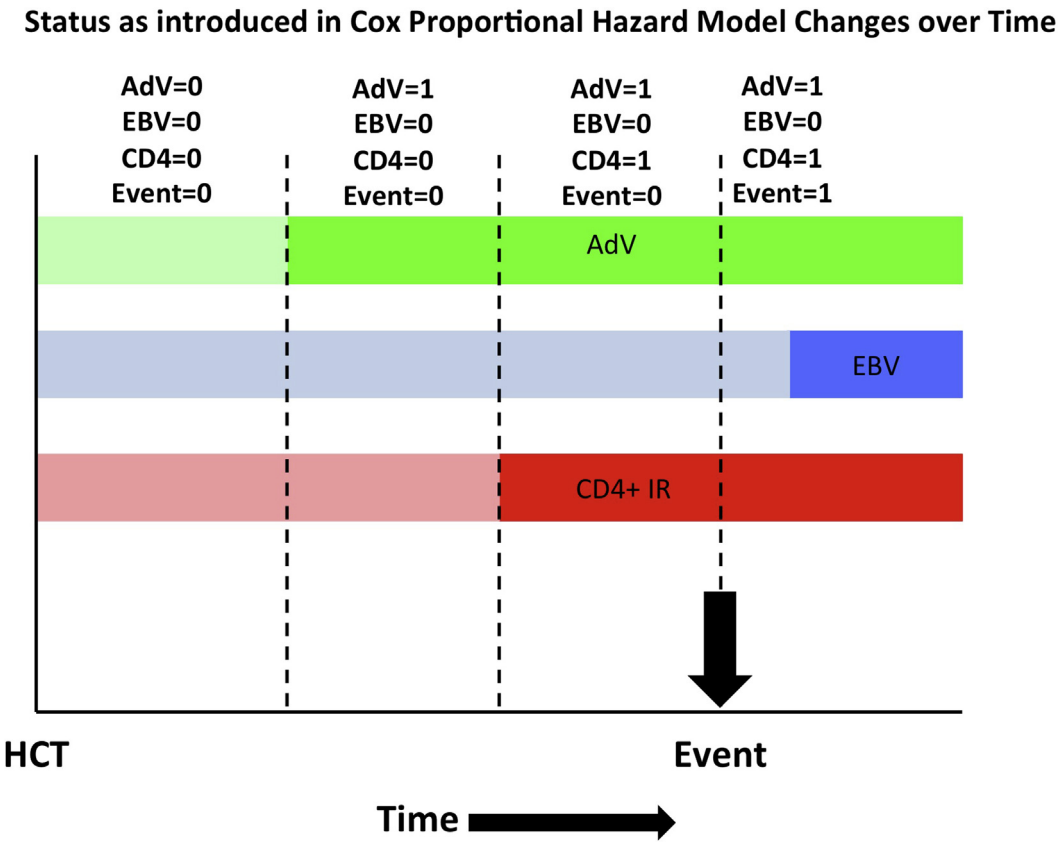

FIG E3. Status as introduced in Cox proportional hazard model changes over time. An overview of the statistical approach schematic representation of events over time in 1 patient is shown. For each clinical outcome measure, predictors were assessed over time. In the current example no reactivations of adenovirus *(AdV)* and EBV occurred at transplantation. At the *first dashed line*, the patient experienced an adenovirus reactivation, and therefore the status of adenovirus was changed in the model input. The patient is now considered to be at risk for the event because of adenovirus reactivation. The same applies for $CD4^+$ T-cell reconstitution and EBV. Note that the event takes place before EBV reactivation, and therefore EBV reactivation in this patient did not contribute to the risk for the event. This approach is superior to non–time-dependent predictors, in which any VR is considered to either to take place starting at the time of HCT or not at all. Additionally, this patient might have been considered EBV positive for the end point, whereas in fact, the reactivation took place after the event, and therefore the event probability was not influenced by EBV.

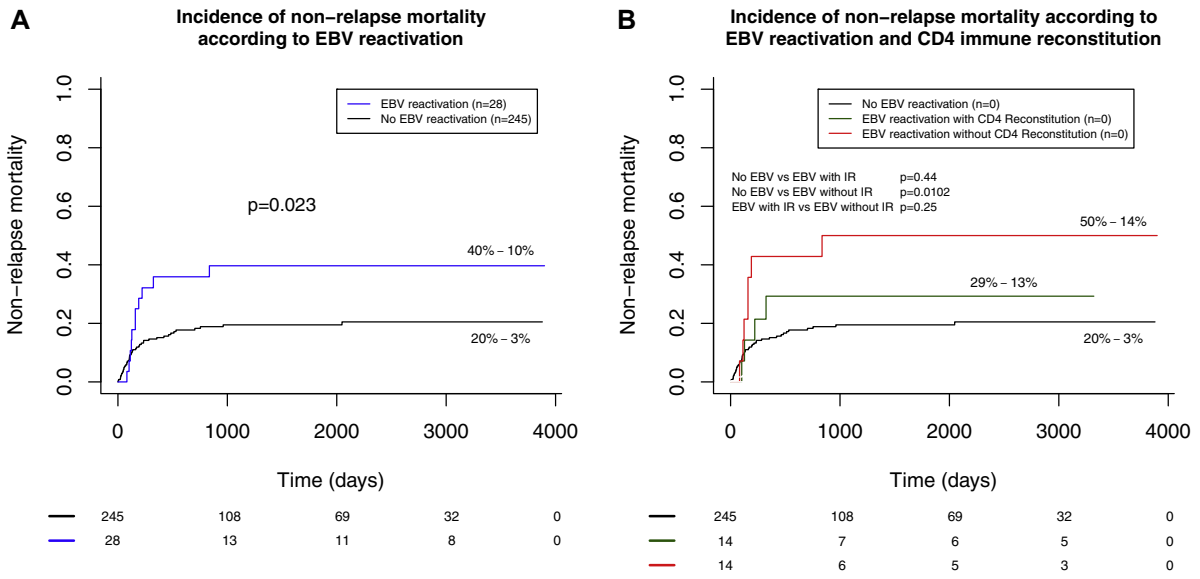

FIG E4. NRM according to EBV reactivation and IR. **A,** NRM in patients with no EBV reactivation *(black lines)* versus those with EBV reactivation *(blue lines)*. VR was defined as having a viral load of greater than 1000 copies/mL. **B,** NRM in patients with no EBV reactivation *(black lines)* versus patients with EBV reactivation subdivided into patients having $CD4^+$ T-cell reconstitution *(green lines)* and patients not having $CD4^+$ T-cell reconstitution *(red lines)*. IR was defined as having 50×10^6 $CD4^+$ T cells/L or greater in 2 consecutive measurements within 100 days after HCT.

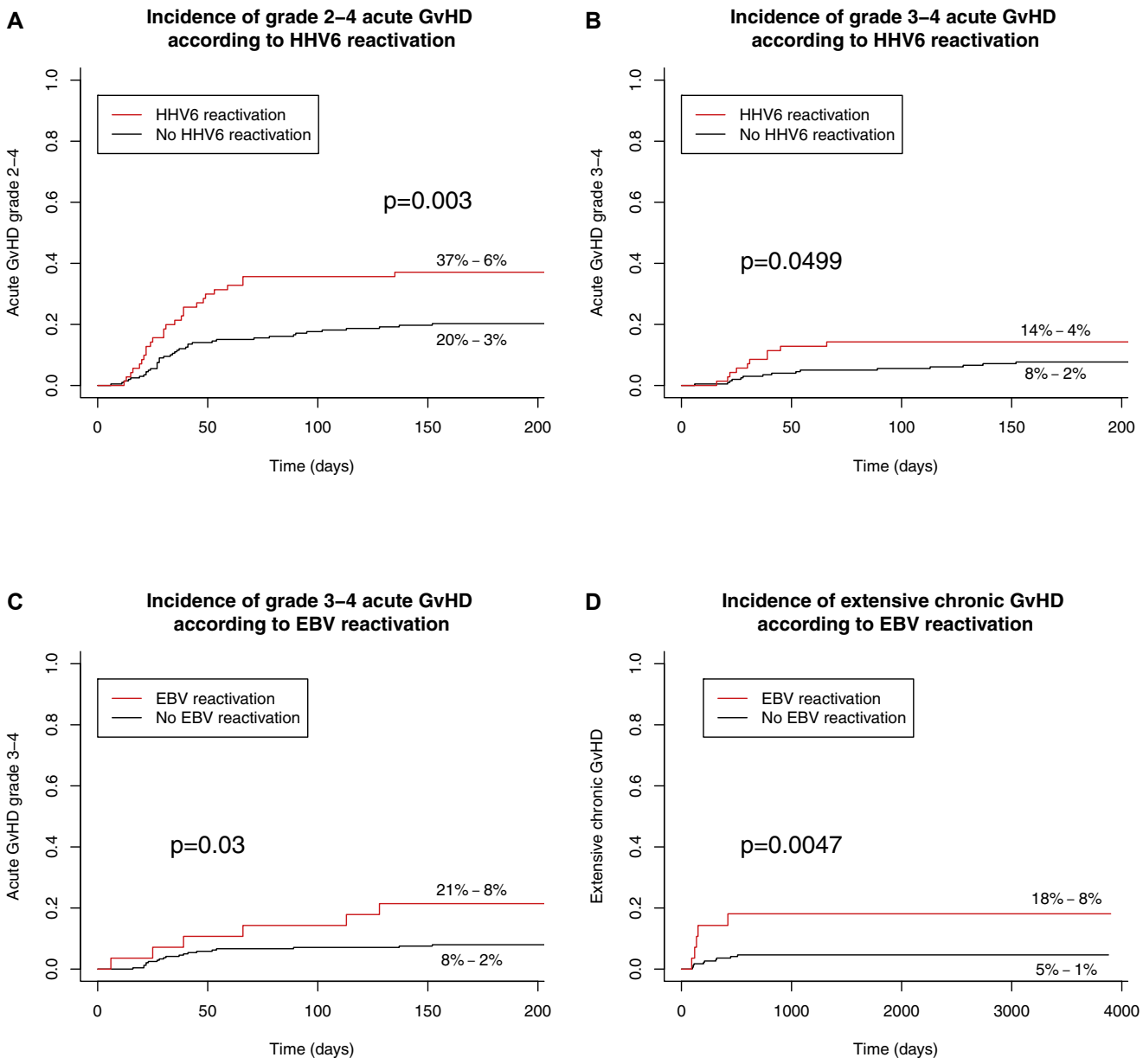

FIG E5. GvHD according to HHV6 and EBV reactivation. **A,** Incidence of grade 2 to 4 aGvHD in patients with no HHV6 reactivation *(black lines)* versus patients with HHV6 reactivation *(red lines)*. VR was defined as having a viral load of greeter than 1000 copies/mL. **B,** Incidence of grade 3 to 4 aGvHD in patients with no HHV6 reactivation *(black lines)* versus patients with HHV6 reactivation *(red lines)*. **C,** Incidence of grade 3 to 4 aGvHD in patients with no EBV reactivation *(black lines)* versus patients with EBV reactivation *(red lines)*. **D,** Incidence of extensive cGvHD in patients with no EBV reactivation *(black lines)* versus patients with EBV reactivation *(red lines)*.

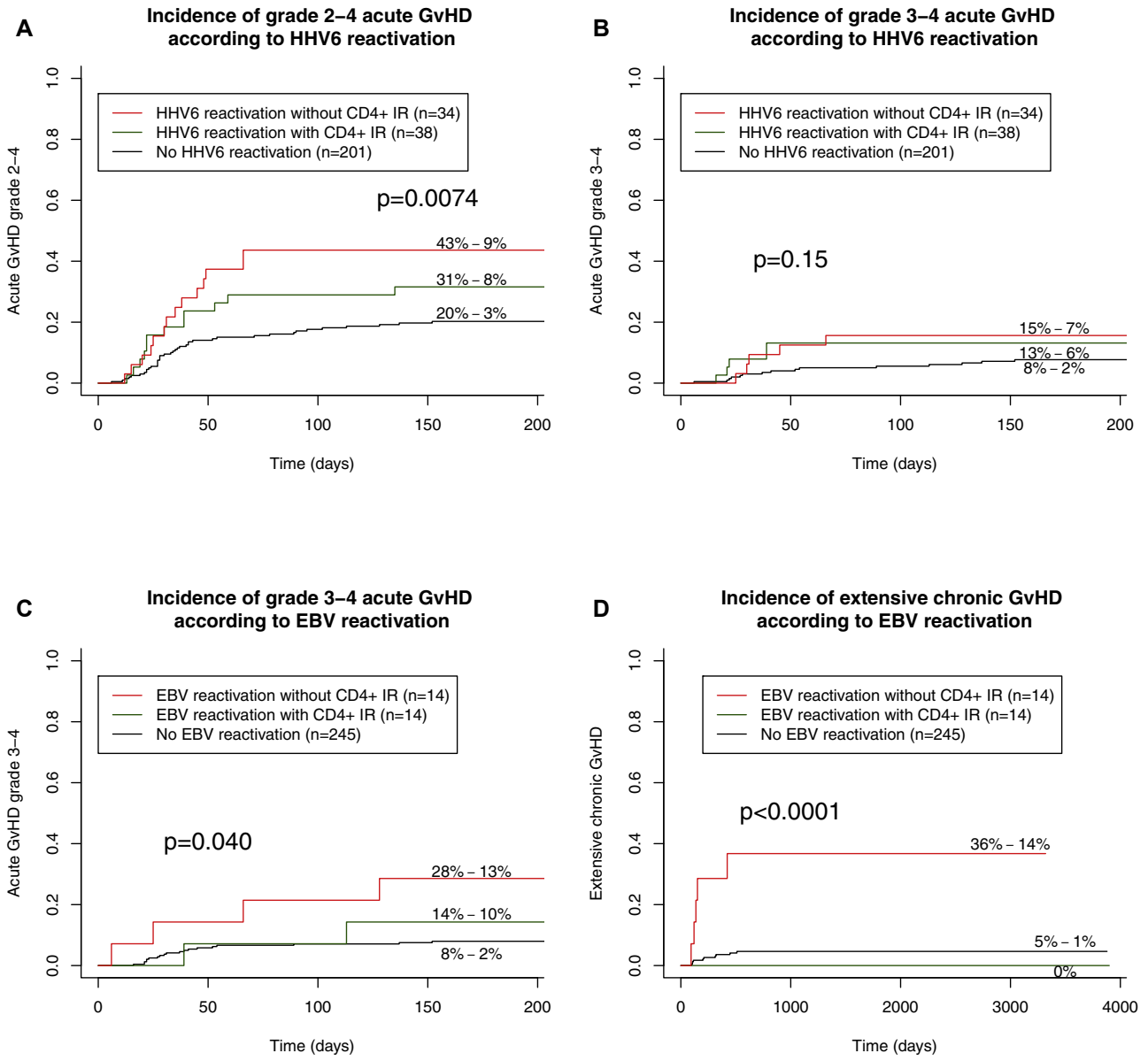

FIG E6. GvHD according to $CD4^+$ IR and immune reactivation of HHV6 and EBV. **A,** Incidence of grade 2 to 4 aGvHD in patients with no HHV6 reactivation *(black lines)* versus patients with HHV6 reactivation with *(green lines)* and without *(red lines)* $CD4^+$ IR. VR was defined as having a viral load of greater than 1000 copies/mL. **B,** Incidence of grade 3 to 4 aGvHD in patients with no HHV6 reactivation *(black lines)* versus patients with HHV6 reactivation with *(green lines)* and without *(red lines)* $CD4^+$ IR. **C,** Incidence of grade 3 to 4 aGvHD in patients with no EBV reactivation *(black lines)* versus patients with EBV-reactivation with *(green lines)* and without *(red lines)* $CD4^+$ IR. **D,** Incidence of extensive cGvHD in patients with no EBV reactivation *(black lines)* versus patients with EBV reactivation with *(green lines)* and without *(red lines)* $CD4^+$ IR.

TABLE E1. Univariate analysis of IR versus VR

	$CD3^+$ T cells	$CD4^+$ T cells	$CD8^+$ T cells	B cells	NK cells
CMV	.56	.31	.74	.13	.18
HHV6	.021*	.012*	.11	.26	.49
BK virus	.82	.27	.93	.87	.23
Adenovirus	.26	.022*	.66	.54	.24
EBV	.45	.029*	.8	.16	.35

IR was considered a continuous time-varying variable. VR was defined as having a viral load of greater than 1000 copies/mL in plasma.
*$P < .05$; significant $CD4^+$ T-cell predictors were selected for multivariate analysis in Table II.

TABLE E2. Univariate analysis of VR versus outcome

	BK virus	HHV6	CMV	Adenovirus	EBV
OS	.34	.015*	.38	.00038*	.017*
EFS	.34	.061*	.43	.0101*	.15
NRM	.2	.018*	.16	<.0001*	.0013*
RRM	.75	.26	.33	.63	.61
aGvHD, grade 2-4	.72	<.0001*	.81	.14	.032*
aGvHD, grade 3-4	NA	.014*	.67	.44	.0085*
Extensive cGvHD	NA	.25	.7	.43	.0298*
GF	.94	.89	.88	.81	NA
Lung injury	.59	.38	.4	.8	.2
IPS	.19	.027*	.95	.095	.23
BO	NA	.3	.33	NA	.4

VR was defined as having a viral load of greater than 1000 copies/mL in plasma and was considered a time-varying-predictor.
BO, Bronchitis obliterans; *IPS*, interstitial pulmonary syndrome; *NA*, because of no events in patients with VR, no exact *P* value could be calculated, although the *P* value lies close to 1.
*$P < .05$; these values were selected for multivariate analysis in Tables III and E3.

TABLE E3. Multivariate analysis of VR and IR versus outcome

End point	HR	95% CI	*P* value	Significance level
OS				
Adenovirus reactivation, >1000 copies/mL	2.17	1.28-3.68	.0039	‡
EBV reactivation, >1000 copies/mL	1.47	0.79-2.74	.2	
HHV6 reactivation, >1000 copies/mL	1.35	0.85-2.15	.2	
Early $CD4^+$ reconstitution	0.52	0.33-0.82	.0045	‡
Mismatched donor (matched/mismatched)	1.27	0.82-1.98	.3	
EFS				
Adenovirus reactivation, >1000 copies/mL	1.77	1.05-2.99	.032	†
HHV6 reactivation, >1000 copies/mL	1.35	0.87-2.09	.2	
Early $CD4^+$ reconstitution	0.51	0.34-0.79	.0021	‡
Mismatched donor (matched/mismatched)	1.41	0.93-2.13	.1	
Therapy-related mortality				
Adenovirus reactivation, >1000 copies/mL	2.95	1.57-5.57	.0008	§
EBV reactivation, >1000 copies/mL	2.03	1-4.11	.049	†
HHV6 reactivation, >1000 copies/mL	1.39	0.77-2.53	.3	
Early $CD4^+$ reconstitution	0.34	0.19-0.63	.0005	§
Diagnosis group: malignant (reference)	1			
Diagnosis group: bone marrow failure	0.58	0.2-1.69	.3	
Diagnosis group: inborn errors	1.26	0.56-2.83	.6	
Diagnosis group: immune deficiencies	1.59	0.82-3.09	.2	
Mismatched donor (matched/mismatched)	1.31	0.75-2.28	.3	
Grade 2-4 aGvHD				
HHV6 reactivation, >1000 copies/mL	3.47	2.11-5.7	<.0001	‖
EBV reactivation, >1000 copies/mL	2.59	0.9-7.45	.076	*
Diagnosis group: malignant (reference)	1			
Diagnosis group: bone marrow failure	0.27	0.1-0.74	.011	†
Diagnosis group: inborn errors	0.49	0.22-1.09	.079	*
Diagnosis group: immune deficiencies	0.42	0.2-0.89	.024	†
Grade 3-4 aGvHD				
EBV reactivation, >1000 copies/mL	4.8	1.31-17.62	.018	†
HHV6 reactivation, >1000 copies/mL	2.74	1.22-6.15	.015	†
Age (continuous)	1.11	1.04-1.18	.0028	‡
Extensive cGvHD				
EBV reactivation, >1000 copies/mL	3.61	1.13-11.53	.03	†
Donor source: identical sibling (reference)	1			
Donor source: matched unrelated donor (BM/PBSC)	0.74	0.3-1.82	.5	
Donor source: cord blood	0.23	0.07-0.79	.02	†
HCT before/after 2009	0.51	0.2-1.27	.1	
Lung injury				
HHV6 reactivation, >1000 copies/mL	1.69	0.91-3.13	.095	*
Diagnosis group: malignant (reference)	1			
Diagnosis group: bone marrow failure	0.47	0.14-1.55	.2	
Diagnosis group: inborn errors	2.49	1.33-4.67	.0045	‡
Diagnosis group: immune deficiencies	1.53	0.73-3.22	.3	
HCT before/after 2009	0.57	0.32-1.02	.056	*
IPS				
HHV6 reactivation, >1000 copies/mL	2.03	0.9-4.57	.089	*
Diagnosis group: malignant (reference)	1			
Diagnosis group: bone marrow failure	1.6	0.31-8.31	.6	
Diagnosis group: inborn errors	8.42	2.78-25.46	.0002	§
Diagnosis group: immune deficiencies	2.21	0.57-8.58	.3	
HCT before/after 2009	0.48	0.22-1.07	.072	*
Mismatched donor (matched/mismatched)	2.24	1-4.99	.05	†
Age (continuous)	0.95	0.88-1.02	.2	

VR and IR were considered time-varying variables. VR was defined as having a viral load of greater than 1000 copies/mL in plasma.
BM, Bone marrow; *IPS*, interstitial pulmonary syndrome; *PBSC*, peripheral blood stem cell.
*$P < .1$.
†$P < .05$.
‡$P < .01$.
§$P < .001$.
‖$P < .0001$.