Anti-T-lymphocyte globulin exposure is associated with acute graft-*versus*-host disease and relapse in pediatric acute lymphoblastic leukemia patients undergoing hematopoietic stem cell transplantation: a multinational prospective study

Lisa V.E. Oostenbrink,[1] Erik G.J. von Asmuth,[1] Cornelia M. Jol-van der Zijde,[1] Anja M. Jansen-Hoogendijk,[1] Carly Vervat,[1] Robbert G.M. Bredius,[1] Maarten J.D. van Tol,[1] Marco W. Schilham,[1] Petr Sedlacek,[2] Marianne Ifversen,[3] Adriana Balduzzi,[4] Peter Bader,[5] Christina Peters[6] on behalf of the FORUM study group, Dirk Jan A.R. Moes[7#] and Arjan C. Lankester[1#]

[1]Willem-Alexander Children's Hospital, Leiden University Medical Center, Leiden, the Netherlands; [2]Motol University Hospital, Prague, Czech Republic; [3]Department of Children and Adolescents Medicine, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark; [4]Pediatric Hematopoietic Stem Cell Transplant Unit, Fondazione IRCCS San Gerardo dei Tintori, School of Medicine and Surgery, Milano-Bicocca University, Monza, Italy; [5]University Hospital Frankfurt, Frankfurt am Main, Germany; [6]St. Anna Children's Hospital, Children's Cancer Research Institute, Vienna, Austria and [7]Department of Clinical Pharmacy & Toxicology, Leiden University Medical Center, Leiden, the Netherlands

[#]*DJARM and ACL contributed equally as senior authors.*

Correspondence: L.V.E. Oostenbrink
V.E.Oostenbrink@LUMC.nl

A.C. Lankester
A.Lankester@LUMC.nl

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Abstract

Anti-T-lymphocyte globulin (ATLG) is used in hematopoietic stem cell transplantation (HSCT) to prevent graft-*versus*-host disease (GVHD) and graft failure. To date, insight in ATLG pharmacokinetics and -dynamics (PK/PD) is limited, and population PK (POPPK) models are lacking. In this prospective study, we describe ATLG POPPK using NONMEM® and the impact of ATLG exposure on clinical outcome and immune reconstitution in a homogeneous cohort of pediatric acute lymphoblastic leukemia (ALL) patients transplanted with a matched unrelated donor and receiving uniform ATLG dosing. Based on 121 patients and 812 samples for POPPK analysis, a two-compartmental model with parallel linear and non-linear clearance and bodyweight as covariate, best described the ATLG concentration-time data. The level of ATLG exposure (day active ATLG <1 AU/mL, median 16 days post-HSCT) was strongly associated with aGVHD grade II-IV, with a lower incidence in patients with prolonged active ATLG exposure (≤day 16 50% *vs*. >day 16 8.2%; *P*<0.001). When stratified for remission state, patients transplanted in complete remission (CR) 2 or 3 with prolonged ATLG exposure had a higher relapse risk, while this effect was not seen in CR1 patients (*P*=0.010). High level ATLG exposure was associated with delayed CD4 T-cell recovery at 4 and 8 weeks post-HSCT, but not at 12 weeks, and overall and relapse-free survival were not influenced by CD4 recovery at 12 weeks post-HSCT. This study underlines the importance of individualized ATLG exposure with the use of model-informed precision dosing in order to optimize the HSCT outcome in pediatric ALL.

Introduction

Rabbit anti-thymocyte globulin is a polyclonal antibody product, produced by the immunization of rabbits with the Jurkat T-cell line (anti-T-lymphocyte globulin [ATLG] Grafalon®; Neovii Pharmaceuticals AG, Rapperswil, Switzerland) or with human thymocytes (anti-thymocyte globulin [ATG], Thymoglobulin®; Sanofi Genzyme, Cambridge, MA, USA). ATLG/ATG is used in the conditioning of patients undergoing hematopoietic stem cell transplantation (HSCT) to reduce the risk of graft rejection and acute and chronic graft-*versus*-host disease (aGVHD/cGVHD). The choice

between ATLG or ATG depends on the preference of the transplantation center and the transplantation/disease specific protocols. Both ATLG and ATG contain antibodies not only against T cells but also against other immune and non-immune cells, such as B cells, natural killer (NK) cells and endothelial cells.[1,2] Due to the manufacturing differences, ATLG and ATG differ in the composition and quantity of T-cell specific antibodies, and therefore their lymphodepleting potency.[3] The lymphodepleting fraction of ATLG/ATG is known as active ATLG/ATG and accounts for only a minor fraction of the total rabbit immunoglobulin (Ig)G. The *in vivo* lympholytic threshold of active ATLG/ATG is 1 arbitrary unit (AU)/mL.[4]

High doses of ATLG and ATG are associated with increased relapse risk and delayed immune reconstitution which may lead to serious viral reactivations and infections,[5-7] emphasizing the delicate balance between effective GVHD prevention and allowing timely immune reconstitution. However, to date, there is no consensus about the optimal total dose or the timing of administration with total dosages used in pediatrics varying between 15 and 60 mg/kg for ATLG and between the 3.75 and 10 mg/kg for ATG.

For ATG, the pharmacokinetics (PK) and -dynamics (PD) in the pediatric HSCT setting have been described in a limited number of studies.[8-11] Admiraal *et al*, described the ATG PK and PD with a POPPK model in a heterogeneous cohort of children.[9,11,12] This led to the development of an individual dosing regimen for ATG in children based on body weight, lymphocyte counts before the start of ATG and stem cell source. Compared to historic control patients receiving a fixed ATG dose, study patients with individualized dosing had better CD4 T-cell recovery within 100 days.[13]

Since ATLG and ATG are not biosimilar, their PK and PD profiles will not be identical.[14] Unlike for ATG, very few studies have been published on ATLG PK/PD and no POPPK model has been published.[15] This study describes the first POPPK model for ATLG and the relationship between ATLG exposure, immune reconstitution and pivotal clinical outcome parameters including relapse rate, aGVHD and the incidence of viral infections in a prospective multicenter cohort of pediatric acute lymphoblastic leukemia (ALL) patients undergoing HSCT.

Methods

Patient population

The generation of the ATLG POPPK model and PK/PD analysis was part of the ATLG/ATG add-on study of the international, randomized, open-label, phase III study For Omitting Radiation Under Majority age (FORUM, EudraCT 2012-003032-22; *clinicaltrials gov. Identifier: NCT01949129*) and approved by the investigational review board and national authorities for each participating center. The FORUM trial was performed in accordance with the Declaration of Helsinki and informed consent was provided by all patients and/or parents/legal guardians.

Patients included in the FORUM study had high-risk ALL, were ≤18 years of age at initial diagnosis and were in complete morphological remission pre-HSCT. The transplantation was performed between September 2014 and July 2020 in one of the FORUM participating centers (*Online Supplementary Appendix*; *Online Supplementary Table S1* and list of participating institutions). More details regarding the patient inclusion criteria can be found in the FORUM study protocol and in the publication of Peters *et al.*[16] All patients transplanted from an HLA-compatible unrelated donor received ATLG with a recommended dose of 45 mg/kg, divided over 3 consecutive days starting at day -3 pre-HSCT. As per FORUM study protocol, conditioning regimen included either 12 Gy total body irradiation (TBI) plus etoposide 60 mg/kg (maximum dose 3.6 g) on day 3 pre-HSCT, or an irradiation-free conditioning with fludarabine 30 mg/m^2 in 5 days, thiotepa 5 mg/kg twice a day in 1 day, and either treosulfan 14 g/m^2 once a day for 3 days or busulfan over 4 days with dosing according to age and bodyweight, often guided by therapeutic drug monitoring. All patients received cyclosporine A and methotrexate as GVHD prophylaxis.

Sampling, anti-T-lymphocyte globulin measurements and pharmacokinetic modeling

A brief summary of the sampling, ATLG measurement assays and even so the complete development of the POPPK model, its validation and the use of the model to calculate different PK exposure metrics, are presented in the *Online Supplementary Appendix*[14,17] *(Online Supplementary Tables S2, S3).*

Statistical analysis

Definitions and details of the clinical outcomes parameters and immune recovery can be found in the *Online Supplementary Appendix*. Time-to-event outcomes, such as overall survival and event-free survival, were estimated using the Kaplan Meier method. Differences in survival were tested using the log-rank test for discrete covariates and Cox model for continuous covariates and multivariate analysis. Viral reactivations/infections (cytomegalovirus [CMV]/ Epstein-Barr virus [EBV] only patients at risk [patient and/or donor with a seropositive status], adenovirus [HAdV] all patients) and aGVHD were both analyzed using competing risk cumulative incidence curves, adjusting for mortality, and tested using Gray's test. Multivariate analysis for aGVHD was performed using Fine-Gray regression. Relapse rate was also assessed with competing risk analysis stratified by remission state and modeled using Fine-Gray regression to include the effect of TBI and age. The influence of aGVHD on relapse and non-relapse mortality was estimated using 100-day landmark competing risk analysis. For univariate analyses, we reported survival

or cumulative incidence percentages, and for multivariate analyses, we reported the hazard ratio (HR). For missing immune reconstitution data on a specific time point (5-14% of all immune recovery data) we estimated cell levels using a linear mixed model including both a general trend and individual variance, if we knew the value to be below the cut-off value for recovery of the specific immune cell population this was included as well. Immune cell counts were compared with a Mann-Whitney U test. These statistical analyses were performed with R statistics (v.4.2.2), cmprsk (v2.2.11) and survival (v.3.5.5).18 A *P* value of <0.05 was considered statistically significant.

Results

Population pharmacokinetic model development and evaluation

ATLG POPPK was best described by a two-compartment model with parallel first-order linear and non-linear (Michaelis-Menten) elimination (*Online Supplementary Figure S1*) and total bodyweight allometrically scaled added as covariate to clearance and central volume of distribution (*Online Supplementary Figure S2*). Adding pre-ATLG lymphocyte numbers, which were low (*Online Supplementary Table S2*) irrespective the conditioning regimen or remission state, as a co-variate did not improve the model. The details of the model building process and the validation are included as *Online Supplementary Appendix (Online Supplementary Table S4; Online Supplementary Figures S3-S6)*. Parameter estimates of the final model are presented in Table 1. In order to demonstrate the practical use of our model, we created a Shiny application (https://doi.org/10.5281/zenodo.7944182). This application performs model simulations, based on ATLG dose and patient weight and predicts the expected ATLG exposure (the day active ATLG concentration <1 AU/mL) with a 90% prediction interval considering the unexplained inter-individual variability. The application allows users to define the infusion rate and the starting day of ATLG (detailed information included in the *Online Supplementary Appendix*).

Anti-T-lymphocyte globulin exposure and clinical outcome

While 121 patients were included for PK model development detailed transplantation and clinical outcome data was available from 101 patients (Table 2). In order to assess the correlation between clinical outcome and ATLG expo-

Table 1. Population pharmacokinetic parameters of the final model and 1,000 bootstrap runs.

	Final model			1,000 bootstrap runs results (91% successful)	
	Mean value	**RSE, %**	**Shrinkage, %**	**Median value**	**95% CI**
CL, L/day	526	7	-	5.24	4.64-5.97
Theta 10	0.788	13	-	0.79	0.58-1.00
V_1, L	28.9	5	-	28.8	26.2-32.0
Theta 11	0.583	16	-	0.59	0.39-0.77
K_{21}	0.0459	10	-	0.046	0.0383-0.0556
T_{max}, AU/day	1,790	58	-	1,790 (fixed)	-
T_m, AU/L	3,540	56	-	3,540 (fixed)	-
V_{max}, AU/day	3.95	23	-	4.05	3.09-5.28
K_m, AU/L	0.261	18	-	0.26	0.192-0.369
Interindividual variability (IIV)					
CL, CV%	58.8	7	5	58	50.1-66.1
CL ~ V1,CV%	-	-	-	49.3	40.2-58.0
V_1, CV%	47.1	8	11	46.3	38.2-55.3
T_m, CV%	78.5	14	25	78.1	59.1-95.5
V_{max}, CV%	29.7	27	63	27.6	5.5-43.3
K_m, CV%	78.7	16	56	78.7	41.7-109.1
Random residual variability					
Proportional, %	32.4	4	17	32.2	29.7-34.6
Additive	0.0098	3	17	0.0098	0.0093-0.0104

RSE: relative standard error; CI: confidence interval; CL: clearance; V_1: volume of distribution; K_{21}: transfer rate constants connecting compartments; AU: arbitrary unit; T_{max}: maximum transport rate of distribution towards peripheral compartment; T_m: Michaelis-Menten constant saturable distribution towards peripheral compartment; V_{max}: maximum elimination rate; K_m: concentration at which the elimination pathway is half saturated; CV%: percentage coefficient of variation.

sure the previously described ATLG exposure metrics were used which all correlated strongly with each other (*Online Supplementary Figure S7*). We selected the day active ATLG concentration reached the lympholytic level of 1 AU/mL (ATLG <1 AU/mL) for the exposure-response analysis, since it best describes the complete exposure period (median day 16 post-HSCT, Interquartile range, 13-20 days post-HSCT). Furthermore, exposure-outcome analysis with our main clinical outcome parameter aGVHD grade II-IV was most significant for the day ATLG <1 AU/mL (P<0.001). In addition, the day ATLG <1 AU/mL had the highest correlation in logistic regression and the best diagnostic distinctiveness according to the AUC of the receiver operating characteristic curve (AUC ROC-curve) (*Online Supplementary Table S5*). Exposure-response analyses below were performed by dividing our patient cohort into two groups defined by the median value of the estimated day active ATLG <1 AU/mL (the "low-exposure" group (≤16 days) and the "high-exposure" group (>16 days); *Online Supplementary Table S6*) or into three groups (tertiles, "low-exposure" [≤15 days], "intermediate-exposure" [16-20 days] and "high exposure" [>20 days]; *Online Supplementary Table S7*).

In order to investigate possible confounders, we determined which patient, disease and transplant characteristics were associated with median ATLG exposure. The only associated factor was the use of TBI *versus* chemoconditioning, with 69% of patients with low exposure receiving TBI while this was 33% in those with high exposure (P<0.001). Age, MRD, HLA mismatches, graft source and remission state were not associated with ATLG exposure (*Online Supplementary Table S8*).

Acute graft-*versus*-host disease and chronic graft-*versus*-host disease

Within 100 days after transplantation, the cumulative incidence of aGVHD grade II-IV, accounting for death as a competing event, was 30% (Figure 1A). Grade II was most common (N=26), while severe aGVHD (grade III N=1, grade IV N=3) was seen in only four patients. Three patients developed late onset aGVHD, defined as aGVHD occurring >100 days post-HSCT and were therefore excluded from our analyses. In exposure-response analysis a significant correlation between all ATLG exposure metrics and the incidence of aGVHD grade II-IV was seen, most profound for the day ATLG <1 AU/mL (P<0.001). The incidence of aGVHD grade II-IV was significantly higher in the low-exposure group (P<0.001; ≤16 days 50% *vs*. >16 days 8%; see Figure 1B; multivariate analysis low exposure *vs*. high exposure hazard ratio [HR]=0.10; 95% confidence interval [CI]: 0.03-0.31; P<0.001). In multivariate analysis, conditioning regimen, remission state, age and stem cell source had no significant effect on aGVHD grade II-IV incidence (*Online Supplementary Table S9*).

Chronic GvHD was observed in seven of the 101 patients (N=1 limited and N=5 extensive cGVHD, and N=1 extensive cGVHD after leukemia relapse), without significant correlation with ATLG exposure metrics (*data not shown*).

Table 2. Patient characteristics.

Characteristics	
Patients, N	101
Sex, N (overall %)	
Male	63 (62)
Female	38 (38)
Patient age in years, median (range)	9.2 (0.6-18.6)
0-4, N (overall %)	20 (20)
>4, N (overall %)	81 (80)
Immunophenotype, N (overall %)	
B-cell precursor ALL	77 (76)
T-cell ALL	16 (16)
Other	6 (6)
Unknown	2 (2)
Remission status, N (overall %)	
CR1	52 (51)
CR2-3	49 (49)
MRD pre-HSCT, N (overall %)	
Positive	28 (28)
Negative	52 (51)
Unknown	21 (21)
Graft source, N (overall %)	
Bone marrow	74 (73)
PBSC	27 (27)
Donor type, N (overall %)	
MUD	101 (100)
HLA-mismatch, N (overall %)	
10/10	63 (62)
9/10	37 (37)
8/10	1 (1)
Conditioning, N (overall %)	
TBI+VP16	52 (51)
Chemo treosulfan	33 (33)
Chemo busulfan	16 (16)
GVHD prophylaxis, N (overall %)	
CSA+MTX	88 (87)
CSA+MTX+other	10 (10)
other	3 (3)
Total nucleated cells x10^8/kg BW, median (range)	4.82 (0.51-28.7)[a]
Bone marrow	4.20 (0.51-12.55)[a1]
PBSC	10.2 (1.92-28.7)[a2]
$CD34^+$ cells x10^6/kg BW, median (range)	5.2 (0.0-33.0)[b]
Bone marrow	4.5 (0.0-16.6)[b]
PBSC	9.2 (2.8-33.0)
Serotherapy ATLG dose, mean (range)	
ATLG Grafalon, mg/kg BW	44 (15-45)
Serotherapy parameters, median (range)	
Start serotherapy, day pre-HSCT	-3 (-5 to -2)[c]
Days ATLG infusion	3 (3-4)[d]
Body weight, kg	30.2 (5.6-111.0)
Lymphocytes pre-ATLG x10^9/L	0.05 (0.0-9.1)[e]

[a]Nucleated cell numbers missing from 18 patients. [a1]Nucleated cell numbers missing from 13 patients. [a2]Nucleated cell numbers missing from 5 patients. [b]$CD34^+$ numbers missing from 3 patients. [c]Start day ATLG missing from 1 patient. [d]Days ATLG infusion missing from 2 patients. [e]Lymphocytes pre-ATLG missing from 3 patients. ALL: acute lymphoblastic leukemia; CR: complete remission; MRD: minimal residual disease; PBSC: peripheral blood stem cell; MUD: matched unrelated donor; HLA: human leukocyte antigens; BW: body weight; ATLG: anti-T-lymphocyte globulin; GVHD: graft-*versus*-host disease; TBI: total body irradiation; CSA: cyclosporin A; MTX: methotrexate.

Overall, event-free and relapse-free survival

Overall survival (OS) in the total patient cohort was 96%, 90% and 84% at 1, 2- and 5-years post-transplant, respectively (*Online Supplementary Figure S8A*) and was not significantly different between the low and high ATLG exposure group (2-year OS probability of 91% and 88%; *P*>0.9; Figure 2A). Event-free survival (EFS), with event defined as relapse (N=24), extensive cGVHD (N=5) and death (N=3, all HSCT related), was 75% and 68% at 1 and 2 years post-HSCT and 64% at 5 years post-HSCT (*Online Supplementary Figure S8B*). Relapse-free survival (RFS) at 1 year after transplantation was 79%, decreasing to 73% and 65% at respectively 2 and 5 years after transplantation (*Online Supplementary Figure S8C*). In the overall cohort, ATLG exposure showed a trend, with a 2-year RFS probability of 81% in the low-exposure group and 65% in the high-exposure group (*P*=0.078; Figure 2B). A higher relapse rate was observed in patients with aGvHD grade 0-I compared to patients with aGvHD grade II-IV, however this was not significant (*P*=0.2) (*Online Supplementary Figure S9A*). AGVHD grade II-IV was not correlated with RFS (100-day landmark analysis; *P*=0.60; *Online Supplementray Figure S9B*), since in the group of patients with aGVHD grade II-IV more patients died due to an HSCT-related cause compared to patients with aGVHD grade 0-I. Remission status had a significant effect on RFS, with a 2-year RFS probability of 82% in patients with CR1 compared to 63% in CR2-3 and a 5-year probability of 79% and 46%, respectively (*P*=0.015; Figure 2C). In multivariate analysis, considering conditioning regimen, patient age, remission state, stem cell source and ATLG exposure, only remission state had a significant effect on the relapse rate (CR1 *vs*. CR2-3, HR=2.64; 95% CI: 1.09-7.01; *P*=0.031; *Online Supplementary Table S9*). However, since the effect of graft source on relapse showed a strong trend we performed an bivariate analysis. We found an effect of ATLG exposure both in patients with bone marrow and PBSC on relapse, this effect was most evident in patients with a PBSC graft (*Online Supplementary Table S10*). When analyzing the effect of ATLG exposure separately within TBI and non-TBI patients, the trend towards more relapse with prolonged ATLG exposure occurred in both groups (*Online Supplementary Table S11*).

Univariate analysis showed a trend for ATLG exposure on RFS and a correlation between remission state and relapse. Combining remission state with ATLG exposure showed a much higher relapse incidence in the CR2-3 patients with high ATLG exposure (incidence of relapse 2 years post-HSCT 51%) compared to the other three groups (low-exposure and CR1 16%, high-exposure and CR1 16% and low-exposure and CR2-3 14%; *P*=0.010; Figure 2D). Death co-occurred more frequently with relapse in the high ATLG exposure group (see *Online Supplementary Table S12*). This result in the CR2-3 group remained per individual conditioning regimen, with a 2.3-times higher 1-year relapse rate in the TBI high ATLG exposure group compared to those with low ATLG exposure. Likewise, a 2.5-times higher 1-year relapse rate was found in the chemotherapy-conditioning group with high ATLG exposure, even though patients in CR2-3 with chemotherapy-conditioning more often had high ATLG exposure than those with TBI (chemotherapy group, low ATLG exposure: N=5, high ATLG exposure: N=15; TBI group: low ATLG exposure: N=20, high ATLG exposure: N=9; *P*=0.0037). Subgroup analysis of the effect of ATLG exposure on aGVHD incidence in the two remission subgroups revealed a much

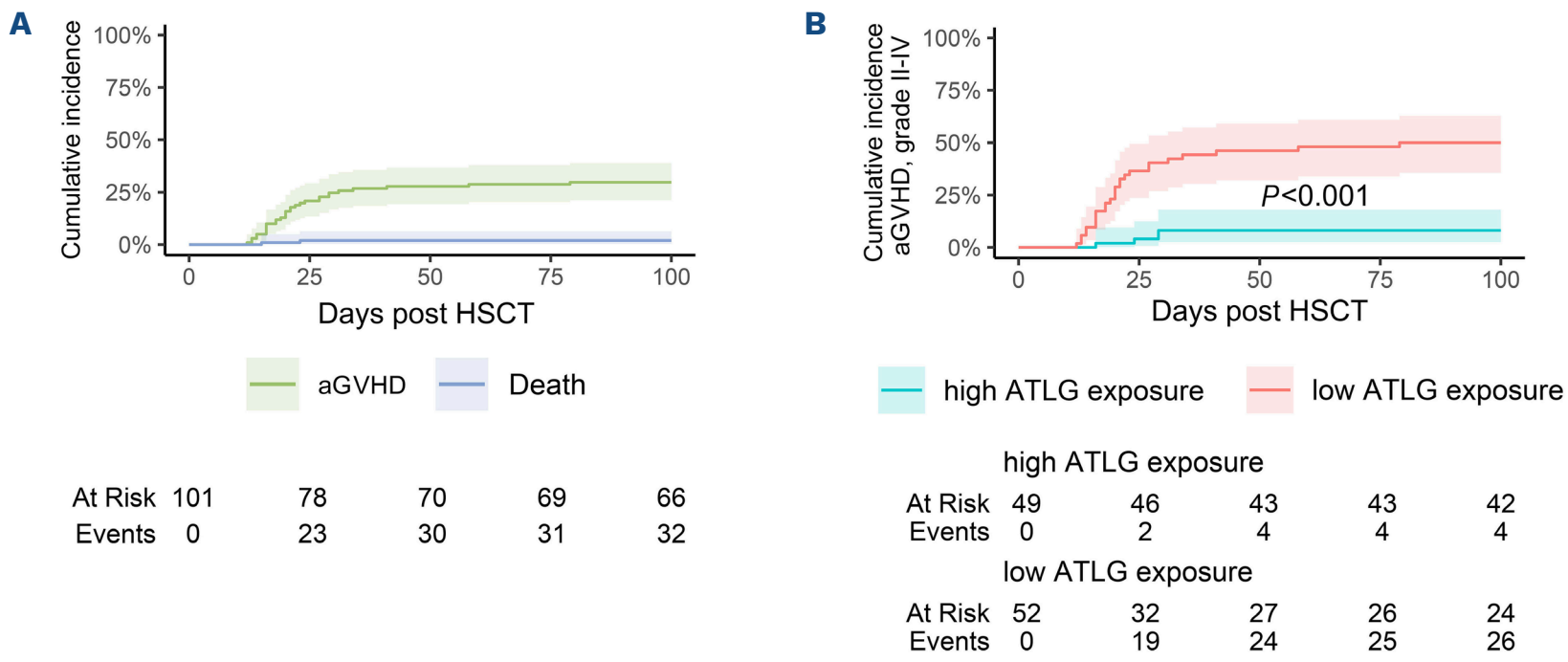

Figure 1. Cumulative incidence plots of acute graft-*versus*-host disease grade II-IV. (A) Competing risk cumulative incidence of acute graft-versus-host disease (aGVHD) grade II-IV curve of the total cohort (green line), adjusting for death (blue line), was 30% at 100 days after the transplantation. (B) Cumulative incidence of aGvHD grade II-IV in the high-exposure anti-T-lymphocyte globulin (ATLG) group (blue line) was 8.2% at 100 days post-hematopoietic stem cell transplantation (HSCT), while in the low-exposure group (red line) this was 50% (*P*<0.001). The exposure groups are defined by the median value of the estimated day active ATLG fell <1 AU/mL (the "low-exposure" group [≤16 days] and the "high-exposure" group [>16 days]).

higher incidence of grade II-IV aGVHD in the low *versus* high ATLG exposure groups in CR1 patients (63% *vs*. 4% at 100 days; *P*<0.001), while in CR2-3 patients, there was only a trend to significance (36% *vs*. 12% at 100 days; *P*=0.053). Preconditioning/pretransplant MRD state was separately analyzed and not included in the multivariate analysis due to missing values (N=21). MRD-positive patients had a higher risk for relapse with 57% 2 year relapse-rate in high ATLG exposure patients and 29% in low ATLG exposure patients compared to 21% and 9% in high and low ATLG-exposed MRD-negative patients (*P*=0.007) (*Online Supplementary Figure S10)*.

Engraftment was not influenced by ATLG exposure, both the cumulative incidence and the timing were comparable in both ATLG exposure groups (*P*=0.13; *Online Supplementary Figure S11*). One patient in the low-ATLG exposure group and three patients in the high-ATLG exposure group relapsed or died before engraftment.

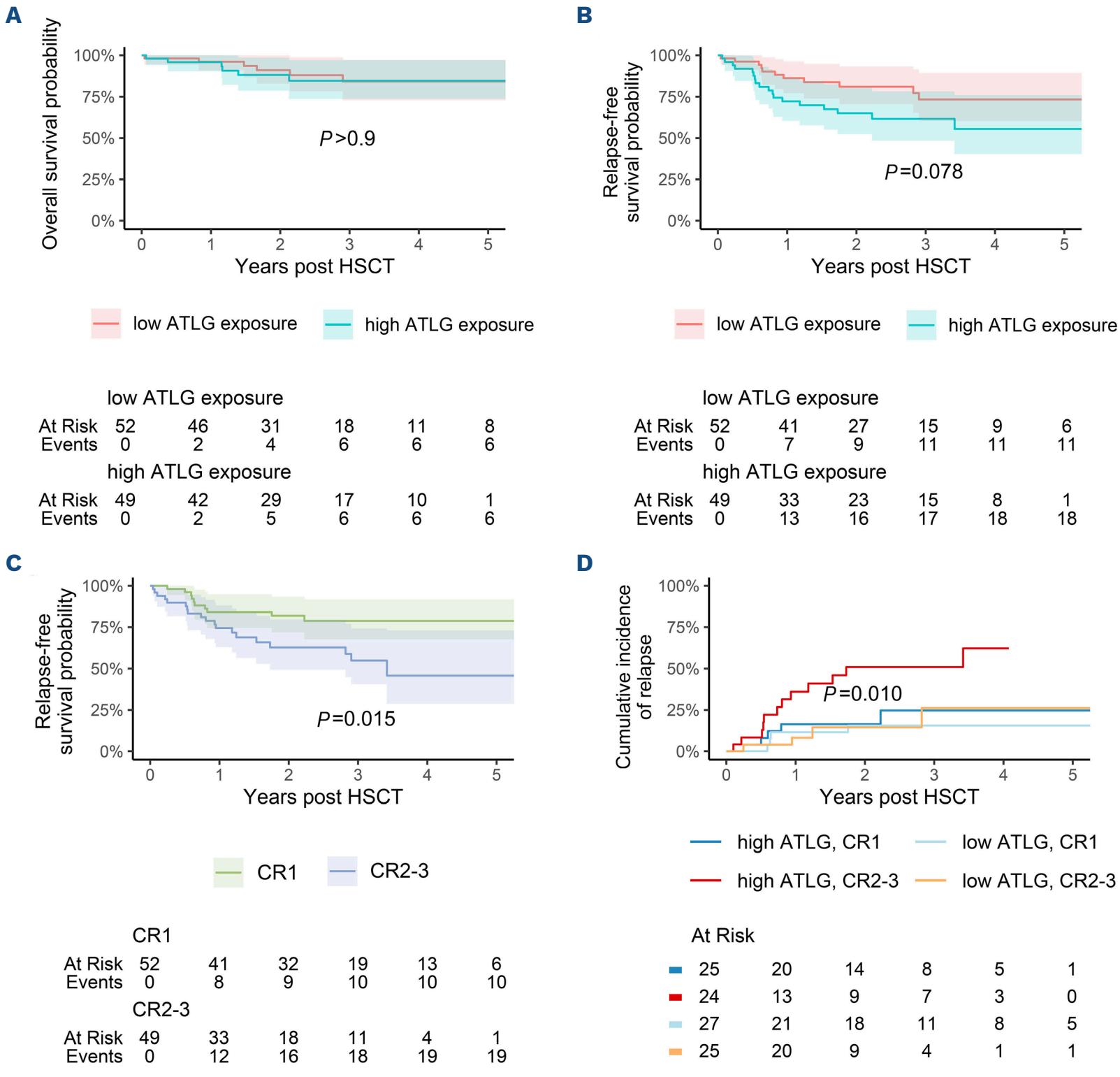

Figure 2. The effect of anti-T-lymphocyte globulin exposure on overall survival, relapse-free survival and relapse incidence. (A) Overall survival was similar in the patients with low anti-T-lymphocyte globulin (ATLG) exposure (red) and high ATLG exposure (blue) (2-year overall survival [OS] probability of 91% and 88%; *P*>0.9). (B) Relapse-free survival in the low ATLG exposure group was higher (respectively 86% and 81% at 1 and 2 years post-hematopoietic stem cell transplantation [HSCT]) compared to patients with high ATLG exposure (72% and 65%), however this was not significant (*P*=0.078). (C) Relapse-free survival was significantly worse in patients in complete remission (CR) 2-3 at the time of transplantation compared to patients in CR1 (63% *vs*. 82% at 2 years post-HSCT; *P*=0.015). (D) Patients in CR2-3 had a significantly higher relapse risk when ATLG exposure was high. By stratifying for remission state, we showed that patients with CR2-3 had a significantly higher relapse risk when ATLG exposure was high, while this effect was not seen in those in CR1 (cumulative incidence of relapse at 2 years post-HSCT; *P*=0.010; low ATLG exposure + CR1: 16%, high ATLG exposure + CR1: 16%, low ATLG exposure + CR2-3: 14%, high ATLG exposure + CR2-3: 51%.)

Viral infections

ATLG exposure-response analysis for viral infections and immune reconstitution performed with tertiles ("low-exposure" [≤15 days], "intermediate-exposure" [16-20 days] and "high exposure" [>20 days]) provided additional information compared to analysis performed with two exposure groups. Ninety of 101 patients were at risk for EBV reactivation/infection, of whom 11 patients experienced an EBV reactivation/infection within 100 days after the transplantation. Cumulative day 100 incidence of EBV reactivation was correlated with ATLG exposure as shown using a Gray's test analysis (low ATLG exposure group 0% *vs*. intermediate ATLG exposure group 11% *vs*. high ATLG exposure group 30%; *P*=0.002; *Online Supplementary Figure S12A*). Eighteen of 76 patients at risk for CMV experienced a CMV reactivation within 100 days, with a trend to significance with duration of ATLG exposure (low ATLG exposure group 6.9% *vs*. intermediate ATLG exposure group 27% *vs*. high ATLG exposure group 38%; *P*=0.054; *Online Supplementary Figure S12B*). Ten patients were diagnosed with an HAdV infection without correlation with ATLG exposure (*P*=0.4; *Online Supplementary Figure S12C*).

Anti-T-lymphocyte globulin effect on immune reconstitution

Immune reconstitution data at 4, 8 and 12 weeks after HSCT was available from 72 of the 101 patients with clinical outcome data (tertiles day active ATLG <1 AU/mL "low-exposure" [≤13 days], "intermediate-exposure" [14-19 days] and "high exposure" [>19 days]). CD4 T-cell recovery at 4 weeks post-HSCT was lowest in the ATLG "high exposure" group (median $CD4^+$ cells/μL at 4 weeks 30 *vs*. 24 *vs*. 3; *P*=0.045; *Online Figure Supplementary S13A*), while this difference was no longer observed at 8 and 12 weeks. CD8 T-cell numbers at 4, 8 and 12 weeks were lower in the high ATLG exposure group compared to the intermediate- and the low-exposure groups with the highest significance at 8 weeks post-HSCT (median $CD8^+$ cells/μL 311 *vs*. 115 *vs*. 65; *P*=0.012; *Online Supplementary Figure S13B*). Conversely, NK-cell reconstitution at 4 weeks was most profound in the high ATLG exposure group (median 64 *vs*. 99 *vs*. 160 cells/μL; *P*=0.015), while no effect of ATLG exposure was seen on B-cell reconstitution (*Online Supplementary Figure S13C, D*). Using a landmark analysis, no difference was seen in OS and RFS between patients with CD4 T cells >50 cells/μL or <50 cells/μL at 12 weeks post-HSCT (OS, *P*=0.8; RFS, *P*=0.7).

Discussion

To our knowledge, this is the first study investigating the PK and PD of ATLG by POPPK modeling. With limited publications addressing the effect of ATLG exposure on clinical outcome and immune reconstitution,[19] especially in children[14,15] we believe that the results of this study provide a unique extension of the current knowledge. Our data reveal a significant correlation between ATLG exposure and the incidence of aGVHD, with high exposure (i.e., lympholytic levels >day +16) decreasing the risk of aGVHD grade II-IV. In the overall cohort, no correlation was found between ATLG exposure and OS or for RFS. However, subgroup analysis revealed that high ATLG exposure in patients transplanted in CR2 or 3 was positively correlated with relapse. Furthermore, high ATLG exposure was inversely correlated with immune reconstitution and was associated with an increased risk for viral infections such as EBV.

In current practice, a fixed dose ATG or ATLG per kg bodyweight is given to children,[5,6,20] with a recent trend towards lower doses. Recent consensus-based recommendations by an international expert panel mentioned that for children ATLG dosing at 15 mg/kg is as effective as 30 mg/kg.[21] However, giving a fixed dose per kg assumes that both the PK and PD of ATG and ATLG show a linear increase with bodyweight and for both brands this is not the case (this study and Admiraal *et al.*[12]), specifically in children. Both our ATLG PK model and the ATG model are two-compartment models with first-order linear and non-linear elimination from the central compartment with bodyweight added as covariate. However, the models differ from each other e.g., in clearance rate, volume of distribution and inter-individual variability. These differences emphasize that even though both products consist of rabbit IgG and are used for the same purpose they are unique and the models cannot be used interchangeably.

The ATG POPPK model generated based on data from patients with malignant and non-malignant diseases contains, besides bodyweight, absolute lymphocyte count (ALC) before the first ATG dose as co-variate.[9,12] Adding the ALC pre-ATLG as a co-variate did not improve our ATLG model, likely due to the relatively low lymphocyte counts pre-ATLG in this ALL patient cohort, with little variability between patients.

Our study has several limitations. First, due to chemotherapy pretreatment in this homogenous ALL cohort, patients were uniformly, lymphopenic therefore excluding, lymphocyte count pretransplant as a possible co-variate which may be different in other, particularly chemotherapy-naïve, disease entities. Secondly, the possible role of MRD as covariate could not be fully elucidated due to missing data and the use of various (i.e., molecular and flowcytometry-based) technologies to determine MRD. Finally, the preferred exposure for favorable outcome observed in this study may be different for other transplant settings, e.g., in haplo-identical HSCT with PT-Cy or TCR a/b approaches. This requires further studies in these specific HSCT platforms.

In line with previous publications, we found no correlation between ATLG exposure and OS even though there was a significant decrease in aGvHD incidence with higher ATLG exposure.[21-23] The fact that less aGVHD does not lead to better OS is often attributed to the increased risk of infections and relapse.[5-7,24] For relapse, reports in matched unrelated donor HSCT are inconsistent, ranging from no

effect[25] of ATLG to an increase in relapse after ATLG in a recent RCT. This increase in relapse after ATLG was observed in patients with low ALC at the time of ATLG administration which correlated with receiving TBI as conditioning. The authors concluded that a low ALC likely resulted in a delayed clearance of ATLG.[24] In our study, lymphocytes pre-ATLG did not explain the variability in ATLG clearance, likely due to the generally low ALC. We found a trend for a correlation between ATLG exposure and RFS and between aGVHD II-IV and RFS. However, stratifying for remission state revealed that patients transplanted in CR2-3 had a significantly higher relapse risk when ATLG exposure was high, while this effect was not seen in those in CR1. This effect was independent of conditioning regimen.

Previous studies focusing on ATLG/ATG and immune reconstitution reported a dose-dependent delay of T-cell recovery but remain unclear about the effect of both drugs on NK-cell reconstitution.[14,26-28] Our current study revealed a delayed recovery of T cells in the first weeks post-HSCT in the high ATLG exposure group compared to the low-ATLG exposure group.

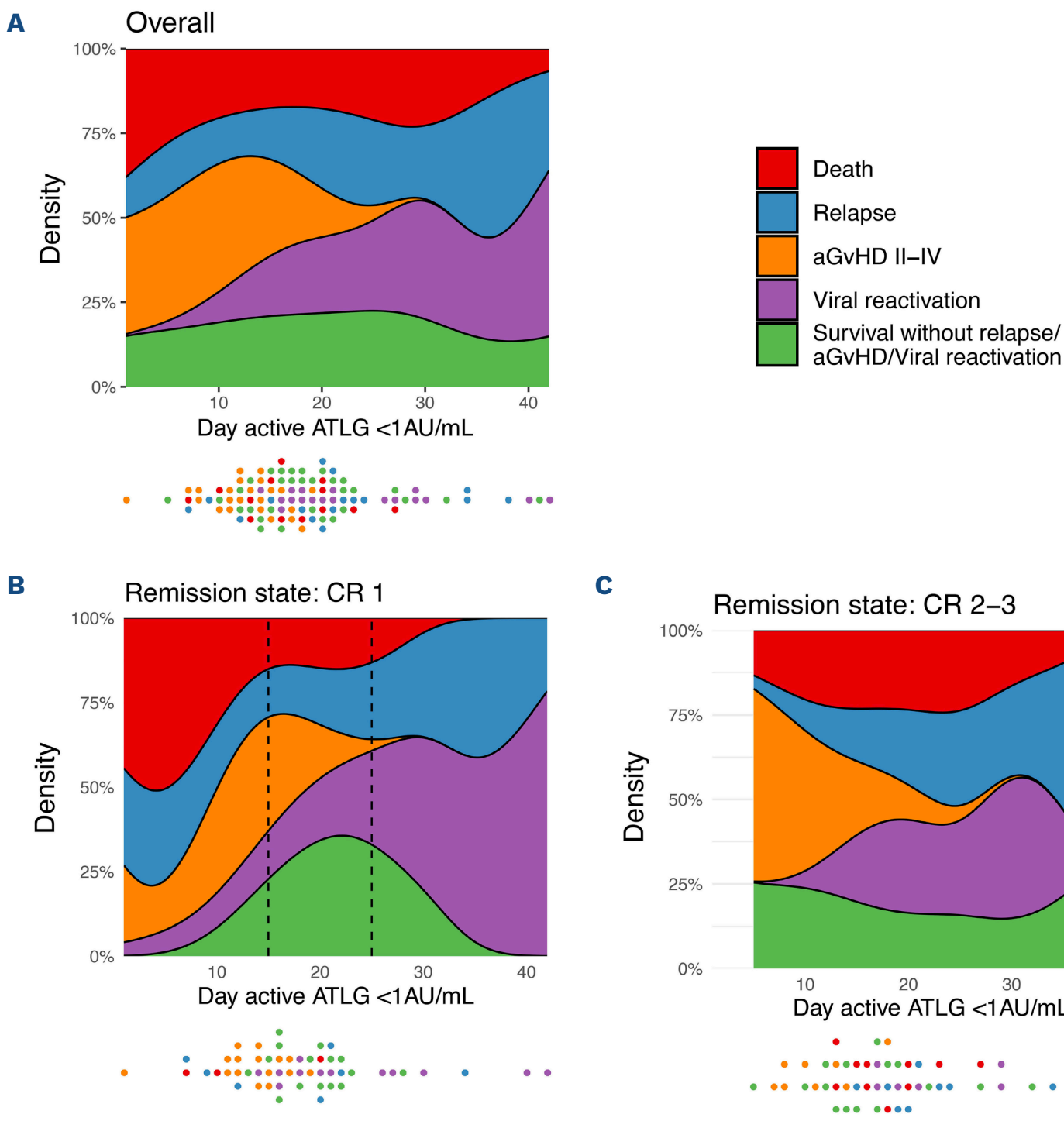

Figure 3. Stacked density plots and bee swarm plot with an overview of anti-T-lymphocyte globulin exposure and clinical outcome parameters. (A) Density plot of the overall cohort (top) with an accompanying bee swarm plot (bottom). Each dot in the bee swarm plot represents an individual patient, color of the dot matches the worst event that occurred (death: red; relapse: blue; acute graft-*versus*-host disease (aGVHD) II-IV: orange; viral reactivation: purple; survival without relapse/aGVHD/viral reactivation: green). The areas in the top plot represent the estimated proportion of patients with a certain worst event given the anti-T-lymphocyte globulin (ATLG) exposure. (B) Subgroup density plot with suggestions for ATLG exposure for 1st complete remission-hematopoietic stem cell transplantation (CR-HSCT) patients. Based on all results in our study, aiming for active ATLG <1 AU/mL between 15-25 days post-HSCT in patients in CR1 at time of HSCT seems a good target to strive for, with reduced risk for aGVHD grade II-IV, no increased risk for relapse and a relatively low risk of getting a viral reactivation. (C) Subgroup density plot for patient with CR2-3. Defining an optimum for ATLG exposure is more difficult for patients with CR2-3, since in this subcohort prolonged ATLG exposure prevents aGVHD incidence, however the relapse incidence increases.

Conversely, NK-cell reconstitution at 4 weeks post-HSCT was more robust in the high-ATLG exposure group. This phenomenon, of potent NK-cell expansion in patients with delayed T-cell recovery, has previously been described for ATG.[29]
Several studies have reported a correlation between immune recovery (often CD4 T-cell recovery) and OS and reduction of relapse and non-relapse mortality.[10,11,30] In our cohort we did not find a correlation between successful CD4 T-cell recovery (defined as >50 CD4 T cells/μL) at 12 weeks post-HSCT and OS or RFS. This might be explained by the different statistical analysis that was performed since some previous publications did not use a landmark analysis,[10,11] or by the primary disease and disease status since successful CD4 T-cell reconstitution by day 100 in the study of Admiraal *et al*. was correlated with decreased relapse-related mortality in patients with myeloid malignancy but not in lymphoid malignancy[11]
While our study has provided the rationale for model-based dosing, finding an optimal dose for ATLG exposure remains challenging, as the risk of aGVHD, relapse and infections need to be balanced. We integrated these risks in a stacked density plot to summarize these findings in a single figure (Figure 3A), but this has several limitations, including not accounting for censoring and length of follow-up, and only visualizing the most impactful event per patient. In patients in CR1, reaching an active ATLG level <1 AU/mL between 15-25 days appears associated with a low incidence of aGVHD, viral infections and relapse (Figure 3B), but for patients in CR2-3, no such optimum is apparent (Figure 3C). This may be explained by the higher relapse risk in CR2-3, which could increase even further with high ATLG exposure[24] outweighing the decreased aGVHD risk. In our study in ALL patients transplanted with matched unrelated donors optimal ATLG exposure depends on disease state at HSCT. Future studies should explore optima for other transplant indications, disease factors and transplant platforms influencing the PK and PD of serotherapy.

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Contributions

CMJ-vdZ, MJDvT, RGMB, PS, MI, AB, PB, CP and ACL designed the study. LVEO, CJM-VdZ and AMJH collected the data and the data was accordingly analyzed by LVEO, EGJvA, AMJH, CV and DJARM. LVEO and EGJvA prepared the original draft of the manuscript, with supervision of MWS, ACL and DJARM. All authors contributed to data interpretation, manuscript revisions and reviewed and approved the final version of the manuscript. A list of contributing clinical centers and their representatives is included in the Online Supplementary Appendix.

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Data-sharing statement

Since this study is part of the ongoing FORUM study (FORUM, EudraCT 2012-003032-22; clinicaltrials gov. Identifier: NCT01949129), the current data used for this manuscript can currently not be shared.

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