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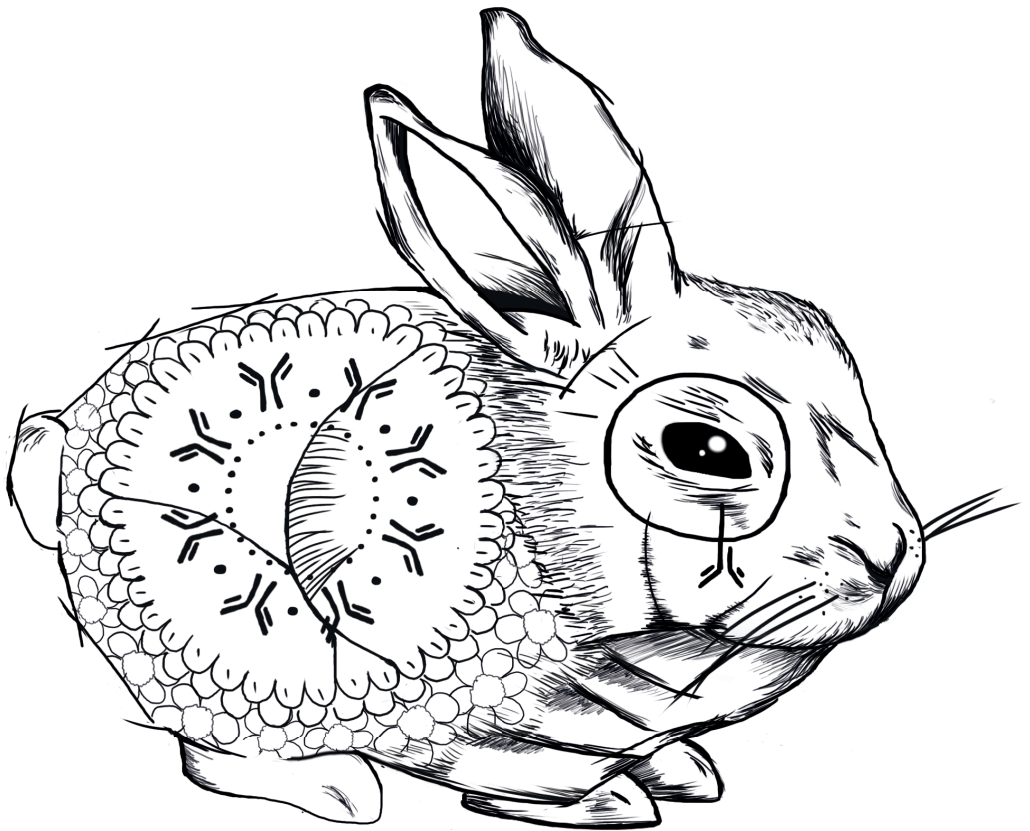


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Chapter 7

Optimizing Anti-Thymocyte Globulin Exposure to Improve Survival Chances

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ABSTRACT

Background

Anti-thymocyte globulin (ATG) is used to prevent graft-versus-host-disease (GvHD) following allogeneic hematopoietic cell transplantation (HCT). However, ATG can also cause delayed immune reconstitution, negatively influencing survival. We studied the relation between exposure to ATG and clinical outcomes in adult patients with acute leukemia and myelodysplastic syndrome.

Methods

In a retrospective analysis, consecutive patients receiving a T-cell repleted allogeneic peripheral blood stem cell-HCT with ATG (Thymoglobulin) as part of reduced intensity conditioning were included (March-2004 to May-2015). Active-ATG levels were measured using a validated bioassay and pharmacokinetic exposure measures were calculated with a validated population PK-model. Main outcome of interest was overall survival (OS); other outcomes were relapse- and non-relapse mortality, acute- and chronic-GvHD and evaluation of current and optimal dosing. Cox proportional-hazard models and Fine-Gray competing risk models were used.

Results

146 patients were included. ATG exposure after HCT was found the best predictor for 5-year OS. Optimal exposure after HCT ($60\text{--}95\text{ AU}\cdot\text{day/mL}$; $69\pm 8\%$) yielded superior OS compared to below ($32\pm 8\%$, hazard ratio [HR] 3.36, 95% confidence interval [CI] 1.69–6.68, $p=0.00057$) and above-optimal exposure ($48\pm 6\%$, HR 2.5, 95%-CI 1.29–4.84, $p=0.0064$). Above-optimal exposure led to higher relapse-related mortality (HR 2.66, $p=0.027$). Below-optimal exposure increased non-relapse mortality (HR 4.17, $p=0.0060$), grade 3–4 acute-GvHD (HR 3.09, $p=0.035$) and chronic-GvHD (HR 2.56, $p=0.048$). Dosing based on absolute lymphocyte counts led to higher optimal target attainment compared to weight-based dosing.

Conclusions

Exposure to ATG impacts survival following HCT in adults, stressing the importance of optimal ATG dosing. Individualizing dosing of ATG, based on lymphocyte counts rather than body weight, may improve survival chances after HCT.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative treatment option for high-risk or relapsed acute leukemia's and myelodysplastic syndrome (MDS). Immunological rejection of residual tumor by donor-derived immune cells (graft-versus-leukemia; GvL) should give disease control including life-long anti-tumor immune surveillance¹. Graft-versus-host disease (GvHD) remains a severe complication of HCT, leading to significant morbidity and mortality. As a strategy to prevent GvHD following HCT, antithymocyte globulin (ATG) was introduced to the conditioning regimens applied before HCT². Although the use of ATG was associated with a decreased incidence of acute-^{3–5} and chronic-GvHD^{4,6}, most studies fail to show a survival advantage^{5–7}. This is probably due to unpredictable ATG-induced in vivo T-cell depletion of the graft resulting in delayed or absent early T-cell immune reconstitution^{5,8,9}. Poor immune reconstitution abrogates the GvL-effect and antiviral activity resulting in increased relapse- and non-relapse mortality¹⁰.

In this delicate balance between preventing GvHD and timely T-cell immune reconstitution, ATG has a pivotal role as T cell depleting antibody of host and donor T cells. A variety of studies have tried to determine the optimal dose of ATG by performing dose-effect studies^{11–14}. A major drawback of these dose-effect studies is the lack of incorporation of the high inter-patient variability in pharmacokinetics (PK) of ATG. Therefore, using standard dosing for all patients and not taking into account exposure, makes the results difficult to interpret^{15,16}. Furthermore, as patients are exposed to ATG before and after transplantation due to the long half-life of 5–14 days^{17–19}, the timing of ATG is an additional important variable⁵. It would be better to correlate actual exposure to outcome rather than dosage. Population PK-modeling can be used to describe pharmacokinetics and determine individual exposure, and is the standard for reporting pharmacokinetic data according to current FDA and EMA guidelines^{20,21}. Although ATG has been used since the early 1980s, the pharmacokinetic of ATG has not been thoroughly described^{17,19,22–25}.

We recently described that ATG exposure impacted outcome in pediatric HCT receiving either bone marrow or cord blood after myeloablative conditioning. High exposure to ATG after HCT was associated with a poor immune reconstitution probability and lower survival, while high exposure before HCT was associated with less GvHD and graft failure²². No studies are available investigating the optimal ATG exposure in adult HCT using mobilized peripheral blood stem cells (PBSC) after reduced intensity conditioning (RIC)^{26,27}. Therefore, we aimed to assess the relation between ATG exposure and clinical outcomes in this RIC-PBSC-setting. To achieve this, the available pharmacokinetic model for ATG in children and young adults was expanded and validated for adults. We subsequently performed a retrospective cohort analysis of consecutive patients to relate different exposure

measures of the pharmacologically active fraction of ATG (hereafter referred to as ATG) to various clinical outcomes of HCT, such as GvHD, relapse and survival.

METHODS

Study design and Patients

We included patients transplanted for an acute lymphoid leukemia (ALL), acute myeloid leukemia (AML) or MDS receiving their first HCT between March 2004 and May 2015 within the adult blood and marrow transplantation unit at the University Medical Center in Utrecht, the Netherlands. Only patients receiving a T-repleted PBSC graft with ATG (Thymoglobulin) as part of RIC conditioning were included. Clinical data and serum samples for ATG concentration measurements were collected prospectively; consecutive patients were included. Minimal follow-up for surviving patients was 6 months. Patients were included and data were collected after informed consent was acquired according to the declaration of Helsinki. Ethical committee approval was given through trial number 11/063.

Procedures

Patients underwent a RIC containing a cumulative dose of 8 mg/kg ATG divided over 4 days, starting on day -8 before HCT, fludarabine 90 mg/m² (day -3, -2 and -1) and 200 cGy total body irradiation (TBI) on day 0. Actual dose of ATG was rounded upwards to 25 mg so that patients received only full vials. Clemastine, paracetamol and 100 mg prednisolone were given prior to ATG infusion. GvHD-prophylaxis consisted of cyclosporin A (CsA) and mycophenolate mofetil (MMF). Start dose of CsA was 4.5 mg/kg/day until day +84 or +120 (target trough levels 200-350 mg/L). Hereafter, CsA was tapered if no GvHD was present. Patients received 15 mg/kg/day MMF (maximum of 3 g/ day) until day +28 or +84, also followed by tapering in the absence of GvHD²⁸. Ciprofloxacin and fluconazole were given as selective gut decontamination, trimethoprim/sulfomethoxazole and valaciclovir were used for infectious prophylaxis until 12-15 months after HCT.

ATG pharmacokinetics and exposure

To describe ATG pharmacokinetics from young children to adult patients, an adult dataset for ATG was combined with a previously published dataset of children and young adults¹⁵. In the adult population, samples were collected weekly after HCT, while samples during infusions were available in 35 patients. The complete development and validation of the population PK-model is described in the Supplemental Methods.

Following the development of the population PK-model, full concentration-time curves were estimated for each individual patient. Based on these, individual pharmacokinetic

exposure measures could be calculated. The pharmacokinetic exposures of interest included the maximum concentration ($C_{\max}$), concentration at time of infusion of the graft (C_{HCT}), time to reach a concentration of 1 AU*day/mL ($T_{C<1}$)¹⁸, the area under the curve (AUC) and the AUC before and after HCT (Figure S9).

To study the most predictive PK-measure for the main outcome of interest, all PK exposure measures were split in four groups according to quartiles. Predictive PK-measure models were selected based on the lowest Akaike Information Criterion (AIC), a criterion to select the best predicting model; in this case the proportional hazard model. Additionally, selection was based on the distribution in estimated survival between groups in Kaplan Meier plots.

Outcomes

Main outcome of interest was 5-year overall survival (OS), defined as days to death of any cause or last follow up. Other outcomes of interest included non-relapse mortality (NRM) and relapse related mortality (RRM), which were defined as days to death due to any cause other than relapse and days to death due to relapse, respectively, or last follow up. Event free survival (EFS) was defined as the days to death, relapse, graft failure (GF), or last follow up, whichever occurred first, while relapse incidence was defined as time to relapse or last follow up. Acute and chronic GvHD were classified according to the Glucksberg²⁹ and Shulman³⁰ criteria. GF was defined as non-engraftment or secondary graft rejection. As we were interested in the predictive power of the various PK exposure measures, we related the outcomes of interest with these PK measures.

Statistical analyses

Duration of follow-up was defined as the time from HCT to last contact or death. Patients were censored at the date of last contact. Factors considered as predictors for outcome included patient variables (age, sex, Epstein-Barr virus [EBV] and cytomegalovirus [CMV] serostatus), disease variables (ALL, AML, MDS), donor related variables (HLA-disparity, EBV and CMV serostatus), year of treatment (before or after median year of HCT), and ATG exposure measures.

Probabilities of survival were determined using the Kaplan Meier estimation; p-values were calculated using a two-sided log-rank test. Variables with a p-value < 0.05 in univariate analysis were included as a predictor in multivariate analysis. For the endpoints OS and EFS, Cox proportional hazard models were used. For the endpoints TRM, RRM, relapse and acute and chronic GvHD, Fine-Gray competing risk models were used³¹. Statistical analyses were performed using R 3.2.4, with packages *cmprsk*, *survival* and *rms*.

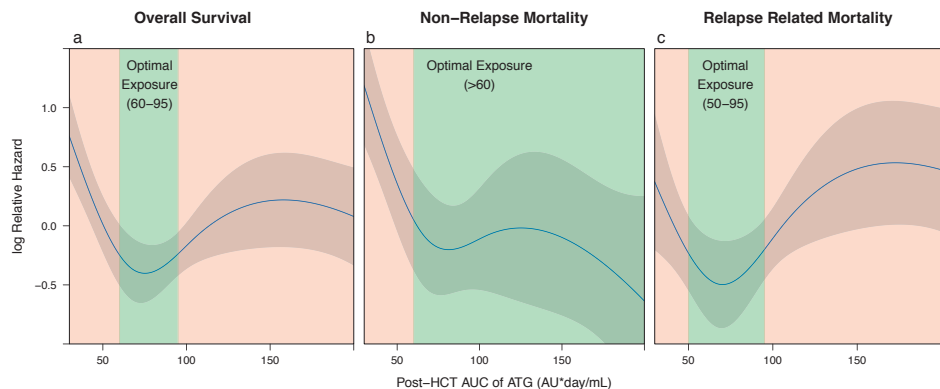


Figure 1. Log relative hazard for overall survival (panel A), non-relapse mortality (panel B) and relapse mortality (panel C) according to ATG exposure after HCT. Blue line: log relative hazard; shaded area: 95% CI for log relative hazard; green areas: optimal exposure range; red areas: sub- optimal exposure range.

	Total
Number of patients (n)	146
Male sex [n(%)]	84 (58)
Baseline lymphocyte count ($\times 10^9$ /L)	0.7 (0.0-3.3)
Age at transplant (years)	49.8 (18.1-69.9)
Cumulative dose of ATG (mg/kg)	8 (4.1-9.1)
Starting day of ATG (days before HCT)	8 (4-12)
Number of blood samples taken per patient	4 (1-12)
Diagnosis [n(%)]	
Acute Myeloid Leukaemia	74 (50)
Myelodysplastic Syndrome	36 (25)
Acute Lymphoid Leukaemia	36 (25)
Stem Cell Source [n(%)]	
Peripheral Blood Stem Cells	146 (100)
Conditioning Regimen [n(%)]	
Flu-TBI	146 (100)
Match grade [n(%)]	
Matched	111 (76)
Mismatched	35 (24)
Follow-up (months)	37 (0.6-139)

Table 1. Patient Characteristics. Values are depicted as median (range), except when otherwise specified. Baseline lymphocyte count: Absolute peripheral blood lymphocyte count before 0-7 days before the first dose of ATG; Flu: fludarabine; TBI: total body irradiation

Optimal dosing regimen

Following the determination of the most predictive PK-exposure measure and the sub-

sequently determined optimal range of exposures, available ATG dosing regimens were evaluated for target attainment. Investigated regimens included current local dosing (8 mg/kg over 4 days, starting day -8), the European society for Bone Marrow Transplantation (EBMT) protocol³² (7.5 mg/kg over 3 days, starting day -3), and a recently published regimen in adult RIC-PBSC³³ (4.5 mg/kg, starting day -2). Additionally, an optimal dosing regimen will be designed and subsequently evaluated using the same approach. Groups of patients were selected based on the predictors for PK. Concentration-time profiles were simulated using the validated PK-model, incorporating 1000 virtual patients in each group,

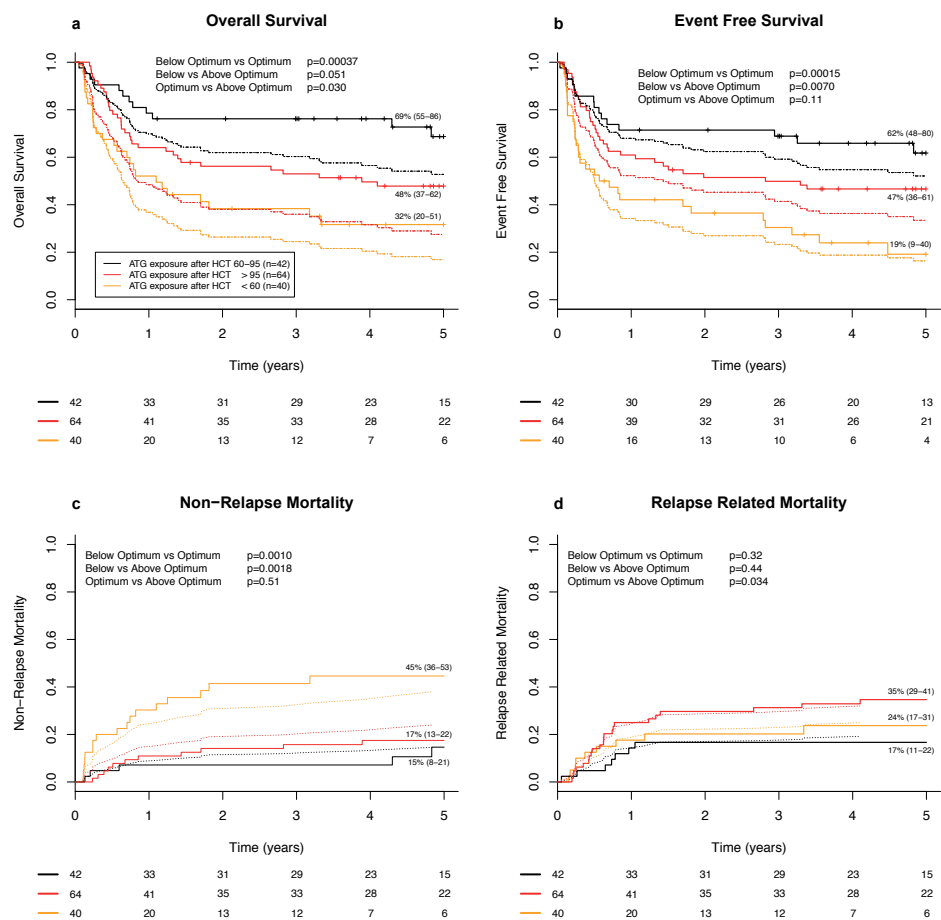


Figure 2. Unadjusted (solid lines) and adjusted (dashed lines) estimations of clinical outcomes according to groups of ATG exposure after HCT. Black lines: optimal exposure; Orange lines: below optimal exposure; Red lines: above optimal exposure. Panel A: Overall Survival; Panel B: Event Free Survival; Panel C: Non-relapse Mortality; Panel D: Relapse Mortality. Adjusted estimations are to be interpreted as the expected outcomes if all ATG exposure groups were the same, on average, with respect to all multivariate predictors (diagnosis [all], age [OS, EFS, NRM] and EBMT risk score [OS, EFS]). P-values are derived from the two-sided log-rank test. .

while taking into account full interindividual variability. For each group, median exposure after HCT was compared to the optimal therapeutic window.

RESULTS

Patients

A total of 146 patients were included; 74 with AML, 36 with ALL and 36 with MDS as indication for HCT (Table 1). Median age was 50 years (range 18.1-69.9 years); 111 patients (76%) received a 10/10 matched graft. Median follow-up of all patients was 37 months (range 0.6-139 months).

Pharmacokinetic analyses

A population pharmacokinetic model was developed that accurately described concentration data and was extensively validated (Figure 1). In the model, body weight was found a predictor for ATG clearance for body weights below 50 kilogram. Above this weight, no increase in clearance was observed with increasing body weight (Figure S5). Absolute lymphocyte counts (ALC) before the first dose of ATG also predicted clearance, which is in line with the pharmacological properties of ATG. Higher number of lymphocytes harbor

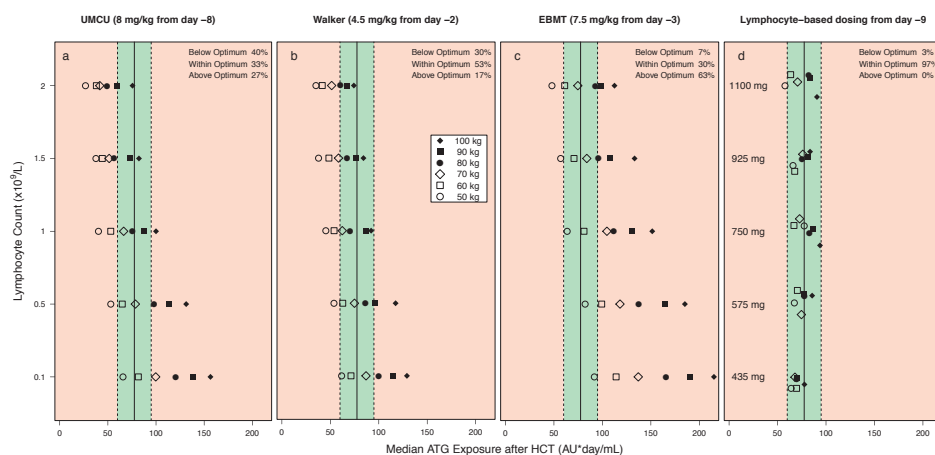


Figure 3. Evaluation of currently used ATG dosing regimens and optimal thymoglobulin dosing regimen, showing median ATG exposure after HCT for several lymphocyte/body weight groups. Panel A: Dosing regimen used for the current cohort; cumulative dose of 8 mg/kg over 4 days, starting day -9. Panel B: Dosing regimen according to Walker et al; cumulative dose of 4.5 mg/kg, 0.5 mg/kg on day -2, 2 mg/kg on day -1, and 2 mg/kg on day +1. Panel C: Dosing regimen proposed by European Bone Marrow Transplant Society; cumulative dose of 7.5 mg/kg over 3 days, starting day -3. Panel D: Dosing regimen based only on lymphocyte count, given over 4 days, starting on day -9. Absolute cumulative dose is given for each lymphocyte level. Symbols depict body weights; open circles: 50kg; open squares: 60kg; open diamonds: 70kg; filled circles: 80kg; filled squares: 90kg; filled diamonds: 100kg.

more targets for ATG binding, leading to increased clearance (Figure S5). ATG exposure measures could be accurately calculated for all patients using the validated PK-model.

Main outcome of interest

Exposure to ATG after HCT was found to be the best predictor for OS. The AIC for exposure after HCT was lowest (Supplemental Table S2), indicating the best fit of the Cox proportional hazard model, as well as the largest distribution of survival curves.

To evaluate the most optimal range of exposure to ATG after HCT, the hazard ratio (HR) for OS, NRM and RRM was plotted against ATG exposure after HCT (Figure 2). The optimal AUC after HCT was determined to be between 65 and 90 AU*day/mL (Figure 2A); below this threshold increased risk for non-relapse mortality was observed (Figure 2B), while both below and above this threshold chances on relapse mortality were higher (Figure 2C). Estimated 5-year overall survival after optimal exposure ($69 \pm 8\%$), was significantly higher than in the groups below ($32 \pm 8\%$; $p=0.00037$) and above ($48 \pm 6\%$; $p=0.030$) optimal exposure (HR 3.36, 95% confidence interval [CI] 1.69-6.68, $p=0.00057$ and HR 2.5, 95% CI 1.29-4.84, $p=0.0064$ for below and above optimum, respectively; Figure 3a, Table 2).

Other outcomes of interest

RRM was higher in patients with above optimal exposure after HCT (HR 2.66, 95% CI 1.12-6.31, $p=0.027$; Figure 3d). Patients with a below optimal exposure had an increased risk for NRM compared to optimal exposure (HR 4.17, 95% CI 1.51-11.54, $p=0.0060$; Figure 3c). EFS was comparably influenced by the exposure groups as OS; patients in the optimal exposure groups have higher EFS chances than those below or above optimal exposure (HR 2.88, 95% CI 1.53-5.43, $p=0.0011$ and HR 1.97, 95% CI 1.07-3.61, $p=0.029$, respectively) (Figure 3b). The incidence of grade 3-4 was higher in patients with below-optimal exposure compared to the optimal exposure group (HR 3.09, 95% CI 1.08-8.78, $p=0.035$). Probability on chronic GvHD were higher in the lower exposure group (HR 2.56, 95% CI 1.01-6.48, $p=0.048$) compared to optimal exposure.

Optimal dosing regimen

Current thymoglobulin dosing regimens and a novel ALC-based nomogram thymoglobulin were evaluated; groups of patients with body weights of 50-100 kg and ALC before first infusion of ATG of $0.1-2.0 \times 10^9/L$ were simulated for three current thymoglobulin dosing regimens (Table S5). Simulated ALC values were in line with the actual lymphocytes of included patients (median $0.72 \times 10^9/L$, range 0.01-3.29). The dosing regimen used in our center is representative for most Dutch centers and affiliated centers participating in HO-VON studies²⁸. It showed high variability in ATG exposure after HCT; median exposure was on target in 33% of the groups (Fig 4a). Practices from other centers utilizing thymoglobulin

Variable	HR	95% CI	p-value	
Overall survival				
<i>Optimal ATG Exposure after HCT</i>	1			
<i>Below Optimal ATG Exposure after HCT</i>	3.36	(1.69-6.68)	0.00057	***
<i>Above Optimal ATG Exposure after HCT</i>	2.5	(1.29-4.84)	0.0064	**
Event free survival				
<i>Optimal ATG Exposure after HCT</i>	1			
<i>Below Optimal ATG Exposure after HCT</i>	2.88	(1.53-5.43)	0.0011	**
<i>Above Optimal ATG Exposure after HCT</i>	1.97	(1.07-3.61)	0.029	*
Non-relapse mortality				
<i>Optimal ATG Exposure after HCT</i>	1			
<i>Below Optimal ATG Exposure after HCT</i>	4.17	(1.51-11.54)	0.006	**
<i>Above Optimal ATG Exposure after HCT</i>	1.63	(0.58-4.62)	0.35	
Relapse mortality				
<i>Optimal ATG Exposure after HCT</i>	1			
<i>Below Optimal ATG Exposure after HCT</i>	1.45	(0.55-3.83)	0.46	
<i>Above Optimal ATG Exposure after HCT</i>	2.66	(1.12-6.31)	0.027	*
Relapse Incidence				
<i>Optimal ATG Exposure after HCT</i>	1			
<i>Below Optimal ATG Exposure after HCT</i>	1.28	(0.57-2.86)	0.55	
<i>Above Optimal ATG Exposure after HCT</i>	1.79	(0.89-3.61)	0.11	
Incidence of grade 2-4				
<i>Optimal ATG Exposure after HCT</i>	1			
<i>Below Optimal ATG Exposure after HCT</i>	1.44	(0.75-2.76)	0.28	
<i>Above Optimal ATG Exposure after HCT</i>	0.79	(0.41-1.51)	0.48	
Incidence of grade 3-4				
<i>Optimal ATG Exposure after HCT</i>	1			
<i>Below Optimal ATG Exposure after HCT</i>	3.09	(1.08-8.78)	0.035	*
<i>Above Optimal ATG Exposure after HCT</i>	1.07	(0.35-3.24)	0.91	
Incidence of chronic GvHD				
<i>Optimal ATG Exposure after HCT</i>	1			
<i>Below Optimal ATG Exposure after HCT</i>	2.56	(1.01-6.48)	0.048	*
<i>Above Optimal ATG Exposure after HCT</i>	1.03	(0.41-2.57)	0.95	

Table 2. Multivariate analyses. Multivariate analyses using Cox proportional hazard and Fine-Gray competing risk models. * p<0.05; ** p<0.01; *** p<0.001

with a different timing and dosing (0.5 mg/kg on day-2, 2 mg/kg on day -1, and 2 mg/kg on day +1)³³ demonstrated an optimal target attainment in 53% of groups (Fig 4b), and the current EBMT-recommended dose (7.5 mg/kg in 3 days, starting day -3) of thymoglobulin for unrelated donors³² led to an optimal target attainment in 30% of groups only (Figure 4c). Based on the developed PK-model, the optimal dosing should be based on ALC, as ATG

clearance in patients (> 50 kg) was not influenced by weight. When targeting to the optimal ATG exposure after HCT, cumulative ATG dosage is calculated using the following formula:

$$\text{Cumulative dose} = 400 + 350 * \text{lymphocyte count (in } 10^9/\text{L)}$$

This cumulative dose should be given over 4 days starting day -9. Simulations of this dosing regimen led to optimal exposure in 97% of groups (Fig 4d); higher than any of the regimens analyzed in current study^{28,32,33}.

DISCUSSION

To our knowledge, this is the first study investigating the pharmacokinetics and pharmacodynamics of ATG in a large, consecutive cohort of adult patients receiving PBSC after RIC for acute leukemia's and MDS. We aimed to determine the therapeutic window of ATG in this setting. Taking into account the limitations of a retrospective study, the data show that the exposure to ATG after HCT impacts survival as well as acute and chronic GvHD. There seems to be an optimal window of exposure to ATG after HCT (60-95 AU*day/mL); lower exposure increases chances on mortality, mostly associated with GvHD, while an exposure above the optimum was related with more relapse-related mortality. The PK-model showed that ALC was found the only relevant predictor for PK. Therefore, ALC-based dosing will result in achieving the optimal AUC in >95% of the simulated patient groups. This may subsequently result in higher survival chances after RIC HCT receiving PBSC.

The importance of T-cell immune reconstitution following HCT is increasingly recognized in recent years. The use of ATG, more particularly exposure of the graft to ATG, has been associated with poor immune reconstitution early after HCT^{5,13,18,22}. In the early phase after HCT, until thymic output, patients depend on graft-infused T-cells undergoing peripheral expansion³⁴. Therefore, in-vivo depletion of these T-cells may result in prolonged T-cell lymphopenia, leaving patients vulnerable for relapse and viral reactivations². Restoration of thymic function can be hampered by age, GvHD, chemotherapy and steroids³⁴. In the current cohort, few data on immune reconstitution shortly following HCT was available. The increased relapse mortality following high ATG exposure however suggests that one of the causes of relapse after HCT may be poor immune reconstitution, as was found in the previous studies^{22,35,36}.

Higher concentrations of ATG following infusion of the graft have been associated with a lower incidence of GvHD, although these studies only investigated single concentra-

tions^{19,25}. However, in a more comprehensive study investigating ATG PD in children, too low exposure before HCT was associated with higher chances on GvHD, while exposure after HCT did not impact GvHD²². This is in contrast with our current results in adults, where ATG levels before HCT did not impact the clinical outcome and a very low exposure to ATG after HCT was associated with an increased incidence of grade 3-4 acute GvHD and chronic GvHD. These differences might be the consequence of the fact that the majority of patients already had a very high exposure to ATG before HCT and we consequently had no power to address the question of levels of exposure before HCT and clinical outcome. In addition, the profound effect of high ATG exposure after HCT on GvHD in the current analyses presumably reflects the at least one log higher numbers in T cells used in PBSC, compared to CB and BM³⁷.

The clearance of ATG in adults is not influenced by bodyweight. In contrast to the current practice, ATG should therefore not be dosed in mg/kg but rather as a fixed dose based on ALC prior to ATG. This is illustrated by the simulation studies: the currently used weight-based dosing regimens for ATG result in poor optimal target attainment of 30-53%. An ALC-based dosing regimen leads to optimal exposure in 97% of patients. However, this proposed dosing nomogram should be evaluated in a prospective study. Moreover, the dosing regimen is only valid for Thymoglobulin, as the various ATG preparations are not biosimilar.

In conclusion, our data show that survival after HCT is highly impacted by ATG exposure after HCT. By using a dosing regimen that aims for optimal ATG exposure, which can best be achieved using an ALC-based dosing, outcomes following HCT could be improved, resulting in higher survival chances.

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SUPPLEMENTAL METHODS

Patients in pharmacokinetic analysis

In order to derive an individualized dosing regimen for ATG for patients of all ages, the pharmacokinetics need to be described. For this purpose, data from adult HCT patients was pooled with data from a previously published PK-analysis in children and young adults¹.

The adult population consisted of all patients treated in the adult hematopoietic cell transplantation program of the University Medical Center in Utrecht between April 2004 and May 2015 who received an HCT with ATG as part of the conditioning. No restriction applied in terms of conditioning regimen, type of donor and underlying diagnosis. Dose and starting day of ATG varied based on the treatment protocol; most patients received a cumulative dose of 8 mg/kg starting day -8 or -12 before HCT given over 4 days. In all patients samples were collected weekly after HCT, while in 35 patients samples before and after infusions were available.

The previously described PK-model consisted PK data of children and young adults receiving a HCT with ATG as part of the conditioning for any indication and conditioning regimen¹. Patients treated in the pediatric hematopoietic cell transplantation programs of the University Medical Center in Utrecht (UMCU) and the Leiden University Medical Center (LUMC) in Leiden, both in the Netherlands, between April 2004 and December 2012 were included. Most patients received a cumulative dose of 10 mg/kg ATG given over 4 days, starting day -5 before HCT, however selected patients received other dosages at different starting times. In patients treated in the LUMC, samples were collected before and after each dose, and weekly thereafter, while patients treated in the UMCU only weekly elimination phase samples where available.

Any patients receiving serotherapy in the 3 months prior to the current HCT was excluded, as well as patients developing IgG anti-ATG antibodies.

Measurements of active ATG concentrations

Active ATG, defined as the fraction of ATG capable of binding to human lymphocytes, was measured in a flow-cytometry based assay².

The samples collected in adult patients were measured in the laboratory for translational immunology in the University Medical Center Utrecht (UMCU). In this assay, the EDTA plasma was filtered and added in different dilutions to Jurkat cell suspensions (ATCC) in 96-well plates (100.000 cells/well). Dilutions of ATG were used to prepare a standard curve and allow quantitative measurements. After 30 min incubation cells were washed and incubated with biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch) and subsequently with streptavidin conjugated to phycoerythrin (BD Biosciences). Acquisition was performed on a FACS CANTO and results were analyzed with BD FACSDiva software (version 8).

The assay used in the previously described population were performed in the pediatric immunology laboratory of the Leiden University Medical Center (LUMC) in the Netherlands, has been previously published^{1,3}. In short, HUT-78 T cells (PHACC, Porton Down, UK) were incubated with patient serum, followed by incubation with goat anti-rabbit IgG labeled with Alexa Fluor 647 (Biosource, Life Invitrogen, Carlsbad, CA, USA). Standards were made by serially diluting ATG in triplicate in a range from 5 to 0.005 AU/ml. Active ATG is expressed in arbitrary units, 1 µg/mL is arbitrarily set to 1 AU/mL. After washing, cells were analyzed by flow cytometry on a FACS-scan (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA).

Population pharmacokinetic analysis

The population approach was used for pharmacokinetic analyses to allow for analysis of sparse and unbalanced data (differing samples times and number of samples per patient). For this purpose, the non-linear mixed effects software package NONMEM version 7.2.0 (Icon Hanover, MD, USA) was used, with Pirana 2.8.2 and R 2.3.4 for workflow management and data visualization, respectively^{4,5}. Within NONMEM, the first order conditional estimation option with interaction was used (FOCE-I). Active ATG concentrations were logarithmically transformed and simultaneously fitted. Concentrations below the limit of quantification for the respective assays (which were only found in the tail end of the concentration-time curve), were set at half the limit of quantification, with any subsequent measurements below the limit of quantification being removed according to method M6⁶.

For the structural model one, two and three compartment models were tested. In addition, as antibodies are mostly eliminated by target binding or protein degradation⁷ with the former being responsible for the pharmacological effects, also non-linear clearance parameters being dependent on the concentration substrate available were tested^{8,9}. Non-linear elimination pathways were explored by incorporating clearance described by Michaelis-Menten kinetics.

Inter-occasion variability was tested for the subsequent doses on all parameters involved in elimination to detect potential time-dependency.

Several criteria applied in the development of the structural and statistical pharmacokinetic model. First, the inclusion of a parameter had to result in a significant improvement of the model fit. This was evaluated using the objective function value (OFV), the sum of all squared differences between observed and predicted values, which is assumed to follow a chi-squared distribution. Lower numbers of OFV represent a better fit of the model; a decrease of 3.84 points in OFV corresponds to a p-value of <0.05. In addition to a significant decline in OFV, the goodness-of-fit plots, which are used to detect any model misspecification,

needed to improve. In these plots, observed concentrations are plotted against individual predictions and population predictions, and conditional weighted residuals (CWRES) is plotted against time and observed concentrations. Finally, the residual standard errors of the parameters and shrinkage of interindividual variability were assessed¹⁰.

Inter-individual variability was assumed to follow a log-normal distribution and was therefore implemented into the model as:

$$P_i = P_{pop} * e^{\eta_i} \quad (\text{Eq. 1})$$

where P_i depicts the individual or post-hoc value of the parameter in the i th individual, P_{pop} the population mean for the parameter, and η_i the inter-individual variability of the i th person, sampled from a normal distribution with a mean of 0 and a variance of ω^2 .

The residual variability was described using a proportional residual error models. Due to the logarithmical transformation of the data, residual error will be inserted into the model as an additive error, however this should be read as a proportional error:

$$Y_{i,j} = C_{pred,i,j} + \varepsilon \quad (\text{Eq. 2})$$

where $Y_{i,j}$ is the observed concentration, $C_{pred,i,j}$ the j th predicted concentration for individual i , and ε the error, sampled from a normal distribution with a mean of 0 and a variance of σ^2 .

Covariate selection

Following development of the structural and statistical pharmacokinetic model, potential predictors (covariates) for pharmacokinetic parameters such as clearance were evaluated. Potential covariates included patients related (body weight, lean body weight (LBW), ideal bodyweight (iBW), body surface area, age, lymphocyte counts, underlying disease), donor related (HLA match, stem cell source) and treatment related (conditioning regimen) and laboratory factors (assay used). Lymphocyte counts were assessed as a potential covariate because it harbors most targets for ATG, and therefore may influence ATG clearance¹. As absolute lymphocyte counts rapidly drop after the first dose of ATG, only samples before the first infusion (-72h – 0h before first infusion) were used. Lean body weight (LBW) was calculated using the formulas developed by Janmahasatian et al. (adults) and Peters et al (children)^{11,12}. IBW was calculated based on actual length and the ideal BMI of 22.5 for adults, while for children, the 50th percentile for the growth charts was used.

To investigate to evaluate the relationship between a potential covariate and a pharmacokinetic parameter such as clearance, potential covariates were plotted against individual predictions of the parameter involved (posthoc values), inter-individual variability, and conditional weighted residuals, both before and after inclusion of a covariate. Continuous covariates such as body weight and lymphocyte counts were tested in a linear and power function (equations 4 and 5):

$$P_i = P_{pop} * (1 + \left(\frac{Cov_i}{Cov_{median}}\right) * l) \quad (\text{Eq. 4})$$

$$P_i = P_{pop} * \left(\frac{Cov_i}{Cov_{median}}\right)^k \quad (\text{Eq. 5})$$

where P_i and Cov_i are the PK-parameter and covariate value for the i th individual, P_{pop} the population mean for the parameter for a patient with a median value for the covariate in the population, and Cov_{median} the median value for the covariate in the population. In the linear relationship equation (eq. 5) l is a fraction of the population value representing the increase of the parameter with the covariate, while in the power-relationship equation (eq. 6) k is the scaling factor. Additionally, more complex variations of equation 5 were explored for body weight as covariate, including a previously published bodyweight-dependent exponent (BDE) for k ^{13,14}:

$$k = a * BW^b \quad (\text{Eq. 6})$$

where k is the scaling factor in equation 5, a is the coefficient and b the exponent. Using a BDE, the exponent k can vary with bodyweight, leading to a sigmoidal relation between bodyweight and the parameter^{13,14}.

Improvement of the model after inclusion of a covariate was evaluated for significance using forward inclusion and backward elimination¹⁰. A significance level of $p < 0.005$ (-7.9 points in OFV) was used for the forward inclusion, and $p < 0.001$ (-10.8 points in OFV) for backward elimination. In other respects, building of the covariate model was comparable to the development of the structural PK-model, with specific emphasis on goodness of fit plots split for age to assure that the model predicts equally well in different age groups. In addition, inclusion of a covariate had to result in a decline in unexplained inter-individual variability and plots for interindividual variability versus the covariate involved needed to improve¹⁰.

Model Evaluation Techniques

As the developed model is used as the basis for dosing in future patients, the model was extensively evaluated for the robustness. Several validation techniques are performed, all in accordance with EMEA and FDA guidelines for population pharmacokinetic analyses^{15,16}. To assess the predictive power and accuracy of the model, a bootstrap analysis was performed. In this validation technique, 1000 replicates of the dataset were created using random sampling with replacement, and the model was fit using each dataset. For each parameter, median and 95% confidence intervals were compared to the parameter estimate and uncertainty in the final model.

In addition, a normalized prediction distribution of errors (NPDE) was performed. The prediction discrepancies between the final model and 1000 simulations of the model were evaluated, taking into account the correlation between observations in the same individual and the predictive distribution¹⁷.

Finally, prediction-corrected visual predictive checks (VPC) were created to assess the predictive performance of the final model as compared to the measured concentrations. The prediction-corrected VPC can handle a large variability in absolute dosing, as is the case in the dataset. In this analysis, the observed concentration data and its median and 95% confidence intervals was compared to the median and 95% confidence intervals of 1000 simulations of the model.

SUPPLEMENTAL RESULTS

Patients and Samples for Pharmacokinetic Analyses

A total of 227 adult patients were included receiving 242 HCT's. Median age was 50 years, all patients received reduced intensity conditioning and the dose of ATG was 7.5-9 mg/kg over 4 days starting at day 7 (range 4-12). A total of 1964 ATG concentrations samples (Figure S1) were available, with a median of 8 samples per patient.

The pediatric cohort consisted of 267 children and young adults receiving 280 HCT's¹. Median age was 6.5 years of age (range 0.2-23 year) and 74% received myeloablative conditioning. Most (83%) patients received a dose of 10 mg/kg over 4 days starting at day -5 before HCT. A total number of 3113 ATG concentration samples were available, mean number of samples per patient was 11¹. The combined cohort existed of 494 adult and pediatric patients (Table S1).

Structural Pharmacokinetic Model

As testing for the best structural model was complicated by the broad range of body sizes resulting in unstable models, body weight was included as a covariate using a power function (equation 5, single exponent) on both clearance and volume of distribution during this phase of model development. Compared to a one-compartment model, the two-compartment model was superior in terms of goodness-of-fit plots and OFV (decrease of 889 points, 4 additional parameters, $p < 0.0001$). A three-compartment model was unstable, giving inaccurate parameter estimates and being highly dependent on initial values. A proportional error model was used. Next, non-linear elimination pathways were explored. Inclusion of a saturable elimination pathway next to the linear elimination resulted in a decline in OFV of 191 points (4 additional parameters, $p < 0.0001$) and improvement in GOF, especially in the lower concentration range. A model with only saturable clearance was unstable. In line with the PK-model in children, in the adult population was also a discrepancy noted in CWRES vs time. To address this under-prediction of concentrations during and shortly after the final infusions of ATG, saturable distribution towards the peripheral compartment was included in the model. This was parameterized using Michaelis-Menten kinetics with the parameters $T_{\max}$ (maximum rate of transport) and T_m (concentration at half maximum rate; Figure S2). Inclusion of saturable intercompartmental transport improved the CWRES vs time, and yielded a decrease of 342 points (2 parameters, $p < 0.0001$; Figure S4). Details of the final structural and statistical model are presented in Figure S2, Figure S3 and Table S2.

Covariate Model

The influence of body weight on clearance and central volume of distribution was further tested using the evaluation of different parameterizations for the influence of bodyweight (see equation 5 and 6) and with the use of different bodyweight metrics such as LBW and iBW. Of these covariates and functions, the use of a Bodyweight Dependent Exponent for k (equation 6) as covariate function for bodyweight for clearance resulted in the best results. Compared to the model with a single exponent k (equation 5), a decrease in OFV of 19 points was noted ($p < 0.005$ considering 1 additional degree of freedom) (Table S2). Of the other covariates tested, lymphocyte counts were found to impact linear clearance with an increase in the absolute lymphocyte count before the first dose of ATG yielding an increase in clearance (decrease in OFV of 37 points ($p < 0.0001$)). No other covariates were identified on any other parameter ($P > 0.05$). Figure S5 shows how clearance changes with bodyweight given different baseline lymphocyte counts for the final model. The figure illustrates that clearance does not change with bodyweight in the adult weight range (> 50 kg) while the influence of baseline lymphocyte counts on clearance are substantial across the population.

After inclusion of body weight and lymphocyte counts, no trends could be observed in the plots of inter-individual variability on clearance and volume of distribution versus the

included covariates (Fig S6, panels a-c). In addition, after correcting for body weight in the model, no effect of age could be identified on any of the PK-parameters (Figure S6, panels d-f). The parameter estimates of the final (covariate) model are included in Table S2.

Model Validation

The final model with body weight and lymphocyte counts before the first dose of ATG was stable in a bootstrap analysis, with 96.1% of runs being successful. Bootstrap medians as well as the 95% confidence interval were well in line with the model estimates and residual standard errors (Table S2). The prediction corrected VPC shows the median and 95% confidence interval of the observed data to follow the predictions of the model (Figure S7). The NPDE-analysis showed a normal distribution, and no trends were observed in the NPDE versus time or predictions (Figure S8).

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SUPPLEMENTAL TABLES

	Adults	Children and young adults ¹	Total
Number of patients (n)	227	267	494
Number of HCTs (n)	242	280	522
Male sex (%)	60	62	61
Age (years)	50 (17.6-70)	6.5 (0.2-23)	17 (0.2-70)
Weight (kg)	74 (43-123)	21 (3.7-96)	55 (3.7-123)
BSA (m2)	1.91 (1.34-2.56)	0.83 (0.14-2.1)	1.55 (0.14-2.56)
Number of samples [n (mean per patient)]	1964 (8)	3113 (11)	5077 (10)
Starting day ATG (days before transplantation)	7 (4-12)	5 (1-19)	7 (1-19)
Lymphocyte count before conditioning (x 10 ⁹)	0.72 (0-10.9)	0.29 (0-10.4)	0.64 (0-10.9)
Cumulative dose (mg/kg)			
<7.5 mg/kg	22	3	12
7.5-9 mg/kg	78	4	38
9-11 mg/kg	0	83	45
>11 mg/kg	0	10	5
Diagnosis (%)			
Malignancy	0	47	72
Immune deficiency	0	19	11
Bone marrow failure	0	6	3
Metabolic disease	0	9	5
Benign hematology	0	18	9
Auto-immune disease	0	1	0
Stem cell source (%)			
Bone marrow	13	49	32
Peripheral blood stem cells	86	15	47
Cordblood	1	34	19
Cordblood plus haplo or 2nd cordblood	0	2	2
Conditioning regimen (%)			
Reduced intensity	55	4	27
Chemotherapy-based	17	74	48
TBI-based	28	22	25

Shown as median (range) unless otherwise specified

Table S1. Patient Characteristics in Pharmacokinetic Analysis

	Dataset [estimate (RSE)]	Shrinkage	1000 bootstrap replicates (96.1% successful)	
			Median	95% confidence interval
Structural model				
$CL_i = CL_{pop} * \left(\frac{WT}{WT_{med}}\right)^{a*WT^b} * \left(1 + \frac{BL}{BL_{med}} * m\right)$				
CL _{pop} (L/day)	2.0 (9%)		2	1.6-2.5
<i>a</i>	0.16 (18%)		0.16	0.1-0.28
<i>b</i>	-1.1 (28%)		-1.1	-2.4--0.5
<i>m</i>	0.32 (26%)		0.33	0.18-0.53
$V_{1,i} = V_{1,pop} * \left(\frac{WT}{WT_{med}}\right)^l$				
V _{1,pop} (L)	16.6 (13%)		16.6	13.2-29.7
<i>l</i>	0.86 (18%)		0.87	0.62-1.43
K _{21,pop} (L-1)	0.67 (19%)		0.68	0.45-1.6
T _{max,pop} (AU/day)	187 (15%)		187	101-266
T _{m,pop} (AU/L)	9.5 (16%)		9.4	5.3-12.5
V _{max,pop} (mg/day)	4.7 (18%)		4.7	2.3-6.6
K _{m,pop} (mg/L)	2.3 (19%)		2.2	1.2-3.3
Random variability				
Inter-individual variability on CL (%)	72 (4%)	15	72	65-79
Inter-individual variability on V1 (%)	57 (11%)	39	57	45-74
Inter-individual variability on T _m (%)	150 (5%)	27	150	118-164
Inter-individual variability on V _{max} (%)	67 (12%)	56	67	50-90
Inter-individual variability on K _m (%)	184 (8%)	38	181	143-219
Proportional residual error (%)	32 (5%)	15	31	28-35

Table S2. Pharmacokinetic Parameter Estimates and Bootstrap Analysis. RSE: residual standard error; CL: clearance; WT: body weight; WT_{med}: median bodyweight of 55 kg; BL: baseline lymphocyte count before first dose of ATG; BL_{med}: median baseline lymphocyte count (0.64 x 10⁹/L); V₁: central volume of distribution; K₂₁: distribution constant towards the central compartment; T_{max}: Maximum rate of distribution towards the peripheral compartment; AU: arbitrary units; T_m: Michaelis-Menten constant of distribution towards the peripheral compartment; V_{max}: Maximum rate of non-linear elimination; K_m: Michaelis-Menten constant of non-linear elimination.

Predictor	AIC
AUC after HCT	507.75
Concentration at time of HCT	508.48
Total AUC	509.02
Time to reach Concentrations < 1 AU/mL	509.88
Maximum Concentration	510.18
AUC before HCT	510.64

Table S3. Akaike Information Criterion (AIC) for all PK-predictors

SUPPLEMENTAL FIGURES

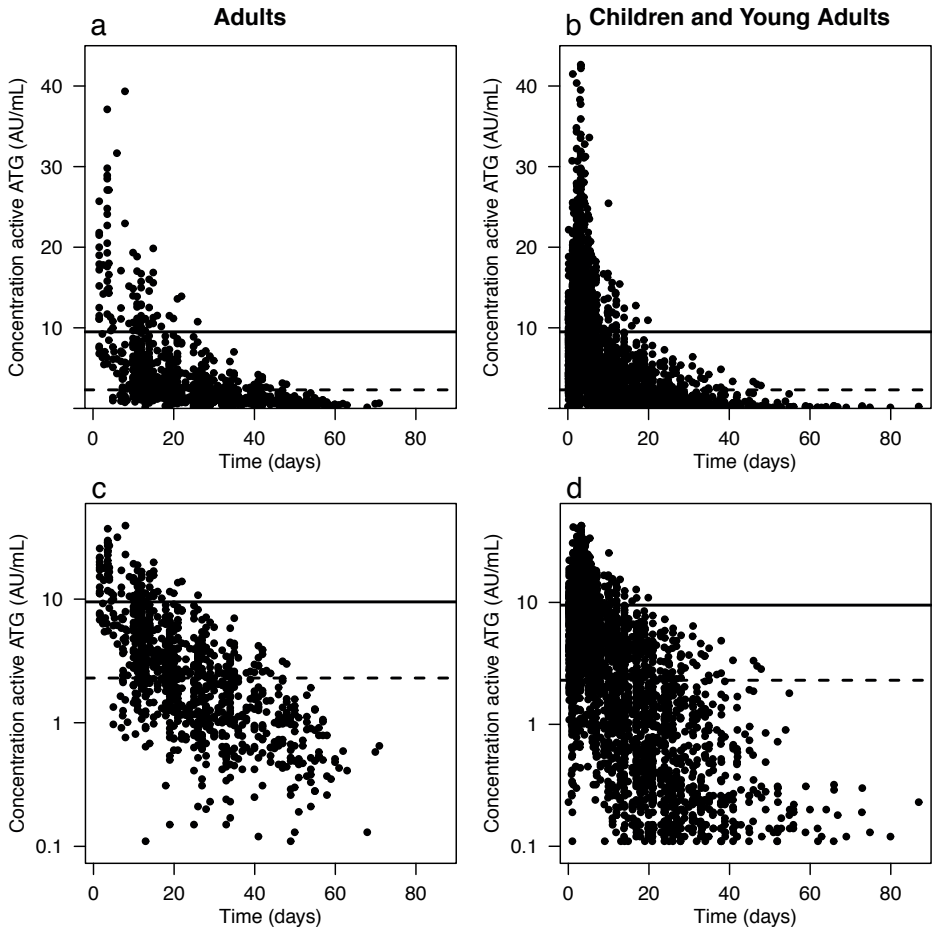


Figure S1. Available active ATG concentration data. Data on linear scale (upper panels) and logarithmic scale (lower panels) over time for adult (left) and children and young adults (right) cohorts. Solid lines: T_m , dashed lines: K_m .

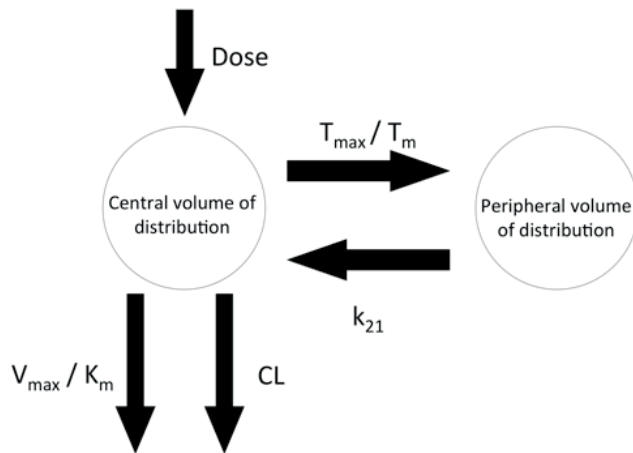


Figure S2. Overview of the final pharmacokinetic model. CL: clearance; $V_{\max}$: Maximum rate of non-linear elimination; K_m : Michaelis Menten constant of non-linear elimination; $T_{\max}$: Maximum rate of non-linear distribution; T_m : Michaelis Menten constant of non-linear distribution; k_{21} : distribution constant towards central compartment.

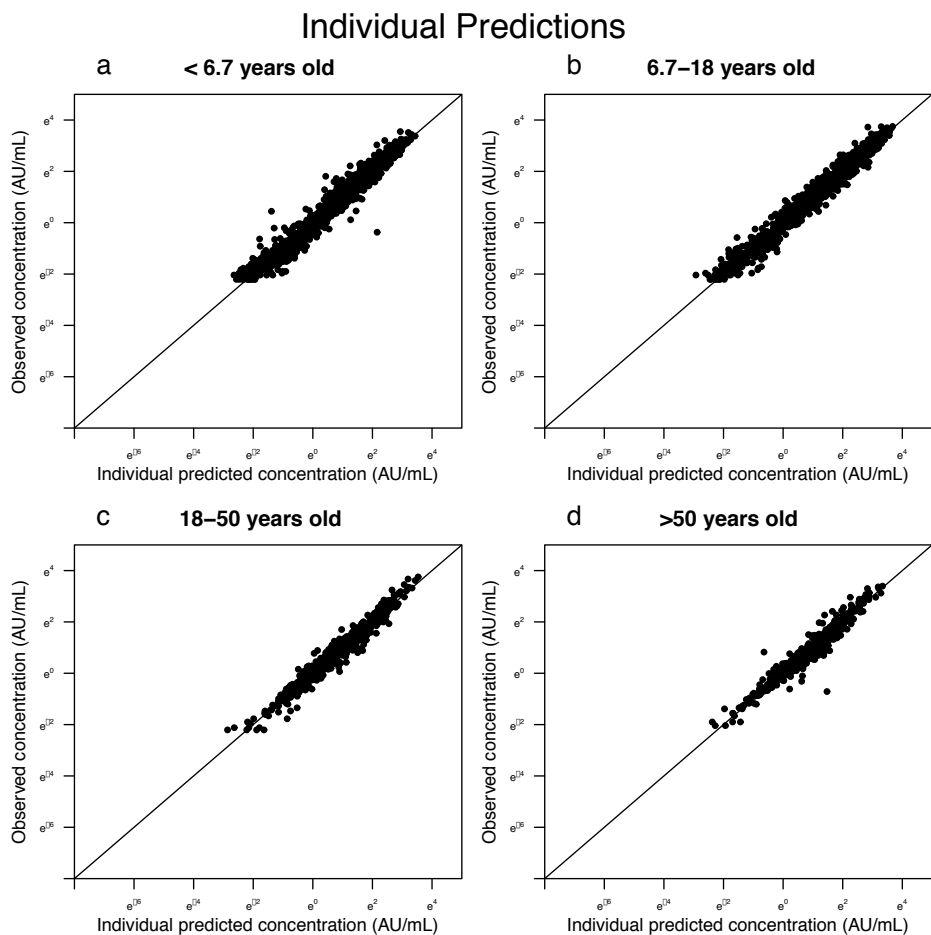


Figure S3.

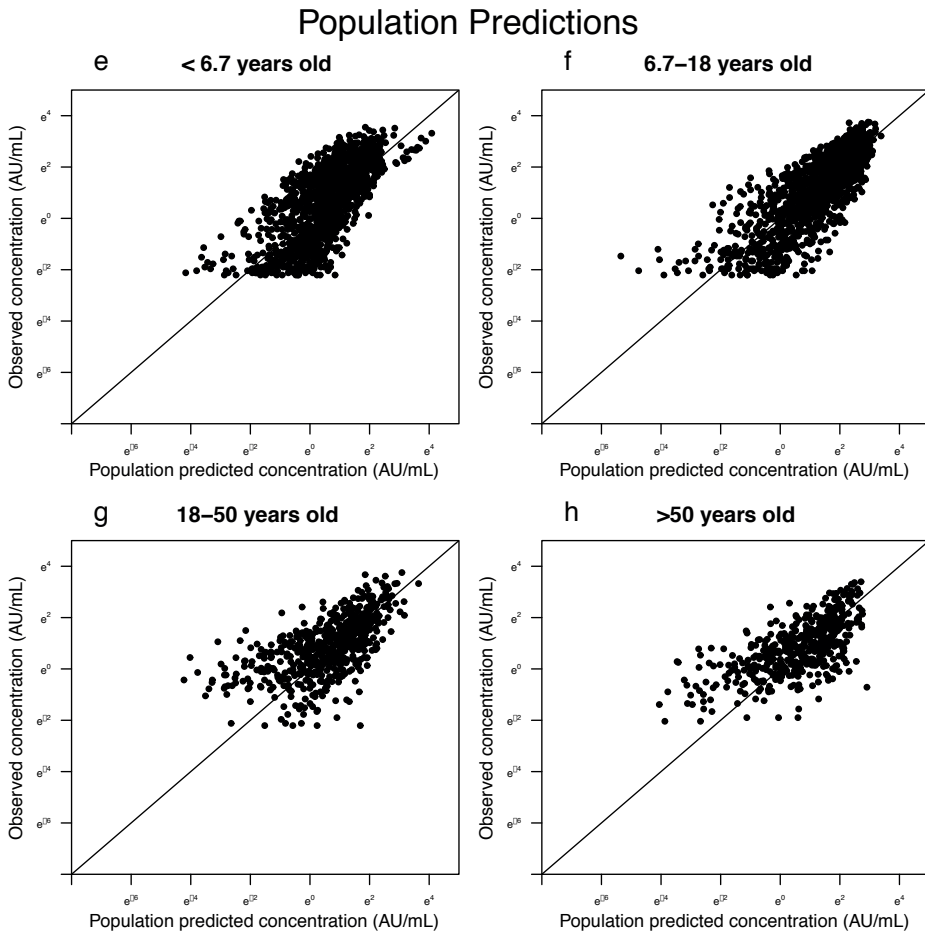


Figure S3. Goodness-of-Fit plots of final model. Individual predictions (panels A-D) and population predictions (panels e-h) for patients < 6.7 years (panels A and E), 6.7-18 years (panels B and F), 18- 50 years (panels C and G) and >50 years (panels D and H). Lines: lines of unity ($x=y$)

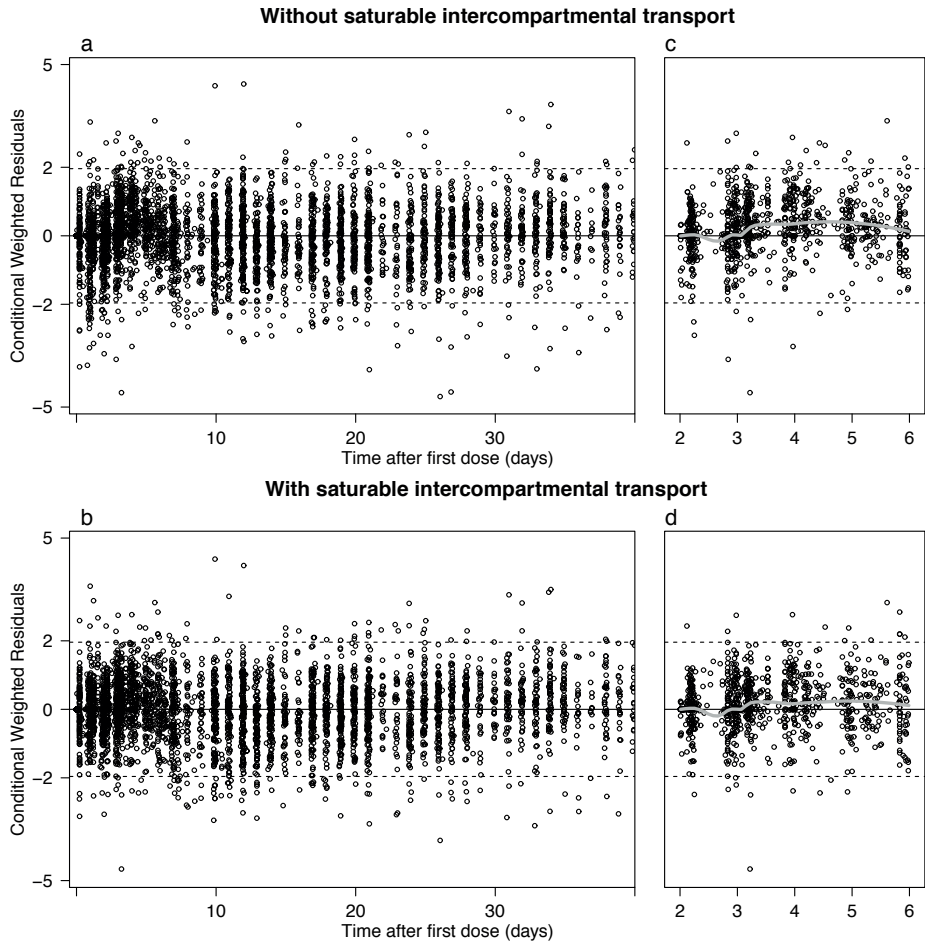


Figure S4. Saturable Intercompartmental Transport. A trend in conditional weighted residuals (CWRES) versus time can be seen before introduction of saturable intercompartmental transport (a+b), which is accounted for after introduction (c+d). a+c: All data; b+d: Zoomed in to 2–6 days. Dots show the CWRES per concentration sample, the solid line shows where CWRES = 0, the dashed lines indicate ± 2 standard deviations, and the curved lines show spline regression

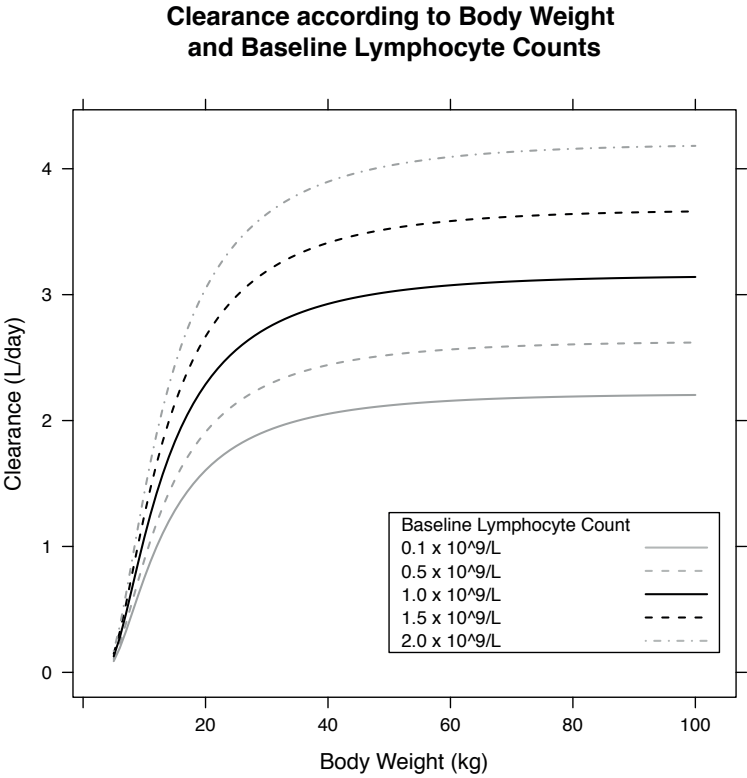


Figure S5. Impact of Body Weight and Lymphocyte Counts on Clearance

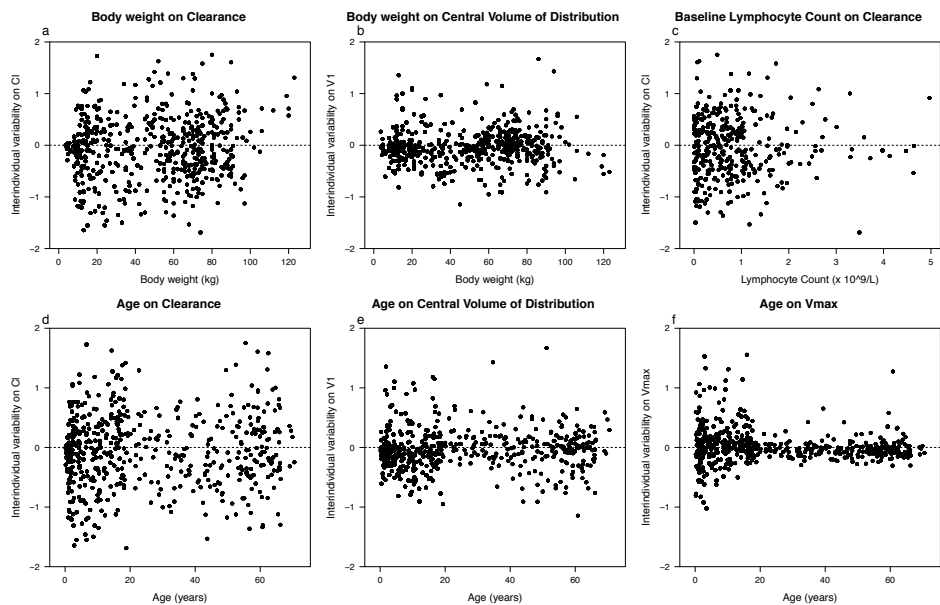


Figure S6. Inter-individual variability versus covariates in final model. Lines: inter-individual variability=0.

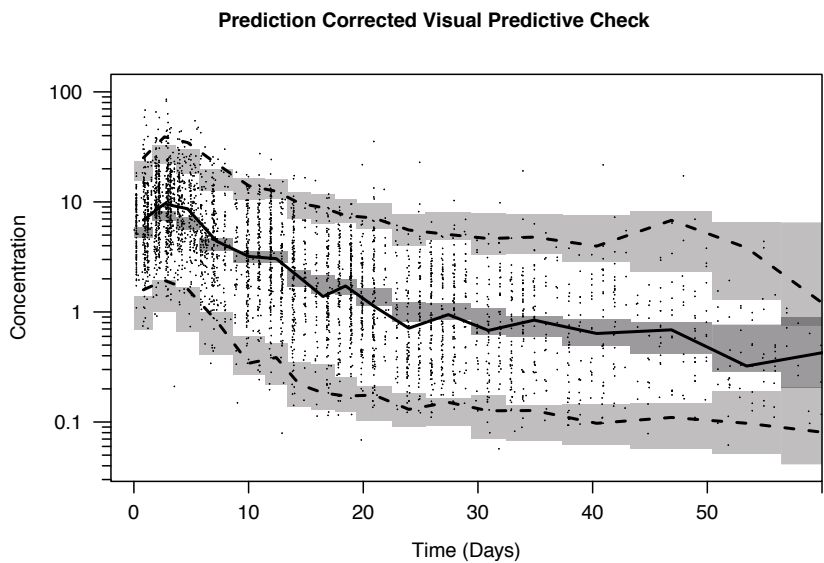


Figure S7. Correction-predicted Visual Predictive Check. Dots: actual concentration data. Solid line: median concentration over time; dashed lines: 2.5 and 97.5% quartiles of concentration of time. Dark grey bars: 95% confidence interval (CI) of median predictions; light grey bars: 95% CI of 2.5 and 97.5% predictions.

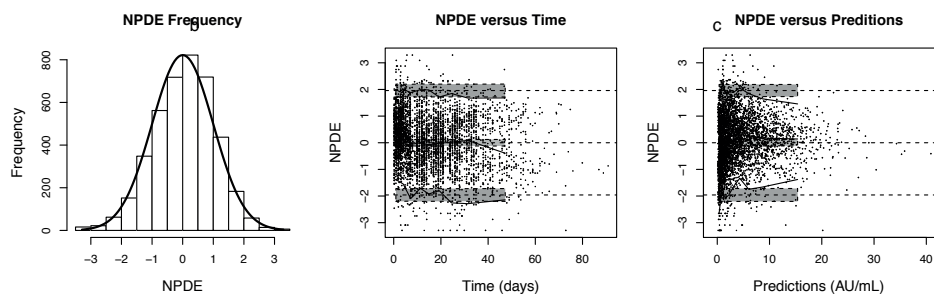


Figure S8. NPDE validation. Panel a: histogram of the NPDE with the solid line representing a normal distribution with a mean of 0 and variance of 1. Panel b: NPDE versus observations, panel c: NPDE versus predictions. Grey blocks: 95% confidence interval of NPDE.

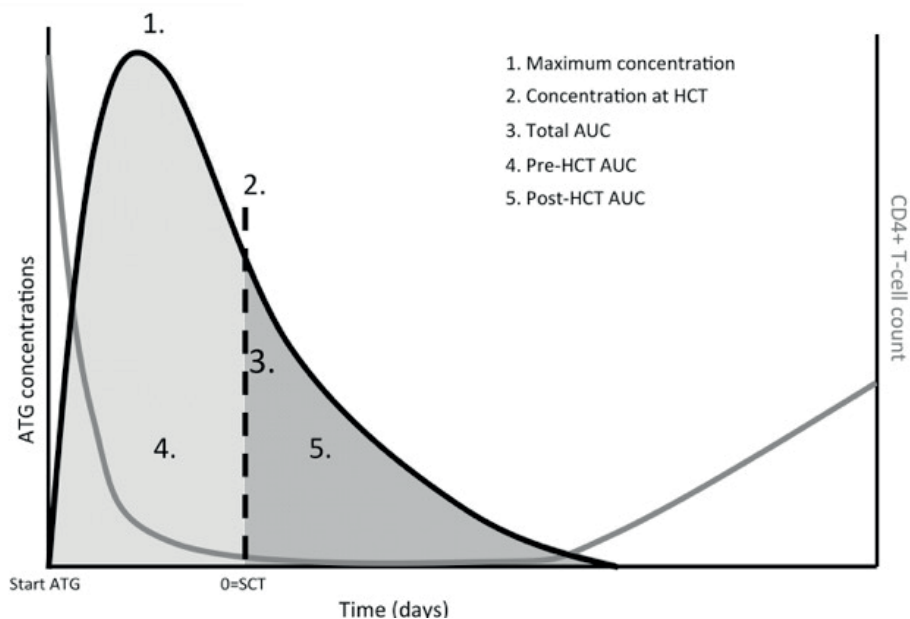


Figure S9. Overview of PK exposure measures