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Association between Busulfan Exposure and Outcome in Children Receiving Intravenous Busulfan before Hematologic Stem Cell Transplantation

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Busulfan, combined with therapeutic drug monitoring–guided dosing, is associated with higher event-free survival (EFS) rates due to fewer graft failures/relapses and lower toxicity. The optimal target area under the curve (AUC) and dosing schedule of intravenous busulfan in children undergoing hematopoietic stem cell transplantation (HSCT) remain unclear, however. We conducted a retrospective analysis of the association between busulfan exposure and clinical outcome in 102 children age 0.2 to 21 years who received busulfan 1 or 4 times daily before undergoing HSCT (46 malignant and 56 nonmalignant indications). EFS and overall survival after a median of 2 years of follow-up were 68% and 72%, respectively. EFS was optimal when the exposure of busulfan (AUC) was 78 mg*h/L (95% confidence interval = 74 to 82 mg*h/L). Acute graft-versus-host disease (aGVHD) grade II–IV occurred more frequently with greater busulfan exposure. The addition of melphalan was an independent risk factor; melphalan use combined with high busulfan exposure (AUC > 74 mg*h/L) was associated with high incidences of aGVHD (58%), veno-occlusive disease (66%), and mucositis grade III-IV (26%). Dosing frequency (1 or 4 times daily) was not related to any outcome. In conclusion, dose targeting of busulfan to a narrow therapeutic range was found to increase EFS in children. Adding melphalan to optimal busulfan exposure is associated with a high incidence of toxicity.

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INTRODUCTION

Busulfan is widely used in preparative chemotherapy-based regimens, being an alternative for total body irradiation in patients undergoing hematopoietic stem cell transplantation (HSCT) for malignant and nonmalignant diseases [1–3]. Until recently, busulfan was available only in oral form and was administered 4 times daily. The therapeutic potential of the oral drug is current under debate, due to the highly variable intraindividual and interindividual and unpredictable systemic exposure related to its pharmacokinetic properties, especially in children [4–7]. To reduce the variability of busulfan exposure, intravenous (i.v.) formulations of busulfan have been developed recently. Despite these formulations, however, considerable interindividual variability in busulfan exposure has persisted in adults and, to an even greater degree, in children [8–16].

An advantage of the i.v. formulation of busulfan is the reduced number of daily doses compared with the oral preparation. The latter must be administered every 6 hours, mainly because only 2-mg tablets are available. In contrast, i.v. busulfan can be given once a day, increasing convenience for both patients and caregivers. In addition, it might be postulated that once-a-day dosing may reduce toxicity, because the patient is not continually exposed to busulfan during the day, given the drug's short half-life (approximately 2 hours in children). This extended period between doses might allow recovery of glutathione-S-reductase

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and glutathione-S-transferase (GST), the enzymes responsible for the metabolism of busulfan, which might reduce toxicity [17–19].

The therapeutic window of busulfan is narrow; a high exposure (expressed as the area under the curve [AUC]) has been associated with increased risk of toxicities, such as mucositis, acute graft-versus-host disease (aGVHD) and veno-occlusive disease (VOD) [2,20-22]. In contrast, low exposure to busulfan has been associated with increased incidence of graft rejection and relapse [23,24]. Dose targeting of oral busulfan based on therapeutic drug monitoring (TDM) has improved clinical outcomes in pediatric HSCT recipients [3,7,25,26]. However, the optimal dose, optimal AUC target, and dosing schedule of i.v. busulfan in children remain undecided [16,27].

We conducted a retrospective analysis of a cohort of pediatric patients who underwent HSCT and had a wide range of exposure to busulfan. The aim was to establish the optimal busulfan exposure in relation to efficacy and toxicity.

METHODS

Setting and Study Population

All pediatric patients who underwent transplantation between August 2000 and March 2007 in the HSCT units of the Leiden University Medical Center (LUMC) and the University Medical Center Utrecht (UMCU) receiving i.v. busulfan–based myeloablative conditioning combined with TDM were included in this study. All data were collected prospectively. Patients were enrolled in the HSCT and research protocol after providing written informed consent and receiving institutional ethical committee approval.

Transplantation Details, Conditioning Regimens, and Supportive Care

HLA-matching was based on high-resolution (HR) typing for class I and class II (10 alleles) for family and unrelated bone marrow (BM) or peripheral blood stem cell (PBSC) donors. For cord blood (CB) donors, intermediate resolution criteria were used (low resolution for loci HLA-A, -B, and -DRB1 by HR typing). A HLA-DPB1 mismatch was not taken into account. For the analyses, the patients were divided into a matched group and a mismatched group, with 1 or more allele or antigen mismatches defined as a mismatch. CB grafts were considered matched (6 antigens) when they were identical according to the aforementioned intermediate resolution criteria.

All patients received conditioning according to applicable international and national protocols. Busulfan (Busilvex; Pierre Fabre Medicament, Boulogne, France) was given as the first-line agent, followed by cyclophosphamide after at least 24 hours. Based on these protocols, melphalan, fludarabine, or etoposide also was given to some patients. In general, a combination of busulfan (Bu), cyclophosphamide (Cy), and melphalan (Mel) was used in patients with myeloid malignancies, myelodysplastic syndrome (MDS), and acute lymphoblastic leukemia (ALL), as well as in some patients with thalassemia or hemoglobinopathy. Bu and Cy, combined with fludarabine (Flu) in some cases, was administered for nonmalignant indications; Bu, Cy, and etoposide (VP16) was administered in patients with hemophagocytic lymphohistiocytosis (HLH), as well as some younger patients (age < 3 years) with ALL.

All patients were cared for in high-efficiency, particle-free, air-filtered, positive-pressure isolation rooms. Unrelated donors received serotherapy (either antithymoglobulin [ATG]-rabbit or alemtuzumab [both from Genzyme,]). GVHD prophylaxis consisted of cyclosporine (Sandimmune iv, Neoral po; Novartis Pharma BV). Methotrexate (10 mg/m^2 on days +1, +3, and +6) was added to the recipients of non–T cell-depleted grafts, and recipients of an unrelated CB donor graft also received prednisone (1 mg/kg) until day +28 (tapered in 2 weeks in patients without aGVHD). Patients who received CB were treated with filgrastim (Neupogen; Amgen Europe BV,) from day +7 until a neutrophil level $> 2000/\mu L$ was achieved.

Gut decontamination and infection prophylaxis was given according to the institutional protocol. No VOD prophylaxis was given. VOD was treated with defibrotide, diuretics, and fluid restriction. Patients received standard antiemetic drugs (ondansetron) and prophylactic anticonvulsive therapy (clonazepam) during busulfan therapy. The conditioning regimen remained fixed throughout the study period, except for the busulfan dosing regimen. From 2003 onward, the frequency of CB transplantations increased, with T cell depletion performed less often. The medical and nursing staff's familiarity with CB transplantation increased during the study period.

Intravenous Busulfan Regimen and Therapeutic Drug Monitoring

Busulfan was administered intravenously over a 4-day period. The dose and target AUC of busulfan were modified 3 times during the study period (as shown in Table 2), producing 3 groups of patients with different dosing schedules. The first group of patients received busulfan 4 times a day for 4 consecutive days. The starting dose was 1.0 mg/kg for patients under age 4 years and 0.8 mg/kg for those age 4 years and older, similar to the adult dose. Dose adjustment was allowed to a limit of 1.0 mg/kg every 6 hours if the target AUC of 4.93 mg hr/L was not achieved [28]. The second group received a once-daily busulfan regimen. The starting dose was 80 mg/m^2 and subsequent doses

were targeted at an AUC of 15.6 mg hr/L [29]. The third group received an initial dose of 120 mg/m^2 (age $\geq$ 1 year) or 80 mg/m^2 (age $<$ 1 year) [30]. These patients were targeted to an AUC of 17.5 mg*h/L. Modifications to the regimen were introduced based on experience and after an initial analysis and publication of data [28-30]. The current study included both those patients reported previously and those treated more recently with i.v. busulfan.

Busulfan concentrations were analyzed by high-pressure liquid chromatography, based on the method described by Cremers et al. [10] and Zwaveling et al. [28]. Calculation of the AUC was based on at least 3 blood samples (obtained 1, 2, and 3 or 4 hours after the end of infusion) on day +1, using a single-compartment model with linear pharmacokinetics established by Cremers and coworkers [10,28,30]. Empirical Bayesian pharmacokinetic parameter estimates (clearance and volume of distribution) were estimated using the pharmacokinetic software package MwPharm [31]. Busulfan dose was adjusted only when the AUC differed by $>$ 10% from the target AUC. If possible, an evaluation of the AUC after dose adjustment was performed on a subsequent day and was used to calculate the total busulfan exposure.

Busulfan exposure (ie, total AUC over 4 days) in all patients was calculated by the sum of the daily (extrapolated) AUC measurements. If 2 AUC measurements were performed, then the AUC after dose adjustment was extrapolated to the other 2 days on which the same dose was used: $AUC_1 + AUC_2 \times 3$. If the AUC after dose adjustment was not measured, then the results of day +1 were extrapolated to days +2, +3, and +4: $AUC_1 + (AUC_1 \times$ new dose/old dose) $\times 3$. If no dose adjustment was performed and the second AUC differed from the first AUC, then both AUCs were extrapolated to the other days on which no blood samples were measured ($AUC_1 \times 2 + AUC_2 \times 2$).

Endpoints

The main study endpoints were event-free survival (EFS) and overall survival (OS) after HSCT, with a follow up of at least 6 months. EFS was defined as alive with engraftment ($>$ 95% chimerism) and without evident relapse in those children who underwent transplantation for malignant disease.

Toxicity endpoints were VOD, moderate/severe ($>$ grade I) aGVHD, severe ($>$ grade II) mucositis, and acute lung toxicity (ie, bronchiolitis obliterans or idiopathic pneumonia syndrome). VOD was diagnosed according to the modified Seattle criteria [30]. Severity of VOD was graded according to the system of Bearman [32]. Baseline VOD risk was defined according to criteria described in a VOD defibrotide prophylaxis study [33]. aGVHD was diagnosed and graded according to the criteria of Glucksberg et al. [34], and oral mucositis was evaluated and scored based on World Health Organization criteria [35]. Because melphalan was used as a second alkylating agent in the conditioning regimen in many patients (42%; Table 1), we studied the inclusion of melphalan as an additional risk factor for toxicity and main endpoints. Chimerism $>$ 95% was considered full donor chimerism; donor chimerism $>$ 10% and $<$ 95% was considered mixed chimerism.

Statistical Analysis

The association between busulfan exposure and the endpoints (EFS, OS, toxicity) were analyzed using univariate and multivariate Cox proportional hazards regression models, stratified by clinical center (UMCU or LUMC), using SPSS version 12.1 (SPSS Inc, Chicago, IL). Because of the different dosing regimens, patients receiving a broad range of doses and having widely varying busulfan exposures were available. To perform covariate analysis, the patients were separated into 5 equal groups (quintiles), each group comprising 20 patients, based on total AUC of busulfan. Univariate predictors of outcome that were statistically significant (P value $<$ .05) were selected for multivariate analysis. Results are expressed as estimates of hazard ratios (HRs) and corresponding 95% confidence intervals (95% CIs). Additional analyses were performed to study the optimal busulfan exposure. In these analyses, the total AUC of busulfan was modeled as a continuous variable in a multivariate Cox regression model. The optimum was estimated for the outcome parameter EFS, using a proportional hazards model of regression in R version 2.6.1. The 95% CI of the optimum was constructed using the delta method with R library "alr3."

RESULTS

Patient Characteristics

Between August 2000 and March 2007, 106 patients received conditioning containing busulfan in the 2 Dutch pediatric HSCT units. Four patients who received a reduced-intensity busulfan regimen were excluded from the analysis; thus, 102 patients (46 with malignant disease and 56 with nonmalignant disease) patients were analyzed in this study (Table 1).

The patients in the 5 groups defined by AUC were comparable for all variables except dosing regimen and center of inclusion (Table 1). The 4-times-daily dose was not evenly distributed among the quintiles, because dosing was maximized in the 4-times-daily regimen, generally resulting in relatively low exposure (median, 56 mg*h/L in the patients receiving the 4-times-daily regimen and 72 mg*h/L in those receiving

Table 1. Patient Characteristics (n = 102)

	Distribution over 5 AUC groups*	
		P value
Follow-up, weeks, median (range)	102.2 (0.3 to 364)	.229
Age, years, median (range)	3.1 (0.2 to 21.0)	.520
Busulfan dosing regimen, n		
Once daily	64	<.001
4 times daily	38	
Sex, n		
Male	57	.763
Female	45	
Diagnosis, n		
Malignant†	46	.993
Nonmalignant‡	56	
Pretreatment, n§		
None	58	.063
Chemotherapy, low intensity	14	
Chemotherapy, high intensity	30	
Conditioning, n¶		
Bu/Cy/Mel	43	.851
Other Bu-based	59	
Serotherapy, n		
No	19	.778
Yes	83	
Stem cell source, n		
BM	60	.563
CB‖	27	
PBSCs	15	
Donor, n**		
Matched	57	.507
Mismatched	45	
T cell depletion, n††		
No	79	.182
Yes	23	
VOD risk, n‡‡		
No	40	.966
Yes	64	
Center, n§§		
LUMC	71	<.001
UMCU	31	
Busulfan total AUC, mg*h/L, median (range)	69.6 (30.6 to110.6)	

Bu, i.v. busulfan; Cy, cyclophosphamide; Mel, melphalan; Flu, fludarabine; Eto, etoposide.

Hemoglobinopathies included thalassemia and sickle cell anemia. Other nonmalignant disorders included paroxysmal biogenesis defect, NOMID-like (Neonatal-onset multisystem inflammatory disease).

*Differences in distributions of patient characteristics over the 5 AUC percentiles were tested with 1-way analysis of variance for continuous normally distributed variables, the Kruskal-Wallis test for not normally distributed continuous variables, and the Pearson χ^2 test for categorical variables.

†Malignancies included (number of patients; disease status): AML (2), infant ALL (9; 4 ALL/complete remission [CR]1, 4 CR2, 1 CR3), lymphoma (1), chronic myelogenous leukemia [CML] (1), MDS (25; 7 RA, [refractory anemia] 5 RAEB, [refractory anemia with excess blasts] 6 RAEBt, [refractory anemia with excess blasts in transformation] 1 RARS, [refractory anemia with ringed sideroblasts] 5 MDS/AML, 1 MDS/CML), JMML (8; 4 patients untreated, 2 patients in CR, 2 received treatment without intent to achieve remission).

‡Nonmalignant disorders included BM failure syndromes (n = 4), inborn errors of metabolism (n = 22), and immunodeficiencies and hemoglobinopathies (n = 30). BM failure included aplastic anemia, Diamond Blackfan anemia, congenital amegakaryocytosis., Shwachman syndrome. Inborn errors of metabolism included osteopetrosis, X-ALD (X-adrenoleukodystrophy), MLD (metachromatic leukodystrophy), Gaucher's disease, and Hurler's disease. Immunodeficiencies included severe combine immunodeficiency, HLH, Chediak-Higashi syndrome, WAS (Wiskott-Aldrich Syndrome), ICF syndrome, X-LPD, (X-linked lymphoproliferative disease) Omenn syndrome, and others.

§All leukemia remission induction and maintenance protocols. HLH induction (HLH1994 and 2004 protocol) was considered high-intensity pretreatment. Low-intensity pretreatment was considered any low-dose chemotherapy with the intent of not to cure but rather to control the disease.

¶Other Bu-based regimens (number of patients): Bu/Cy (47), Bu/Cy/Flu (6), Bu/Cy/Eto (5), and Bu/Flu (1). In general, a combination of Bu, Cy, and Mel was used for myeloid malignancies, MDS, and ALL and in some patients with thalassemia or hemoglobinopathy. Bu and Cy and, in selected cases, Flu was given for nonmalignant indications, and Bu, Cy, and etoposide (VP16) was given to patients with HLH as well as some younger patients (age < 3 years) with ALL.

‖Median number of nucleated cells in CB grafts was 8.6 $\times$ 10^7/kg (range, 2.7 to 23 $\times$ 10^7/kg).

**Related donors (number of patients): matched sibling (18), matched family (4), mismatched family (2), haploidentical (6). Unrelated donors (number of patients): matched (34), mismatched unrelated (38). Mismatches (number of patients): BM, 1-antigen mismatched (9), 2-antigen mismatched (4); CB, 1-antigen mismatched (16), 2-antigen mismatched (7); PBSCs, 1-antigen mismatched (3). Sixteen patients (72%) with mismatched unrelated (BM or PBSC) donors received T cell depletion, versus 7 patients (21%) with matched unrelated (BM/PBSC) donors.

††T cell depletion was performed by various methods, including CD34+ selection or the addition of Campath in the bag, and was performed for various reasons, including haploidentical transplants, young infants, and large mismatched donors. In the matched donor group, 7 patients (12%) had T cell depletion, whereas in the mismatched group, 16 patients had T cell depletion (36%). None of the CB transplants were T cell depleted.

‡‡VOD risk = preexistent liver disease ≥ second myeloablative HSCT, previous treatment with gemtuzimab ozogamicin, leukemia beyond second relapse, osteopetrosis, conditioning with Bu + Mel, macrophage-activating syndromes, adrenoleukodystrophy.

§§Further analyses were stratified for the center of inclusion.

the once-daily regimen). Univariate analysis found that neither the 4-times-daily nor the once-daily regimen influenced the main outcome or the toxicity outcome (data not shown). The LUMC center included more patients in the low total AUC percentiles; therefore, stratified Cox regression was performed with center of inclusion as a stratification factor.

Therapeutic Drug Monitoring of Busulfan

The results based on the 3 dosing regimens are given in Table 2. The study population exhibit a wide range in total AUC, from 31 mg*h/L to 110 mg*h/L (~1860 to 6740 μmol*min/day). Large interindividual variations in the pharmacokinetics of busulfan were evident, necessitating varying dose adjustments at day +2 to +4 of busulfan treatment. Dose adjustments differed among the 3 dose-targeting regimens, as shown in Table 2. The AUC was measured after dose adjustment in 50% of the patients; 48% received a total AUC within 10% of the total targeted AUC after 4 days of busulfan treatment (Table 2).

Main Endpoints: Overall Survival and Event-Free Survival

Overall EFS was 68%, and OS was 72%. All patients engrafted except 3 patients who died within the first 3 weeks after transplantation. Eleven patients

Table 2. Characteristics of Dosing Regimens

Dosing Characteristic	Dose regimen 1	Dose regimen 2	Dose regimen 3
Number	38	16	48
Total target AUC in 4 days, mg*h/L	78.8	62.4	70
Starting dose, mg/day	Age < 4 years: 4 mg/kg in 4 doses Age ≥ 4 years: 3.2 mg/kg in 4 doses (maximum subs. dose 4 mg/kg)	80 mg/m^2 once daily	Age < 1 year: 80 mg/m^2 once daily Age ≥ 1 year: 120 mg/m^2 once daily
Follow-up, weeks, median (range)	249 (2 to 364)	120 (5 to 196)	62 (0.3 to 148)
Total AUC, mg*h/L, median (range)	56.3 (31 to 94)	57.1 (35 to 65)	77.5 (46 to 111)
AUC within 10% of total target AUC	30%	75%	52%
% decreased doses	5%	0%	51%
% increased doses	47%	84%	15%
% no dose change	47%	16%	34%

The 3 dosing regimens are shown. For each of these groups, the percentage of dose adjustments performed and the total AUC calculated within the various groups are shown.

(11%) had mixed chimerism (median, 50% to 75%), of whom 2 died and 9 experienced graft failure.

In univariate analysis, busulfan exposure, HLA disparity, serotherapy, cell source, and age were predictors influencing EFS and OS ($P < .05$). In multivariate analysis, a busulfan exposure of 72 to 80 mg*h/L (~4400 to 4900 μmol*min/day) was associated with the highest EFS and OS ($P = .028$ and .021, respectively). This is demonstrated in the Kaplan-Meier curve of EFS in association with increasing exposure to busulfan shown in Figure 1A. An increased AUC of busulfan was associated with a lower incidence of graft failure and relapse (HR = 0.47; $P = .004$), as shown in Figure 1B. Multivariate analysis found that 2 other covariates independently influenced EFS and OS: HLA disparity (HR = 3.79; $P = .001$) and age at time of HSCT (HR = 1.12 for each year of age; $P = .001$).

Optimum Busulfan Exposure

Figure 2 shows the results of further analysis evaluating busulfan exposure as a continuous variable rather than a categorical variable in relation to EFS. The events experienced by all patients demonstrate that the model generally describes the data well. The Cox regression model produced an optimum AUC of 74 to 82 mg*h/L (~4500 to 5000 μmol*min/day), very similar to the analysis using AUC as categorical variable.

In addition, 2 other models were designed to demonstrate how the covariates could influence the concentration–response relationship: one for the mismatched donors and one for matched donors (curves are shown in Addendum 2), with an optimum of 75 to 85 mg*h/L for mismatched donors and 65 to 77 mg*h/L for matched donors (in children age < 10 years). Age affected all models similarly. EFS was linearly associated with age, being lower in older children.

Toxicity Endpoints

Moderate/severe aGVHD (≥ grade II) occurred in 16% of the patients, VOD occurred in 22%, and mucositis grade III-IV occurred in 12%. Figure 3 shows the association between exposure to busulfan and the incidence of adverse effects. In both univariate and multivariate analyses, busulfan exposure was a significant predictor for aGVHD (HR = 1.56; $P = .019$), but not for VOD (HR = 1.11; $P = .508$) or mucositis. Lung toxicity was seen in 11 patients (3 with bronchiolitis obliterans and 8 with idiopathic pneumonia syndrome), all independent of the AUC of busulfan.

The addition of melphalan was an independent risk factor for the occurrence of aGVHD (HR = 3.04; $P = .042$), VOD (HR = 2.23; $P = .069$), and mucositis (HR = 9.02; $P = .005$) in multivariate analysis. The relationship between the busulfan exposure and toxicity was compared in patients in whom melphalan was and was not included in the conditioning regimen. The incidences of aGVHD and VOD were approximately 3-fold higher (HR = 3.6: $P = .019$) in the 12 patients treated with a combination of busulfan (> 74 mg*h/L), cyclophosphamide, and melphalan (58% aGVHD; 66% VOD) compared with the 23 patients who received the same AUC of busulfan but without melphalan (17% aGVHD; 17% VOD) (Figure 4). Severe mucositis occurred in 26% of the patients who received melphalan, but in only 3% of those who did not receive melphalan, independent of busulfan exposure.

In the patients treated with busulfan, cyclophosphamide, and melphalan, busulfan exposure correlated with aGVHD and VOD (Figure 4). The incidences of both were higher in the patients receiving this conditioning regimen with a high busulfan exposure (> 74 mg*h/L) compared with those treated with the same conditioning regimen but with a low busulfan exposure (< 74 mg*h/L) (VOD, 66% vs 16% [HR = 4.1; $P = .012$]; aGVHD, 58% vs 13% [HR = 4.5; $P = .016$]).

DISCUSSION

Our findings demonstrate that the therapeutic window for i.v. busulfan in children is rather narrow when used in an ablative conditioning regimen before

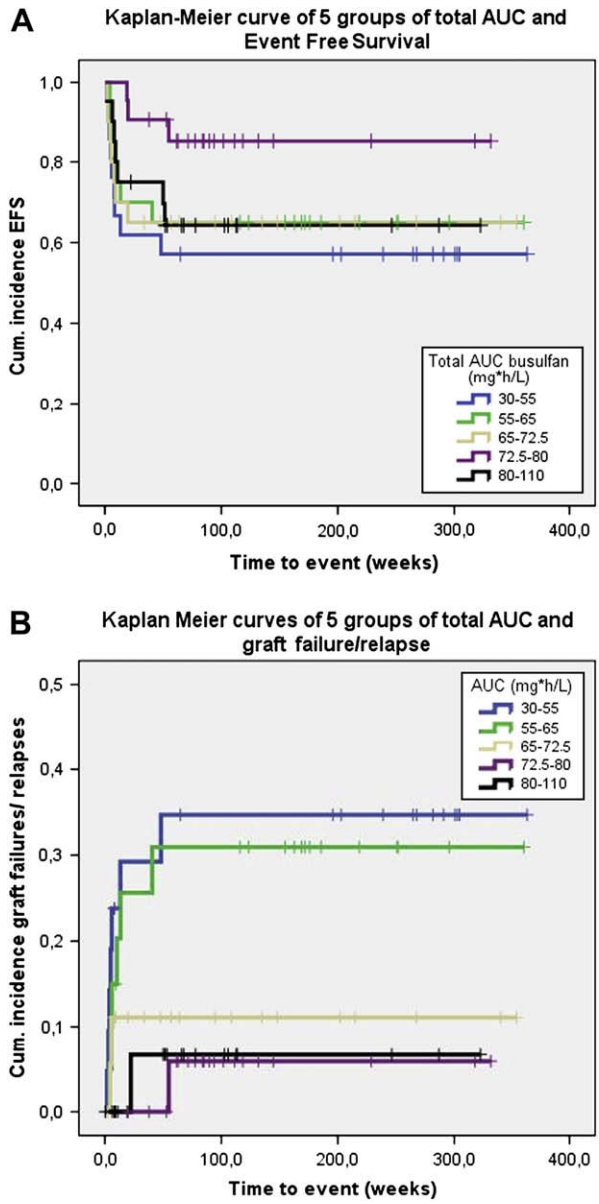

Figure 1. (A) Kaplan-Meier curves of 5 groups based on total AUC and EFS. (B) Kaplan-Meier curves of 5 groups based on total AUC and graft failure/relapse.

HSCT. A total AUC of 74 to 82 mg*h/L (~4500 to 5000 μmol*min/day) was associated with the highest EFS in children with malignant and nonmalignant diseases. Two additional covariates influencing EFS and OS were HLA disparity and age. Increased toxicity was associated with higher busulfan exposure, especially when melphalan was part of the conditioning regimen.

The optimal therapeutic window in these children was found to be 74 to 82 mg*h/L, which is in line with the range in adults (60 to 100 mg*h/L [3600 to 6000 μmol*min/day]) [8]. The optimum value resulting from the analysis of the continuous function was quite similar to that resulting from the analysis using AUC

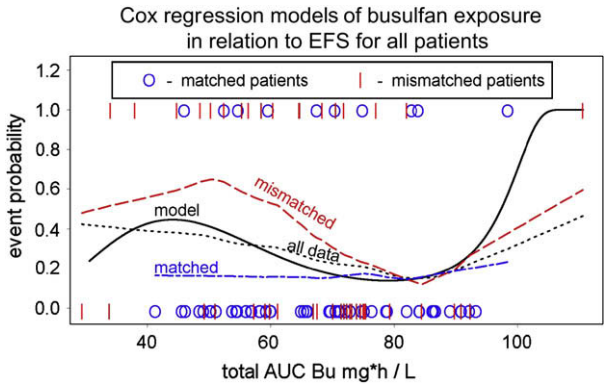

Figure 2. Cox regression models of busulfan exposure in relation to EFS for all patients. The event (death, relapse, or graft failure) in the matched and mismatched patients and these patients corresponding exposure to busulfan are shown separately (1, event; 0, no event). The Loess curves (displays of the mean data) for the EFS of all data are shown for the matched and mismatched groups. The events occurring in all patients during the first 26 weeks of follow-up (the minimum follow-up time) are shown. After this 26-week period, 5 additional events occurred: 3 patients relapsed, and 2 patients died due to TRM. All events are included in the model, in which the total follow-up time is described. A third-order polynomial model, β_1*AUC + β_2*AUC^2 + β_3*AUC^3, can describe these data (black line).

as a categorical variable, demonstrating the consistency of the analyses.

Below a total AUC of 74 mg*h/L, EFS was negatively influenced by graft failure or relapse, whereas above this range, no graft failures occurred, and only 1 patient relapsed. The relatively high rate of rejection (9%) could be due to T cell depletion in this population, however. The patients with a busulfan exposure > 82 mg*h/L exhibited a very high incidence of toxicity, leading to increased mortality [16,36].

The incidence of toxicity in this study was high compared with similar studies [15,16]. Toxicity was related mainly to the addition of melphalan to the conditioning regimen in combination with high busulfan

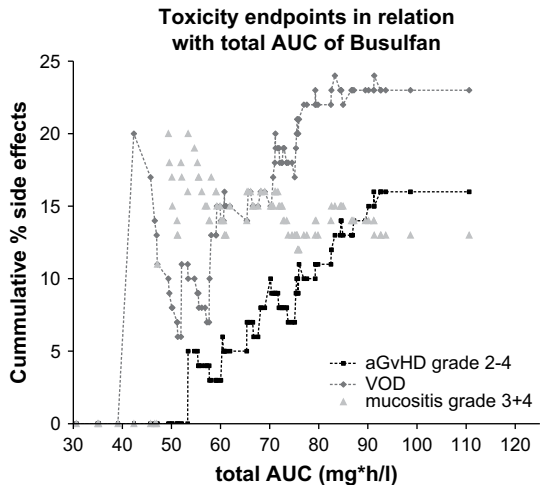

Figure 3. Toxicity endpoints in relation to total AUC of busulfan. Each symbol represents a new patient added to the busulfan exposure–toxicity relationship.

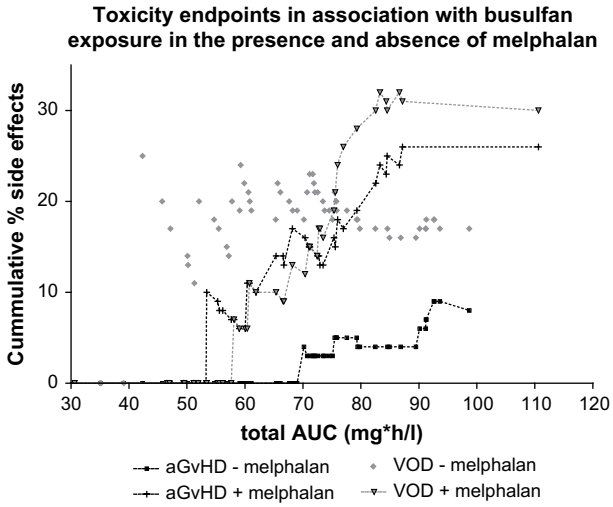

Figure 4. Toxicity endpoints in relation to busulfan exposure in the presence and absence of melphalan. Each symbol represents a new patient added to the busulfan exposure–toxicity relationship of patients treated with and without melphalan.

exposure. Other studies using this combination also found a high toxicity rate [26,30,37]. The high incidence of toxicity possibly could be linked to depletion of GST by high exposure to busulfan, because the metabolites of cyclophosphamide and melphalan are metabolized by the same enzyme system (GST) [26]. This mechanism potentially could increase the combined toxicity of busulfan and cyclophosphamide and melphalan metabolites, as has been demonstrated in a model using murine hepatocytes [38]. The high incidence of toxicity in the patients who received conditioning with busulfan, cyclophosphamide, and melphalan also may be associated with the indication for transplantation. In the present study, the choice of drugs used in the conditioning regimen depended on indication; thus, we cannot totally exclude the covariables of indication or disease risk on the exposure–outcome relationship. The patients who did not receive melphalan (those receiving busulfan plus cyclophosphamide, fludarabine, or etoposide) demonstrated no association between VOD and busulfan exposure, in line with previous studies [16,36]. Lung toxicity was not associated with busulfan exposure. Busulfan is associated mainly with later-onset lung toxicity, however. Given our limited follow-up period (2 years for the whole group), we are unable to comment on the risk of late-onset lung toxicity related to busulfan exposure [33,39-42].

Optimal busulfan exposure apparently leads to higher EFS, but the combination of busulfan and melphalan proved to be highly toxic in the patients treated in this study. In the past, melphalan was added to busulfan (given orally, without TDM) and cyclophosphamide in patients with MDS to reduce the risk of graft failure and relapse [43]. Because of the variable bioavailability of busulfan, the exposure to oral busulfan was generally much lower [6] than the optimum level defined in this study. A subgroup of our patients with myeloid leukemia (acute myeloid leukemia [AML]/MDS or juvenile myelomonocytic leukemia [JMML]), all of whom received melphalan, demonstrated a relationship between the AUC of busulfan and the incidence of failure or relapse; 7 of 27 patients (25%) with a busulfan exposure < 74 mg*h/L had relapse or graft failure, compared with 0 of 8 patients with a busulfan exposure ≥ 74 mg*h/L ($P = .11$). These data suggest that sufficient ablation might already be reached with optimal busulfan exposure; thus, it is tempting to speculate that melphalan might not be necessary in the era of targeted busulfan. Similarly, in adults, conditioning with targeted busulfan with cyclophosphamide and, more recently, the combination of busulfan and fludarabine has proven very effective in patients with AML/MDS. This combination has a safer toxicity profile compared with cyclophosphamide and melphalan [44,45] and appears to be more effective (ie, fewer relapses and lower TRM) [44,46]. Fludarabine inhibits lymphocyte proliferation, resulting in potent immune suppression. In addition, it prevents alkylator-induced DNA repair and thus could be synergistic with busulfan in its tumor-killing effect. Further analyses and optimization of busulfan exposure in reduced- toxicity regimens, such as busulfan and fludarabine, as well as validation in randomized trials, could provide more definitive answers to these questions.

In addition to busulfan exposure, 2 other covariates influenced the outcomes in this study: HLA disparity and age. Patients with a mismatched donor had a much lower EFS than those with a matched donor. This influence of HLA disparity on the outcome of HSCT is in line with the literature [47-52], but, to the best of our knowledge, this has never before been related to the optimal busulfan exposure. Our findings suggest that patients receiving transplants from matched donors might require a lower busulfan exposure, whereas in a mismatched setting, optimal ablation is more important to prevent rejection and autologous reconstitution. We considered that with less busulfan exposure in the mismatch setting, more residual, potentially alloreactive cells (host-versus-graft direction) may remain in the recipient, with the potential for clinical rejection/relapse. Because of the high EFS, the matched model was not as accurate as the mismatched model. The inclusion of more (young) patients may clarify the optimal busulfan exposure in matched donors.

Older age negatively affected OS, in line with most previous pediatric HSCT studies. Older children seemed more prone to TRM, such as aGVHD and reactivation of viral infections (data not shown).

Although a prospective, randomized trial is the best study design for determining the optimal

exposure to busulfan, such a study is made complicated by important ethical issues. Changes to the busulfan dosing regimen were introduced based on available experience; therefore, we felt that a retrospective study of patients in whom all data were prospectively documented was the best available method. All patients were treated in the same way with regard to indication-associated conditioning regimens and supportive care. Over time, 2 changes were made in the protocol; T cell depletion was decreased, and the number of CB transplantations was increased. Although T cell depletion was not found to influence the study endpoints, CB transplantation negatively influenced OS. But CB transplantations were divided equally among the quintiles of busulfan exposure and thus were not expected to influence the exposure–outcome relationship. In addition, the wide range of diseases included and the retrospective character of the study represent limitations in this respect.

The busulfan dosing regimen (4 times daily or once daily) did not influence any of the outcomes. In earlier studies, it was hypothesized that reducing the exposure to busulfan may be associated with less toxicity; the use of once-daily dosing could allow enzyme recovery of glutathione-S-reductase and GST between doses [17-19]. But our findings suggest that the dosing regimen had no influence on outcomes, which is in line with a recent randomized study in adults [19]. Once-daily dosing has the same outcome, is feasible, and certainly is much more convenient for the patient, the pharmacy, and caregivers.

A large interindividual variation in AUC of busulfan was found in this study, similar to the findings of other studies with intravenous busulfan [11,53]. Many patients required dose changes based on the results of TDM and the target AUC. Our data show that OS and EFS may be improved by narrow targeting to a therapeutic window of busulfan exposure, necessitating TDM. The importance of TDM was highlighted in a study in adults in which a dose based on lean body mass resulted in a 4-fold range in AUC among patients and a significant increase in toxicity when an exposure $>$ 100 mg*h/L was reached [1,2,5,6,12,36]. Although TDM showed improved results, some patients were still exposed to an AUC that differed from the targeted AUC. Thus, it seems that analysis of the variability in pharmacokinetics (PK) and pharmacodynamics (PD) using population PKPD modeling, in combination with an identification of covariates that account for this variability, is needed. This will lead to a predictable AUC and effectivity/safety profile in these pediatric patients.

In conclusion, our findings indicate that the outcome of HSCT in pediatric patients can be improved by TDM-adjusted dose targeting to a narrow therapeutic range of busulfan of 74 to 82 mg*h/L ($\sim$ 4500 to 5000 μmol*min/day). Once-daily dosing is feasible, is associated with similar outcomes as for 4-times-daily dosing, and is more practical and patient- and staff-friendly than 4-times-daily dosing. Melphalan in combination with an optimally targeted busulfan dose was associated with severe toxicity, suggesting that in this era of high AUC targeted busulfan dosing, the role of melphalan as a second alkylating agent should be reconsidered. Prospective studies with busulfan targeting this narrow AUC in less toxic regimens, such as busulfan and fludarabine, should help further improve the outcomes of HSCT in children.

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Appendix

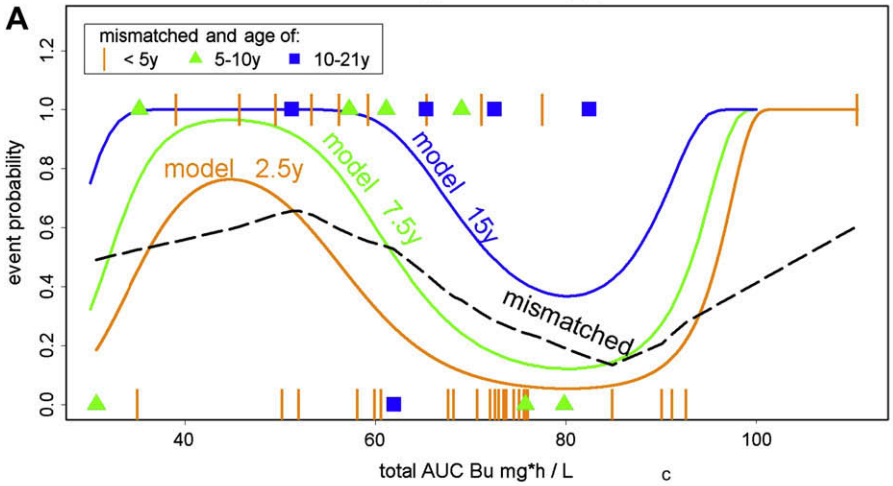
APPENDIX 1: Univariate Analysis of Covariates of EFS and OS

			EFS				OS			
Covariate		n	Events, n (%)	*P*	HR	95% CI	Deaths, n (%)	*P*	HR	95% CI Lower
AUC busulfan	30 to 55 mg*h/L	21	9 (43)		1		8 (38)		1	
	55 to 65 mg*h/L	20	7 (35)	.529	0.73	0.27 to 1.96	6 (30)	.476	0.68	0.24 to 1.96
	65 to 72.5 mg*h/L	20	7 (35)	.545	0.72	0.25 to 2.06	5 (25)	.395	0.61	0.19 to 1.92
	72.5 to 80 mg*h/L	21	3 (14)	.035	0.21	0.05 to 0.90	2 (11)	.042	0.18	0.03 to 0.94
	80 to 110 mg*h/L	20	7 (35)	.400	0.63	0.21 to 1.86	7 (35)	.751	0.84	0.28 to 2.54
HLA disparity	Matched	57	12 (21)		1		10 (18)		1	
	Mismatched	45	21 (47)	.004	2.90	1.40 to 5.98	18 (40)	.019	2.58	1.17 to 5.66
Conditioning	No melphalan	59	15 (25)		1		13 (22)		1	
	Melphalan	43	18 (42)	.083	2.11	0.91 to 4.91	15 (35)	.128	1.82	0.84 to 3.92
Busulfan regimen	4 daily doses	38	12 (32)		1		12 (32)		1	
	1 daily dose	64	21 (33)	.736	1.15	0.52 to 2.56	16 (25)	.563	0.77	0.31 to 1.88
T cell depletion	No	79	23 (29)		1		19 (24)		1	
	Yes	23	10 (43)	.148	1.82	0.80 to 4.10	9 (39)	.176	1.84	0.76 to 4.44
Serotherapy	No	19	2 (10)		1		2 (11)		1	
	Yes	83	30 (36)	.035	4.69	1.11 to 19.8	26 (31)	.082	3.62	0.85 to 15.4
Age				.008	1.09	1.02 to 1.16		.020	1.09	1.01 to 1.17
Sex	Male	57	20 (35)		1		16 (28)		1	
	Female	45	13 (29)	.525	0.79	0.39 to 1.62	12 (27)	.917	0.96	0.44 to 2.07
Source	BM	60	15 (25)		1		11 (18)		1	
	CB	27	11 (41)	.095	2.06	0.88 to 4.80	10 (37)	.038	2.74	1.06 to 7.10
	PBSC (+ BM)	15	7 (47)	.073	2.28	0.93 to 5.62	7 (47)	.019	3.14	1.21 to 8.14
Indication	Malignancy	46	18 (55)				16 (57)			
	Nonmalignancy	56	15 (45)	.225	0.65	0.33 to 1.30	12 (43)	.164	0.59	0.28 to 1.24

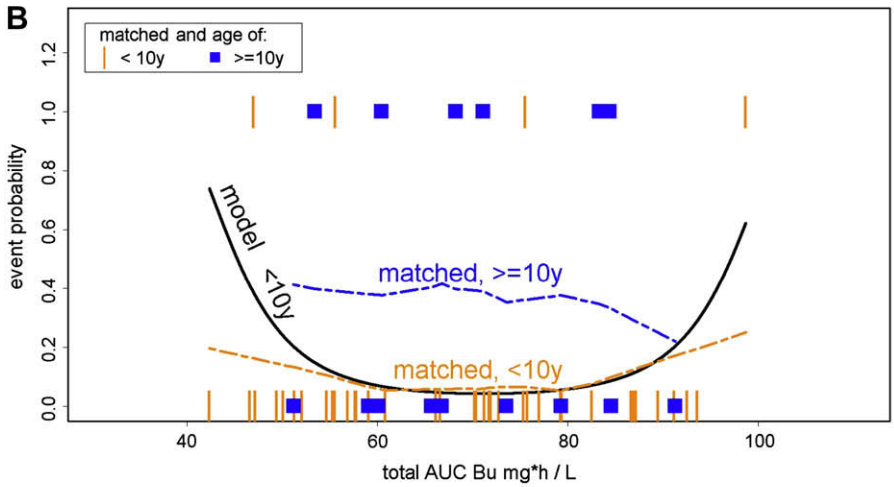
APPENDIX 2: Cox regression models of busulfan (Bu) exposure in relation to EFS for mismatched (A) and matched (B) patients.

Cox regression models of busulfan (Bu) exposure in relation to EFS for mismatched (A) and matched (B) patients. (A) The event (death, relapse, or graft failure), which occurred in each mismatched patient, divided into 3 age categories and their corresponding exposure to busulfan (1, event; 0, no event). The Loess curve (which is a display of the mean data) for the EFS of all data is shown. A third-order polynomial model, β_1*AUC + β_2*AUC^2 + β_3*AUC^3 + age, describes these data (black line). (B) The event (death, relapse, or graft failure) occurring in each matched patient, divided into 2 age categories and their corresponding exposure to busulfan (1, event; 0, no event). The Loess curves (which is a display of the mean data) for the event free survival of the data in both age categories is shown. A parabola (β_1*AUC + β_2*AUC^2) describes these data of patients under agen 10 years (black line).

APPENDIX 2 (continued): Cox regression models of busulfan exposure in relation to event free survival for mismatched (a) and matched (b) patients.



(A) The event (death, relapse, or graft failure), which occurred in each mismatched patient, divided into 3 age categories and their corresponding exposure to busulfan (1, event; 0, no event). The Loess curve (which is a display of the mean data) for the EFS of all data is shown. A third-order polynomial model, b1*AUC 1 b2*AUC2 1b3*AUC3 1 age, describes these data (black line). BU = intravenous busulfan, AUC = Area under the curve



(B) The event (death, relapse, or graft failure), which occurring in each matched patient, divided into 2 age categories and their corresponding exposure to busulfan (1, event; 0, no event). The Loess curves (which is a display of the mean data) for the event free survival of the data in both age categories is shown. A parabola (b1*AUC 1 b2*AUC2) describes these data of patients under agen 10 years (black line). BU = intravenous busulfan, AUC = Area under the curve