Treosulfan pharmacokinetics and dynamics in pediatric allogeneic stem cell transplantation

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CHAPTER 04

IMPACT OF TREOSULFAN EXPOSURE ON EARLY AND LONG-TERM CLINICAL OUTCOME IN PEDIATRIC ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION RECIPIENTS: A PROSPECTIVE MULTICENTRE STUDY

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ABSTRACT

Treosulfan-based conditioning has gained popularity in pediatric allogeneic hematopoietic stem cell transplantation (HSCT) because of its presumed favourable efficacy and toxicity profile. Treosulfan is used in standardized dosing regimens based on body surface area. The relationship between systemic treosulfan exposure, early and long term clinical outcome in pediatric patients undergoing allogeneic HSCT for non-malignant diseases is as yet unresolved. In this study we assessed the association between treosulfan exposure and early, and in particular, long term clinical outcomes. We conducted a multicentre, prospective observational study and included 110 pediatric patients with non-malignant diseases transplanted between 2011 and 2019 in Leiden, The Netherlands and Rome, Italy. Blood samples were collected and treosulfan area under the curve ($AUC_{0-\infty}$) was estimated as a measure of exposure. Cox proportional hazard survival analyses were performed to assess the relationship between treosulfan exposure, OS and EFS. The predictive value of systemic treosulfan exposure for the occurrence of toxicity within 28 days is evaluated using a multivariable logistic regression analysis. In the overall cohort, overall survival (OS) and event-free survival (EFS) at 2 years were 89.0% and 75.3%, respectively, with an excellent OS of 97% in children under the age of 2 years. The occurrence of grade II-IV aGvHD, the level of 1-year whole blood chimerism, and 2-year OS and EFS were not correlated with treosulfan exposure. The occurrence of skin toxicity (odds ratio (OR) 3.97, 95% confidence interval (CI) 1.26-13.68, p=0.02) and all grade mucositis (OR 4.43, 95%CI 1.43-15.50, p=0.02), but not ≥ grade 2 mucositis (OR 1.51, 95%CI 0.52-4.58, p=0.46) was related to high treosulfan exposure (>1750 mg*h/L). Our study demonstrates that standardized treosulfan-based conditioning results in a favourable OS and EFS in infants and children with non-malignant diseases, independent of interindividual variation in treosulfan exposure. These outcomes can be achieved without the need for therapeutic drug monitoring (TDM), thereby emphasizing the advantage of treosulfan use in this category of patients. Although higher treosulfan exposure increases the risk of skin toxicity, there is no absolute necessity for therapeutic drug monitoring if proper preventive skin measures are taken. More research is needed to assess whether deescalation of treosulfan doses is possible in order to minimize early and long-term toxicity without compromising efficacy.

INTRODUCTION

Over the past decade, treosulfan has been increasingly used as part of conditioning regimens in pediatric allogeneic hematopoietic stem cell transplantation (HSCT) for both malignant and non-malignant diseases [1-4]. Treosulfan (Trecondi®) is a prodrug and a water-soluble alkylating agent. It is non-enzymatically, pH-dependently converted into a monoepoxide- and a diepoxide derivative, which are thought to cause DNA alkylation [5, 6]. Treosulfan has gained popularity, because of its myelo- and immunoablative properties, which are combined with an apparent favourable toxicity profile. This makes treosulfan an interesting backbone of conditioning regimens, particularly in patients with non-malignant diseases. In recent years, pharmacological studies have provided evidence that therapeutic drug monitoring (TDM) is an important tool to optimize efficacy and limit toxicity of chemotherapeutic agents, especially in pediatric patients. Large interindividual variation of busulfan exposure while using uniform dosing regimens and the relationship between exposure and clinical outcome and toxicity have resulted in individualized treatment regimens [7, 8]. Building on this experience, similar approaches have been used to investigate interindividual variability in drug exposure and its impact on clinical outcome for anti-thymocyte globulin, alemtuzumab and fludarabine [9-12]. Treosulfan is used in standardized dosing regimens, both in children and adults, mostly based on body surface area. In a retrospective pediatric study, no correlation was found between total dose and clinical outcome [13]. However, in various single and multicentre studies, large interindividual variability in treosulfan exposure has been reported in patients [14-18]. So far, only three studies, including a study from our group, have analysed the relation between treosulfan exposure, treatment-related toxicity and clinical outcome [19-21]. These studies showed associations between treosulfan exposure, toxicity and survival, although results were not consistent. We previously reported the pharmacokinetic behaviour of treosulfan and its relationship with early toxicity, in a pediatric cohort transplanted for malignant and non-malignant diseases. In the present, so far largest, multicentre prospective observational study in pediatric patients with non-malignant diseases only, we assessed the association between treosulfan exposure and early and, in particular long-term clinical outcomes.

METHODS

Study design and patients

A prospective, observational, multicentre study was conducted between June 2011 and January 2019. Pediatric patients who received conditioning with treosulfan prior to their first allogeneic HSCT for a non-malignant disease in the Willem-Alexander Children's Hospital/Leiden University Medical Center in The Netherlands (n=69) and the Children's Hospital Bambino Gesù (OPBG) in Rome, Italy (n=41) were included in this study. The LUMC institutional Ethics Committee approved the study protocol (P12.267) which was subsequently approved in OPBG. Written informed consent for participation in the study was obtained from the parents or legal guardians, as well as consent from patients when they were older than 12 years according to the Helsinki Declaration (last amended in 2013, Fortaleza Brazil). The short term outcome of 61 patients in this cohort was already described in an earlier paper of a more heterogenous cohort [20]. In this study, 49 new patients were added resulting in this large cohort which exclusively includes non-malignant diseases.

Procedures

Patients received HSCT according to institutional protocols and in line with the EBMT Inborn Errors Working Party recommendations. Patients older than 1 year received treosulfan in a total dose of 42 g/m^2, administered over 3 consecutive days (14 g/m^2 per day). Children under the age of 1 year received 30 g/m^2 per day, administered over 3 consecutive days (10 g/m^2 per day). Treosulfan (day -5 to day -3) was combined with fludarabine (total dose of 150-160 mg/m^2, day -6 to day -2), with or without thiotepa (total dose 8-10 mg/kg, day -6). Serotherapy consisted of anti T-lymphocyte globulin (ATLG), anti-thymocyte globulin (ATG) or alemtuzumab. In patients with a mismatched related donor, peripheral blood stem cell grafts were processed by either CD34-positive selection or selective elimination of αβ+ T and CD19+ B cells [22]. Pharmacological graft versus host disease (GvHD) prophylaxis was given to patients receiving an unmanipulated graft according to institutional guidelines. Granulocyte colony-stimulating factor (G-CSF) was routinely given in cord blood transplants from day +8 onwards. Both transplant units are JACIE accredited and supportive care was according to institutional guidelines.

Pharmacokinetics of treosulfan

Blood sample collection was as previously described [17, 20]. Because intra-variability of treosulfan pharmacokinetics was low, blood samples were only collected on day 1 as a good representation of total exposure, as previously demonstrated [20, 23]. Treosulfan concentrations were measured with two different assays, the first part was measured in serum with an HPLC-UV method as described previously [17, 20]. The second part was measured with a validated LC/MS-MS assay. This assay was developed and validated according to EMA guidelines on bioanalytical method validation [24]. Both methods were cross-validated using a large set of study samples and it was concluded that the methods were interchangeable and therefore it was not necessary to reanalyse all samples with one method. Subsequently, the patients who were included after this validation were measured with the new LC/MS-MS method. Details regarding sample preparation, quantification and cross-validation can be found in Supplemental Material 1. A previously developed treosulfan pharmacokinetic model was used to estimate treosulfan area under the curve ($AUC_{0-\infty}$) as a measure of exposure using the posthoc estimation function in NONMEM with the final model [23].

Outcomes

Event-free survival (EFS) at 2 years was defined as survival without either primary or secondary graft failure, death due to any cause, or extensive chronic GvHD (cGvHD). Secondary outcomes were 2-year overall survival (OS), regimen-related toxicity, engraftment, donor chimerism, acute GvHD (aGvHD) and cGvHD. Overall survival was defined as survival from HSCT to last follow-up with death considered as the only event. Engraftment was defined as the first of three days with a neutrophil count of $\geq 0.5 \times 10^9$/L. Primary graft failure was defined as alive on day +28 with neutrophil count $<0.5 \times 10^9$/L. Secondary graft failure is defined as loss of previously functioning graft resulting in cytopenia involving at least two lineages. For hemoglobinopathies, this is recurrence of transfusion dependency. Acute and cGvHD were classified according to standard criteria [25, 26]. Data on chimerism determined in either whole blood or peripheral blood granulocytes and mononuclear cells by VNTR polymorphism at 1 year after transplantation were used in this analysis. When chimerism was determined in both

granulocytes and mononuclear cells the mean percentage was used for the final analysis. Mixed chimerism was defined as a donor chimerism <90%. Early toxicity endpoints were evaluated until +28 days after HSCT and included mucosal, skin, hepatic and neurological toxicity assessed according to CTCAE criteria and Bearman et al. [27]. The relationship between treosulfan exposure ($AUC_{0-\infty}$) and the outcomes of interest were evaluated.

Statistical analysis

Cox proportional hazard survival analyses were performed to assess the relationship between treosulfan exposure, OS and EFS. The predictive value of systemic treosulfan exposure for the occurrence of toxicity within 28 days is evaluated using a multivariable logistic regression analysis. $AUC_{0-\infty}$ is tested as discrete variable, considering 3 exposure groups based on tertiles: low [<1350 mg*h/L (1st tertile)], medium [1350-1750 mg*h/L (2nd tertile)] and high [>1750 mg*h/L (3th tertile)], age was tested as 2 groups (<2 years and ≥2 years old). This age cut-off point was used, because children under the age of 2 years old have immature renal and metabolic drug elimination pathways, which could influence the pharmacokinetics of treosulfan [28]. All statistical considerations are described in detail in Supplemental Material 2. All *p*-values were 2-tailed and considered significant when $p < .05$. Statistical analyses were performed with R (version 4.0.0) and R studio version 1.2.5042 with packages cmprsk, survival, car and rms.

RESULTS

Patient, donor, and transplantation characteristics

A total of 110 pediatric patients were included in the study between June 2011 and January 2019 with a median follow-up of 41 months (range 12-97 months). Clinical and demographic characteristics are detailed in Table 1. Seventy-one males and 39 females were included. Median age at HSCT was 5.2 years (range 0.2-18.8 years). Underlying disease categories were inborn errors of immunity (IEI) (n=38,35%), hemoglobinopathies (HBP) (n=55, 50%) and bone marrow failure disorders (BMF) (n=17, 15%). Thirty-four patients (31%) were conditioned with treosulfan and fludarabine (TF) and 76 patients (69%) were conditioned with treosulfan, fludarabine and thiotepa.

Table 1. Patient characteristics

	Total (N=110)
Characteristic	
Age (years, median (range))	5.2 (0.2-18.8)
Weight (kg, median (range))	18 (3.8-75.0)
Sex (n: M/F)	71/39
Diagnosis for HSCT	
Inborn errors of immunity (%)	38 (35)
Hemoglobinopathies (%)	55 (50)
Bone marrow failure (%)	17 (15)
Donor	
MSD (%)	32 (30)
MUD (≥ 9/10) (%)	50 (45)
MMFD (haplo) (%)	28 (25)
Stem cell source	
BM (%)	73 (66)
PB	
T cell replete (%)	5 (5)
TCR αβ/CD19 depletion (%)	19 (17)
CD34 enrichment (%)	3 (3)
CB (%)	10 (9)
Conditioning	
TFT (%)	77 (68)
TF (%)	37 (32)
Treosulfan dose	
14 g/m^2 (%)	92 (84)
10 g/m^2 (%)	18 (16)
Treosulfan pharmacokinetics	
$AUC_{0-\infty}$, mg*h/L (10 g/m^2) median (IQR)	1776 (1129-1977)
$AUC_{0-\infty}$, mg*h/L (14 g/m^2) median (IQR)	1562 (1140-1860)
Serotherapy	
Yes	
ATG (%)	55 (50)
ATLG (%)	35 (32)
Alemtuzumab (%)	12 (11)
No (%)	8 (7)
Pharmacological GvHD prophylaxis	
CsA (%)	6 (6)
CsA / MTX (%)	61 (55)
CsA / Pred (%)	5 (4)
Other (%)	4 (4)
None (%)	22 (20)
Post-Cy / CsA / MMF (%)	12 (11)

MSD: matched sibling donor, MMFD: mismatched family donor, MUD: matched unrelated donor, BM: bone marrow, PB: peripheral blood, CB: cord blood, TF: treosulfan-fludarabine, TFT: treosulfan-fludarabine-thiotepa, AUC: Area under the Curve, ATG (Thymoglobulin): Anti thymocyte globulin, ATLG (Grafalon): Anti T lymphocyte globulin, GvHD: Graft-versus-Host Disease, CsA: Cyclosporine A, MTX: methotrexate, Pred: prednisolone, Post-Cy: Post transplantation cyclophosphamide, MMF: mycophenolate mofetil

Treosulfan pharmacokinetics

Eighteen patients (< 1 year old) received a treosulfan dose of 10 g/m^2 and 92 patients (≥ 1 year old) a dose of 14 g/m^2 on three consecutive days. Median day 1 treosulfan $AUC_{0-\infty}$ was 1776 (IQR 1129-1977) and 1562 (IQR 1140-1860) mg*h/L in patients receiving 10 g/m^2 and 14 g/m^2, respectively, and showed large interindividual differences. Treosulfan clearance was lower in younger patients (Figure 1). Median age at transplant was significantly lower in the IEI group (1.5 yrs), compared to HBP (8.5 yrs) and BMF group (7.2 yrs) (p<0.001), therefore treosulfan clearance was also significantly lower in the IEI group (p<0.001). Median age was also significantly lower in the treosulfan-fludarabine (TF) group than the treosulfan-fludarabine-thiotepa (TFT) (3.6 vs 7.6 years (p=0.011)), resulting in corresponding higher treosulfan $AUC_{0-\infty}$ in the TF group (1800 vs 1443 mg*h/L (p<0.001).

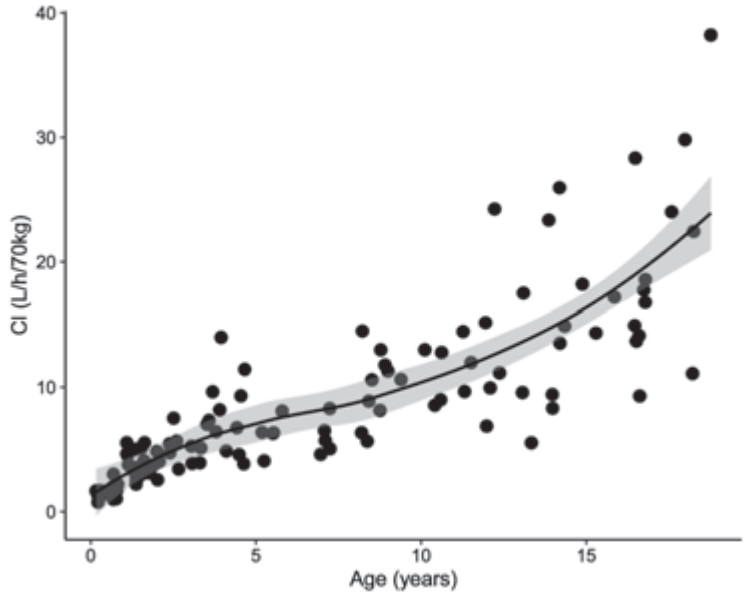

Figure 1. Treosulfan clearance versus age. Each dot represents the clearance of treosulfan (L/h/70 kg) of a patient plotted against age.

Treosulfan exposure and clinical outcome

Engraftment and chimerism

The cumulative incidence of engraftment was 97.1% (95%CI 93.5-100.0), with a median time to neutrophil and platelet engraftment of 20 days (11-43) and 24 days (8-94), respectively. Three patients died before engraftment (on day 0, +11 and +17), 7 patients experienced primary graft failure (3 HBP, 2 IEI, 2 BMF). Mean $AUC_{0-\infty}$ in patients with primary graft failure (1310 mg*h/L) and patients with successful engraftment (1586 mg*h/L) showed no significant difference (p=0.20). Three of the primary graft failure patients subsequently died because of transplant-related complications, 4 patients underwent a second transplantation. Three were successful, one patient rejected again and required autologous reinfusion. Eight patients, all with hemoglobinopathy as underlying disease (14,5% of the hemoglobinopathy group (n=55)), experienced secondary graft failure. Six of them experienced secondary graft failure within 6 months, two patients lost the graft after 2 and 5 years respectively. Four patients received a subsequent transplantation, of which two were successful and two rejected again. The four other patients did not receive a second transplantation or are scheduled for a new transplantation. More detailed information can be found in Supplemental Material 3. Mean $AUC_{0-\infty}$ was 1699 mg*h/L versus 1558 mg*h/L for patients with and without secondary graft failure, respectively (p=0.31).

Eighty-nine patients (81%) were evaluable for 1-year chimerism. Fifty-nine (66%) achieved ≥90% donor chimerism, 14 patients (16%) 50-90% and 16 patients (18%) <50%. Treosulfan $AUC_{0-\infty}$ was not correlated with either donor chimerism at 1-year in whole blood (p=0.87), nor with granulocyte chimerism in a subgroup (n=53) in which these data was available. In contrast, use of TF conditioning (OR 4.96; 95%CI 1.50-18.18, p=0.01) and age < 2 years old (OR 7.69; 95%CI 2.00-35.82, p=0.005) were significantly correlated with mixed chimerism at 1-year.

Graft-versus-host disease

The cumulative incidence of grade II-IV aGvHD was 12.4% (95% CI 7.4-20.7) and 5.1% (95% CI 2.2-12.0) of grade III-IV aGvHD. Eight patients developed grade II (7%), 4 patients grade III (3.6%) and 1 patient grade IV (0.9%).In the TF and TFT groups the cumulative incidence of grade II-IV aGVHD was 8.8% (95%CI 2.9-26.5) and 14.0% (95%CI 7.8-25.0, p=0.36), respectively. No relationship was found between treosulfan $AUC_{0-\infty}$ and the occurrence of aGvHD (p=0.42). Chronic GvHD was reported in 6 patients (CI 5.5% 95%CI 2.5-11.9) of whom three had extensive cGvHD including two patients with bronchiolitis obliterans. Treosulfan $AUC_{0-\infty}$ was not a significant risk factor for cGvHD (p=0.32).

EFS and OS

The cumulative incidence of 2-year OS was 89.0% (95% CI 83.3-95.1) (Figure 2). Nine patients died of TRM (8%) due to severe infections (n=4), toxicity (n=4) and GvHD (n=1). Two patients died because of progressive disease and one patient with TTC7A deficiency died 2.5 years after HSCT because of complications after bowel transplantation. OS in children under 2 years of age was high (97%) and no TRM was seen in this group.

Multivariable Cox regression analysis demonstrated that treosulfan exposure was not correlated with 2-year OS (HR 1.09 (95% CI 0.22-5.46, p=0.92 for treosulfan exposure >1750 mg*h/L) (Table 2; Figure 2). Underlying disease was a significant predictor for OS with the most favourable outcome for HBP (HR 0.13 (95% CI 0.03-0.64, p=0.01).

Estimated 2-years EFS was 75.3% (95%CI 67.6-83.8) (Figure 3). In multivariable Cox regression analysis, treosulfan exposure was not independently correlated with 2-year EFS, nor were any of the other variables (Table 2).

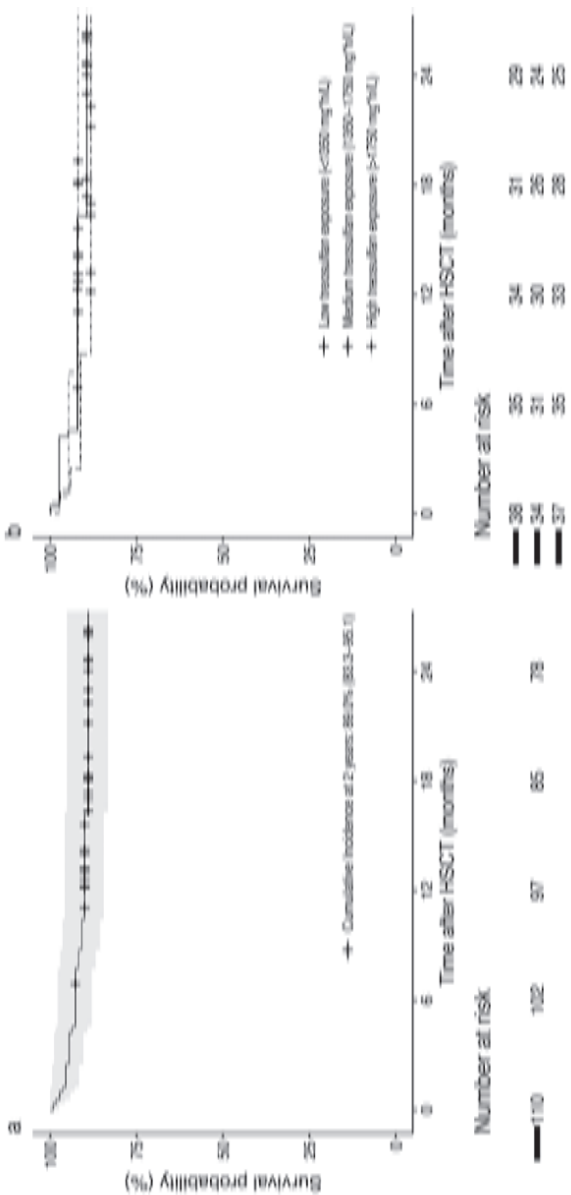

Figure 2. Overall survival. Kaplan Meier-plots of OS (a) and OS, stratified for low (<1350 mg*h/L; solid line), medium (1350-1750 mg*h/L; dashed line) (p=0.36) and high (>1750 mg*h/L; dotdashed line) (p=0.92) treosulfan exposure (b).

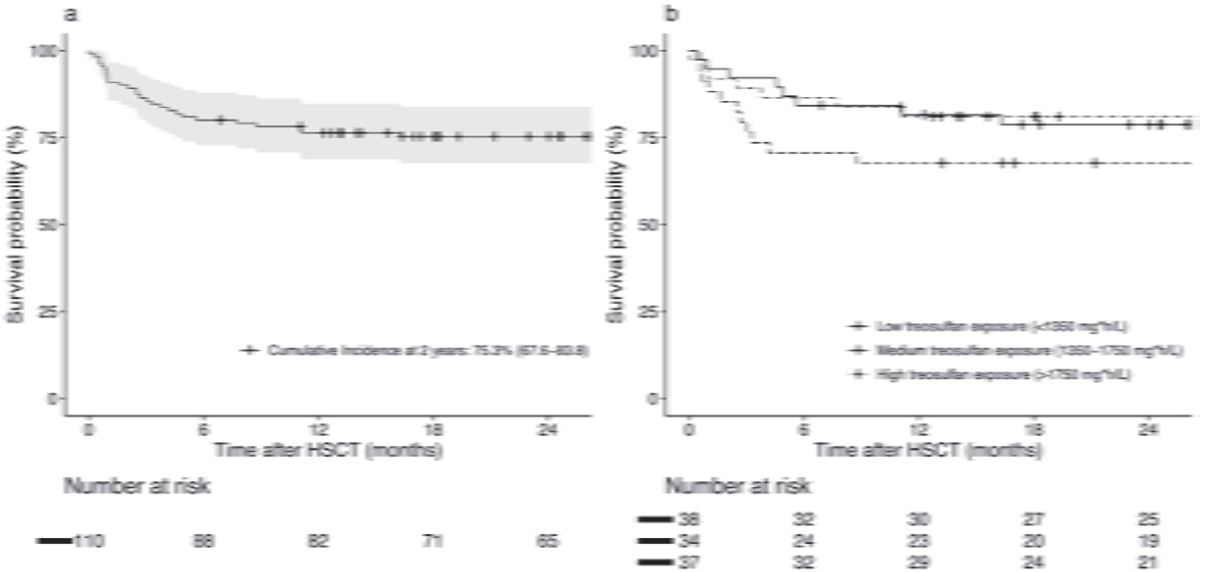

Figure 3. Event free survival. Kaplan Meier-plots of EFS (b) and EFS, stratified low (<1350 mg*h/L; solid line), medium (1350-1750 mg*h/L; dashed line) (p=0.29) and high (>1750 mg*h/L; dotdashed line) (p=0.95) treosulfan exposure (b).

Table 2. Multivariable analysis of event free survival and overall survival

Covariate	EFS 2 years Hazard ratio (95% CI)	P	OS 2 years Hazard ratio (95% CI)	P
Treosulfan $AUC_{0-\infty}$, mg*h/L				
Low (<1350)	1.00 (ref)		1.00 (ref)	
Medium (1350-1750)	1.67 (0.65-4.31)	0.29	2.08 (0.44-9.96)	0.36
High (>1750)	1.03 (0.37-2.89)	0.95	1.09 (0.22-5.46)	0.92
Age				
<2 years old	1.00 (ref)		1.00 (ref)	
≥2 years old	0.83 (0.32-2.18)	0.71	4.81 (0.92-25.28)	0.06
Conditioning regimen				
TF	1.00 (ref)		1.00 (ref)	
TFT	1.17 (0.46-2.97)	0.74	1.77 (0.38-8.30)	0.47
Donor				
MSD	1.00 (ref)		1.00 (ref)	
MUD (≥ 9/10)	1.58 (0.59-4.26)	0.36	1.51 (0.27-8.54)	0.64
MMFD (haplo)	2.65 (0.92-7.69)	0.07	3.24 (0.61-17.34)	0.17
Underlying disease				
Inborn errors of immunity	1.00 (ref)		1.00 (ref)	
Bone marrow failure	1.09 (0.28-4.33)	0.90	0.22 (0.04-1.29)	0.09
Hemoglobinopathies	1.36 (0.44-4.21)	0.60	0.13 (0.03-0.64)	0.01

Adjusted multivariable analyses were done using a Cox proportional hazard model. MSD: matched sibling donor, MMFD: mismatched family donor, MUD: matched unrelated donor, TF: treosulfan-fludarabine, TFT: treosulfan-fludarabine-thiotepa, AUC: Area under the Curve

Early regimen-related toxicity

Mucositis occurred in 50% (n=55) of patients of which 33% (n=36) had grade 2 or higher. In the TF group grade ≥ 2 mucositis occurred in 29% (n=10) versus 34% (n=26) in the TFT group. For the different disease groups this was 37% (n=14) for IEI, 18% (n=3) for BMF and 35% (n=19) for HBP. In multivariable analysis, high treosulfan exposure (>1750 mg*h/L) (OR 4.43 95% CI 1.43-15.50, p=0.01) and age above 2 years (OR 5.69 (95% CI 1.90-19.44, p=0.003) were independent risk factors to develop all grade mucositis while BMF as underlying disease was correlated with significantly less mucositis (OR 0.13 95% CI 0.03-0.57, p=0.01) than IEI and HBP. However, mucositis grade 2 or higher, which is clinically more relevant, was not significantly correlated with high treosulfan exposure (OR 1.51 95%CI 0.52-4.58, p=0.46) (Table 3).

Moderate to severe skin toxicity (≥ grade 2) occurred in 31% of patients, with high treosulfan exposure (>1750 mg*h/L) as risk factor (OR 3.97 95% CI 1.26-13.68, p=0.02). The addition of thiotepa to the conditioning regimen did not significantly increase the risk of skin toxicity (OR 1.85 95% CI 0.61-6.06, p=0.29). Grade 2 or higher hepatic and neurological toxicity occurred in 33% and 6% of patients, respectively, and was not correlated with treosulfan exposure (p=0.67 and p=0.60, respectively), nor with age and conditioning regimen.

Table 3. Early regimen related toxicity

Covariate	Mucositis all grade Odds ratio (95% CI)	P	Mucositis ≥ grade 2 Odds ratio (95% CI)	P	Skin toxicity ≥ grade 2 Odds ratio (95% CI)	P
Treosulfan $AUC_{0-\infty}$, mg*h/L						
Low (<1350)	1.00 (ref)		1.00 (ref)		1.00 (ref)	
Medium (1350-1750)	1.61 (0.56-4.79)	0.38	0.62 (0.20-1.88)	0.40	1.61 (0.53-4.99)	0.40
High (>1750)	4.43 (1.43-15.50)	0.02	1.51 (0.52-4.58)	0.46	3.97 (1.26-13.68)	0.02
Age						
<2 years old	1.00 (ref)		1.00 (ref)		1.00 (ref)	
≥2 years old	5.69 (1.90-19.45)	0.003	4.02 (1.30-14.16)	0.02	2.71 (0.86-9.55)	0.10
Conditioning regimen						
TF	1.00 (ref)		1.00 (ref)		1.00 (ref)	
TFT	2.13 (0.74-6.46)	0.17	1.30 (0.44-4.00)	0.64	1.85 (0.61-6.06)	0.29
Underlying disease						
Inborn errors of immunity	1.00 (ref)		1.00 (ref)		1.00 (ref)	
Bone marrow failure	0.13 (0.03-0.57)	0.01	0.22 (0.04-0.99)	0.06	1.17 (0.26-5.20)	0.84
Hemoglobinopathies	0.66 (0.20-2.05)	0.48	0.59 (0.18-1.85)	0.37	1.66 (0.50-5.93)	0.41

Adjusted multivariable analyses were done using logistic regression. MSD: matched sibling donor, MMFD: mismatched family donor, MUD: matched unrelated donor, TF: treosulfan-fludarabine, TFT: treosulfan-fludarabine-thiotepa, AUC: Area under the Curve

DISCUSSION

In this large prospective multicentre study in children with non-malignant diseases treated with standardized treosulfan-based conditioning, we studied the correlation between treosulfan exposure and both early and long-term clinical outcome after HSCT. The main findings are that treosulfan-based conditioning is safe and results in excellent clinical outcome, despite large interindividual differences in treosulfan exposure. Although treosulfan exposure is correlated with the occurrence of early toxicity, it does not have a significant impact on outcomes such as engraftment, chimerism, GvHD, and OS and EFS.

Treosulfan clearance was correlated with age, thus confirming our initial report developing the population pharmacokinetics model of treosulfan [23]. Clearance increases with age, reflecting maturation of organs and increase in bodyweight. There was a difference in $AUC_{0-\infty}$ between the TF and TFT groups, which could suggest an impact of thiotepa on treosulfan clearance. However, since age was also significantly different between these groups (patients receiving TF were younger), this is the most probable explanation for the observed difference in $AUC_{0-\infty}$.

An important observation in our study was the lack of correlation between the level of donor chimerism at 1-year and treosulfan exposure, while a positive correlation was found for conditioning regimen, i.e. TF versus TFT, and age. Chiesa et al. [19] reported in IEI patients treated with TF a trend toward an association between low level (≤20%) myeloid chimerism and low treosulfan $AUC_{0-\infty}$, but only in univariable analysis. We found a higher risk of mixed donor chimerism (<90%) in the TF compared to the TFT group, however the risk was independent of treosulfan exposure. This information could be of value when deciding between these two regimens in diseases where higher levels of chimerism are preferred. In addition, early toxicity was not significantly increased with the addition of thiotepa to the TF regimen in our patients. However, it has to be noted that the impact of adding thiotepa to TF on long-term toxicity, especially fertility, is currently unresolved.

We demonstrate that high treosulfan exposure is significantly correlated with the risk of skin toxicity, confirming our previous observations [20]. Despite the fact that the use of thiotepa may also lead to skin toxicity [29], similar levels of skin toxicity were observed in the TF and TFT groups, indicating that in this pediatric cohort thiotepa has probably made only a minor contribution to the skin toxicity. Moreover, in multivariable analysis, treosulfan exposure was identified as an independent risk factor. Of note, Chiesa et al. [19] also reported the relationship between treosulfan exposure and skin toxicity in a cohort of 57 children with TF conditioning, thus confirming our observation. While skin toxicity occurs frequently, taking preventive measures can help reduce the incidence of cutaneous complications. Preventive care guidelines for thiotepa-induced skin toxicity, such as suggested by Van Schandevyl and Bauters, could also be implemented for treosulfan [29].

Interestingly, while we previously observed a relationship between high treosulfan exposure and the risk of grade 2 or higher mucositis in a smaller and mixed cohort [20], in the present study on patients with non-malignant diseases exclusively, this correlation was just observed for all grade mucositis, which is clinically less relevant. This difference is probably due to lack of patients with malignant diseases of which 50% experienced ≥ grade 2 mucositis. Our findings are in accordance with Chiesa et al. who did not report a relationship of treosulfan exposure with mucositis. Mohanan et al. [21] reported an incidence of 39% of all grade mucositis and 20% of grade 3-4 mucositis but found no relationship between treosulfan exposure and regimen-related toxicities.

Two-year overall survival was 89.0%, similar to other reports on patients with non-malignant diseases treated with treosulfan-based conditioning [1, 4, 13, 19, 21]. Remarkably, OS of infants under the age of 2 years was 97%, emphasizing the excellent efficacy and safety profile of treosulfan-based regimens in this vulnerable category of patients. Both in the TF and TFT group treosulfan exposure was not correlated with 2-year OS. However, Chiesa et al. [19] found a relationship between treosulfan $AUC_{0-\infty}$ and mortality; in particular a cumulative treosulfan $AUC_{0-\infty}$ >6000 mg*h/L (corresponding with a daily exposure of > 2000 mg*h/L) was associated with higher

transplant-related mortality. Mohanan et al. [21] found that low treosulfan clearance showed a higher risk towards poor OS, however this was not reflected in a similar correlation with $AUC_{0-\infty}$. The differences between our results and those of Chiesa et al. [19] and Mohanan et al. [21] could be explained by the substantial differences in interpatient variation in treosulfan exposure. Chiesa et al. [19] reported daily exposure $AUC_{0-\infty}$ values ranging between 733-4882 mg*h/L and Mohanan et al. [21] reported $AUC_{0-\infty}$ values between 129-4267 mg*h/L. While our patients were treated with similar dosing regimens, the $AUC_{0-\infty}$ values ranged between 366-3368 mg*h/L and thus lacked exposures in the very high region. Therefore, we speculate that the limited interpatient variation and the lack of high levels in our patient cohort, may explain the absence of a correlation between treosulfan exposure and EFS or OS in our study. The other studies did not report whether the patients with high or low $AUC_{0-\infty}$ had specific characteristics (e.g. comorbidities) that could be co-factors explaining the unfavourable outcome.

The EFS rate in our study was very favourable with 75.3% at two years after HSCT, especially if we consider that previous studies (in contrast to ours) did not count cGvHD as an event [1, 4, 13]. An important observation in our study is that EFS was not correlated with treosulfan exposure. This is in accordance with the study of Mohanan et al. [21], who did not find a relationship between treosulfan exposure and EFS in 87 thalassemia patients treated with the same TFT regimen. Our combined results containing more than two hundred patients with non-malignant diseases demonstrate that with current dose regimens treosulfan exposure has no significant impact on EFS, thus supporting the use of these regimens in this category of patients without the need for therapeutic drug monitoring. Whether disease-free survival in children with malignant diseases is similarly independent of treosulfan exposure remains to be demonstrated. Moreover, the correlation between treosulfan exposure and the occurrence of late effects (e.g. growth disorders, gonadal insufficiency and infertility) in children treated with treosulfan-based conditioning has yet to be established.

In the last several years it has become evident that there is a clear relationship between busulfan exposure, clinical outcome and toxicity, resulting in established therapeutic

windows for busulfan exposure. In contrast, our study provides evidence that the impact of treosulfan exposure on clinical outcome is low and, to our opinion, PK-guided dosing is not required to optimize outcome in the majority of children. PK-guided dosing may be instrumental to prevent early toxicity, but since the toxicity profile of treosulfan is relatively mild, the added value and clinical relevance of the introduction of individualized dosing will be limited. Our findings may raise the question whether a lower treosulfan $AUC_{0-\infty}$ can be sufficient to achieve effectiveness. Also, lower treosulfan exposure could be beneficial when it comes to limiting late effects of conditioning, especially gonadal insufficiency. These questions, however, require more (prospective) research and need to be addressed in future studies.

In conclusion, the use of a treosulfan-based conditioning regimen in children with non-malignant diseases translates into very favourable clinical outcomes. Our data demonstrate that standardized dose regimens can be applied in the vast majority of patients to achieve favourable OS and EFS.

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Supplemental Material 1. Sample preparation, quantification and cross-validation

Blood sampling was collected in serum tubes, which were centrifuged at 2500 x *g* for 5 min. The resulting serum samples were stored in the -80°C freezer while waiting for analysis.

For sample preparation, a 50 µl serum aliquot was combined with 200 µl of internal standard (IS) solution in an Eppendorf tube. The IS solution consisted of 10 mg/L treosulfan D4 in acetonitrile. The tube was vortex-mixed (2000 rpm) and centrifuged (13.000 rpm) for 5 and 5 min, respectively. Subsequently, a 100 µl aliquot of supernatant was transferred to an autosampler vial and combined with 500 µl of Mobile Phase A. The final mixture was then vortex-mixed for 5 s, after which 5 µl was injected onto the Thermo LC-MS/MS system.

Quantification of treosulfan with LC-MS/MS was performed using a Thermo Endura UPLC-MS/MS system, consisting of an Ultimate 3000 series UHPLC system, coupled to a TSQ Endura triple stage quadrupole mass spectrometer, all from ThermoFisher Scientific. The UPLC system consisted of a dual gradient pump, autosampler and column heater, also from ThermoFisher Scientific. Data was acquired and processed using ThermoFisher Scientific Chromeleon software version 7.2. Chromatographic separation was achieved using a Zorbax Eclipse Plus C18 5 µm; 2,1 x 12,5 mm precolumn coupled to a Zorbax Eclipse Plus C18 3,5 µm; 2,1 x 100 mm column, both from Agilent. Mobile phase eluents were Mobile Phase A: 0,1% v/v formic acid + 10 mM ammonium acetate in water = 1000 ml water + 1,0 ml formic acid + 0,80 gr. Ammonium acetate and Mobile Phase B: 0,1% v/v formic acid + 10 mM ammonium acetate in methanol = 1000 ml MeOH + 1,0 ml formic acid + 0,80 grams ammonium acetate. The elution gradient was 90%A/10%B from initiation to 0.50 min, followed by 10%A /90%B for 2 min at a constant flow of 0.3 ml min^{-1}, followed by 90%A/10%B for the remaining 1.00 min at a constant flow of 0.5 ml min^{-1} and concluded with the initial settings for the remaining 2.00 min, at a constant flow of 0.3 ml min^{-1}. The injection volume was set to 5 µL, the column temperature was set at 40 °C and sample manager operated at room temperature. The MS was operated in the ESI+ mode. The following mass transitions were used for MRM acquisition (m/z): treosulfan 296-279 and treosulfanD4 300-283.

The HPLC-UV assay and the LC/MS-MS assay were cross-validated using 33 samples divided over the studied concentration range. The obtained mean accuracy by the different methods were within 15% and also meet the specific cross validation requirements described in the EMA guidelines on bioanalytical method validation section 4.3. Furthermore, Passing-Bablok and Bland-Altman analysis showed that both methods were interchangeable in a 1:1 manner.

Supplemental Material 2. Statistical considerations

Normally distributed continuous parameters are shown as mean ± standard deviation, all log-normally continuous distributed parameters as median (IQR) and categorical variables as frequency (percentage). Differences in exposure between different groups was tested with the Kruskall-Wallis or Wilcoxon rank test. The predictive value of systemic treosulfan exposure for the occurrence of toxicity within 28 days is evaluated using a multivariable logistic regression analysis for mucosal, skin, hepatic and neurological toxicity events, with age, conditioning regimen (treosulfan-fludarabine and treosulfan-fludarabine-thiotepa) and underlying disease (inborn errors of immunity (IEI), bone marrow failure disorders (BMF) and hemoglobinopathies (HBP)) as other possible predictors. $AUC_{0-\infty}$ was tested as discrete variable, considering 3 exposure groups low [<1350 mg*h/L (1st tertile)], medium [1350-1750 mg*h/L (2nd tertile)] and high [>1750 mg*h/L (3th tertile)], age was tested as 2 groups (<2 years and ≥2 years old). This age cut-off point was used, because children under the age of 2 years old have immature renal and metabolic drug elimination pathways, which could influence the pharmacokinetics of treosulfan.

The cumulative incidence of engraftment and acute GvHD (aGvHD) was estimated using the method of Fine and Gray for censored data subject to competing risks, taking into account graft failure, death without engraftment and subsequent HSCT as competing risk for engraftment and death before day +100 as competing risk for aGvHD. The association between treosulfan exposure and aGvHD and engraftment was tested with the Gray test. The relationship between treosulfan exposure and chimerism at 1 year after HSCT (≥90% chimerism) was determined

with multivariable logistic regression analysis with age, conditioning regimen and underlying disease as other possible predictors.

Survival curves were drawn by the Kaplan-Meier method. Duration of follow-up was defined as time from HSCT to last contact or death. Patients were censored at the date of last contact. For the endpoints overall survival and event-free survival (EFS), Cox proportional hazard survival analyses were performed. Factors considered as predictors for outcome were treosulfan $AUC_{0-\infty}$, age, conditioning regimen (treo-flu and treo-flu-thiotepa), donor source and HLA matching (MSD, MUD or MMFD) and underlying disease (IEI, BMF and HBP) in multivariable analysis. $AUC_{0-\infty}$ was tested as discrete variable, considering the 3 exposure groups mentioned above, age was tested as 2 groups (<2 years and ≥2 years old).

All *p*-values were 2-tailed and considered significant when $p < .05$. Statistical analyses were performed with R (version 4.0.0) and R studio version 1.2.5042 with packages cmprsk, survival, car and rms.

Supplemental Material 3. Characteristics of patients with primary and secondary graft failure

Patient	Underlying disease	Sex	Age at HSCT (years)	Donor	Stem cell source	Conditioning	Treosulfan dose (g/m2)	Treosulfan AUC (mg*h/L)	Outcome	Comments
1	Beta thalassemia	F	13	MMFD	PB	TFT	14	1567	Primary graft failure	TRM
2	Beta thalassemia	M	1.4	MUD	BM	TFT	14	1396	Primary graft failure	second transplant unsuccesful
3	Bare lymphocyte syndrome	M	1.1	MUD	CB	TFT	14	1318	Primary graft failure	second transplant successful
4	SAA	M	4.4	MMFD	PB	TFT	14	1482	Primary graft failure	second transplant successful
5	Bone marrow failure	M	4.6	MMFD	PB	TF	14	1893	Primary graft failure	second transplant successful
6	SCID	F	3.6	MMFD	PB	TFT	14	1145	Primary graft failure	TRM
7	Beta thalassemia	F	16.3	MUD	BM	TFT	14	366	Primary graft failure	TRM
8	Sickle cell disease	F	5.2	MMFD	BM	TF	14	1726	Secondary graft failure	second transplant is considered/planned
9	Beta thalassemia	M	1.9	MUD	BM	TFT	14	2110	Secondary graft failure	second transplant unsuccesful
10	Beta thalassemia	F	2.4	MUD	BM	TFT	14	1628	Secondary graft failure	second transplant unsuccesful
11	Sickle cell disease	M	5.5	MMFD	BM	TF	14	1656	Secondary graft failure	second transplant is scheduled
12	Beta thalassemia	F	1.1	MSD	BM	TFT	14	1851	Secondary graft failure	second transplant successful
13	Beta thalassemia	M	1.5	MUD	BM	TFT	10	846	Secondary graft failure	second transplant is considered/planned
14	Beta thalassemia	M	1.5	MSD	BM	TFT	14	1573	Secondary graft failure	second transplant successful
15	Beta thalassemia	F	5.3	MSD	CB	TFT	14	2205	Secondary graft failure	second transplant is considered/planned

SAA: Severe Aplastic Anemia, SCID: Severe Combined Immunodeficiency, MSD: matched sibling donor, MMFD: mismatched family donor, MUD: matched unrelated donor, BM: bone marrow, PB: peripheral blood, CB: cord blood, TF: treosulfan-fludarabine, TFT: treosulfan-fludarabine-thiotepa, AUC: Area under the Curve, TRM: transplant-related mortality