Individualized therapeutics

in allogeneic stem cell transplantation

Marloes ten Brink

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Aan mijn ouders

Contents

Chapter 1

General introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an established treatment in various hematologic malignancies, non-malignant hematologic diseases, immune disorders and metabolic disorders in both adult and pediatric patients. Allo-HSCT has the potential to replace the affected stem/progenitor cells, resulting in a properly functioning immune-hematologic system. In certain metabolic diseases, donor hematopoiesis will restore delivery of leukocyte-derived enzymes.

Pharmacotherapy is an essential part of the allo-HSCT procedure. When a patient receives an allo-HSCT, first the patient is prepared for the transplantation in the so-called conditioning phase. In this phase the patient receives immunosuppressive drugs with the goal to cause suppression of the host immune system to allow donor cell engraftment and prevent rejection of the transplant. Furthermore, the patients receives myelosuppressive drugs to create "space" in the bone marrow of the patient for donor hematopoietic stem/progenitor cells and if applicable, maximum reduction of malignant cells. Figure 1.1 gives an overview of the HSCT procedure and the risks involved.

Following conditioning, the patient reaches aplasia and the donor stem cell graft is administered. In the post-HSCT phase the goal is engraftment of donor cells and restoring the immune system and hematopoiesis. During aplasia and the early post-transplant period patients are highly susceptible to infectious complications. Therefore, patients are cared for in isolation rooms and receive antibacterial, antiviral and antifungal prophylaxis.

Next to infections, graft-versus-host disease (GvHD) is another major risk after allo-HSCT which affects 15-25% of pediatric HSCT recipients, despite routine administration of pharmacological prophylaxis.[1,2] The immune cells of the graft react with host tissue cells especially in the skin,

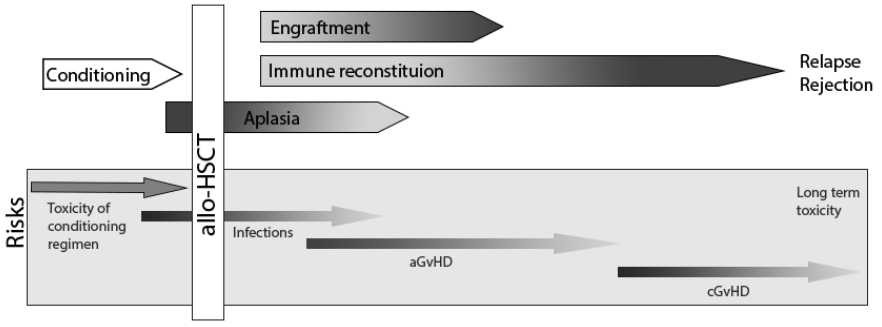

Figure 1.1 Overview of the allo-HSCT procedure and risks involved. Allo-HSCT: Allogeneic hematopoietic stem cell transplantation, aGvHD and cGvHD: acute and chronic graft-versus-host disease.

gastro-intestinal tract and liver.[3] Systemic treatment with high-dose glucocorticoids is currently the gold standard as first line treatment. Unfortunately, approximately half of the patients respond to this therapy.[4]

In recent years, improvements in different parts of the transplantation procedure have indeed made an allo-HSCT a curative treatment for an increasing number of diseases. Improvements in HLA-typing, and with that donor matching, treatment of infectious complications and optimization of conditioning regimens have improved the safety of allo-HSCT. Despite these improvements infections, conditioning regimen related toxicity and aGvHD are still major causes of severe side effects and transplant related mortality.[5]

Optimization of current drug therapies holds the potential to improve the outcome and safety of allo-HSCT. The goal of this thesis is to optimize busulfan- and treosulfan-based conditioning regimens and acute GvHD treatment with glucocorticoids by applying pharmacokinetic and pharmacogenetic profiling. Studies are performed in adults and pediatric patients. It is known that drug disposition and the pharmacodynamic effect can vary between these two populations, due to differences in body composition, metabolic capacity and maturation of enzyme function.[6] Therefore, findings in adults cannot be directly extrapolated to pediatric patients.

In the first part of this thesis the focus is on individualization of busulfan- and treosulfan-based conditioning. In **chapter 2** an overview is given of the current strategies to optimize busulfan and treosulfan therapies in pediatric allo-HSCT. Evidence has been provided that busulfan exposure is related to both HSCT efficacy and toxicity.[7,8] Therefore, therapeutic drug monitoring is applied to target busulfan exposure in the individual patient. However, a significant interpatient variability in pharmacokinetics of busulfan remains, which might be explained by pharmacogenetic variation between patients.[9–11] In **chapter 3** the role of single nucleotide polymorphisms in genes encoding for glutathione-S-transferases on busulfan clearance is investigated in adults patients undergoing an allo-HSCT. In **chapter 4**, a comprehensive pharmacogenetic analysis of busulfan PK in adult patients is performed; the DMET (drug metabolism and transport) array is applied to identify genetic markers involved in drug metabolism and transport. Based on the results in adults in chapter 4, subsequently a selection of genetic markers is analyzed in pediatric patients, this is presented in **chapter 5**. **Chapter 6** and **7** focus on treosulfan-based conditioning in pediatric patients. Treosulfan is an alkylating agent and it has a similar structure to busulfan. It is currently more often applied in allo-HSCT due to its beneficial toxicity profile in comparison with busulfan.[12] The experience with treosulfan prior to allo-HSCT is limited and only a few studies were performed to investigate treosulfan pharmacokinetics in pediatric patients. Clinical outcome of HSCT using busulfan is

associated with the exposure of the drug. Therefore, we presume that clinical outcome after HSCT applying a treosulfan-based regimen might be dependent on exposure as well. In order to study the dose-effect relation of treosulfan in HSCT, both a method of bioanalysis and a population pharmacokinetic model for treosulfan are essential. In **chapter 6** the development of a reversed phase high pressure liquid chromatography (RP-HPLC) bioanalytical method to detect treosulfan in serum is described. Furthermore, a population pharmacokinetic model and limited sampling strategy were developed. **Chapter 7** demonstrates a pilot study in which pharmacokinetic parameters of treosulfan in 21 pediatric patients were related to patient characteristics, such as age, disease, etc. Next to this, early clinical outcome is studied in relation to treosulfan exposure in this pilot study. **Chapter 8** focuses on the involvement of genetic markers in glucocorticoid responsiveness. In a retrospective cohort of pediatric patients with acute GvHD, the relation between genetic markers and glucocorticoid responsiveness is studied. In this analysis the genetic markers are investigated in both donor and recipient DNA, since both sources of immune cells are involved in the process of aGvHD. **Chapter 9** describes a case of pharmacogenetic testing in an allo-HSCT patient. The case highlights the importance of proper quality control in pharmacogenetic analysis and the challenge of pharmacogenetic testing in patients that received an allo-HSCT and have a mixed hematopoietic system. The thesis ends with concluding remarks and future perspectives in **chapter 10**.

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Chapter 2

Personalized busulfan and treosulfan conditioning for pediatric stem cell transplantation: the role of pharmacogenetics and pharmacokinetics

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ABSTRACT

Busulfan- and treosulfan-based conditionings are the cornerstone of pediatric allogeneic hematopoietic stem cell transplantation (HSCT). Although both drugs are alkylating agents, their mechanisms of action, pharmacokinetics (PK) and toxicity profiles are different. Experience with busulfan in pediatric HSCT is broad and the knowledge on the pharmacodynamics (PD), PK and, to a lesser extent, pharmacogenetics (PG) has resulted in a more effective therapy. Treosulfan has only recently been introduced in pediatric HSCT and is considered a promising new therapy because of its beneficial toxicity profile. However, knowledge of the PK and PG of treosulfan is limited. In this review, we describe the pharmacology of both agents and discuss factors causing variability in PK in relation to therapeutic outcome in HSCT.

INTRODUCTION

Allogeneic HSCT (allo-HSCT) is a potential curative treatment for a range of hematological malignancies and non-malignant diseases in pediatric patients. The main reasons for treatment failure are relapse and treatment-related mortality (TRM). TRM can occur in up to 5–20% of patients,[1–3] mostly caused by infections, graft-versus-host disease (GvHD) or toxic organ damage, which is dependent on the underlying disease, donor matching, graft source, supportive care and conditioning regimen administered before the HSCT. A conditioning regimen usually comprises a combination of immunosuppressive and myeloablative drugs with the goal to cause suppression of the host immune system to prevent rejection, create 'space' in the bone marrow of the recipient to allow donor cell engraftment, and, if applicable, eliminate the underlying malignancy. The alkylating agents busulfan (1,4-butanediol-dimethylsulfonate, Busilvex®) and treosulfan (L-threitol 1,4-bismethanesulphonate, Ovastat®) are commonly applied in different conditioning regimens before HSCT. Both drugs have different profiles in relation to the toxicity and mechanism of action in HSCT. In this review, we describe the PD, PK and PG profiles of both drugs. The experience with busulfan-based conditioning in pediatric patients is extensive compared with that of treosulfan. In recent years, a large series of PK, PD and PG studies has reported on the optimization of busulfan treatment. We provide an overview of the most important findings and of future perspectives on how to further optimize busulfan dosing. Dose-optimization studies for treosulfan are scarce and lessons might be learned from previous studies in busulfan.

Mechanism of action of busulfan

Busulfan is a bifunctional alkylating agent of the alkylsulfonate type, comprising two instable methanesulfonate groups (Figure 2.1). Busulfan is hydrolyzed in aqueous environments and releases the methanesulfonate groups, leading to a reactive carbonium ion that alkylates DNA. Busulfan is only slightly soluble in water, although an intravenous formulation became available in 2000. It was first applied in the palliative treatment of chronic myeloid leukemia (CML) for its myelosuppressive properties and antitumor effects.[4] Furthermore, busulfan is mainly cytotoxic for proliferating tissues and depletes non-cycling primitive stem cells. Although busulfan is a strong myelosuppressive drug, it is only weakly immunosuppressive; at a dose causing 50% decrease in myelopoiesis, only a mild decrease in lymphocyte numbers is observed.[5]

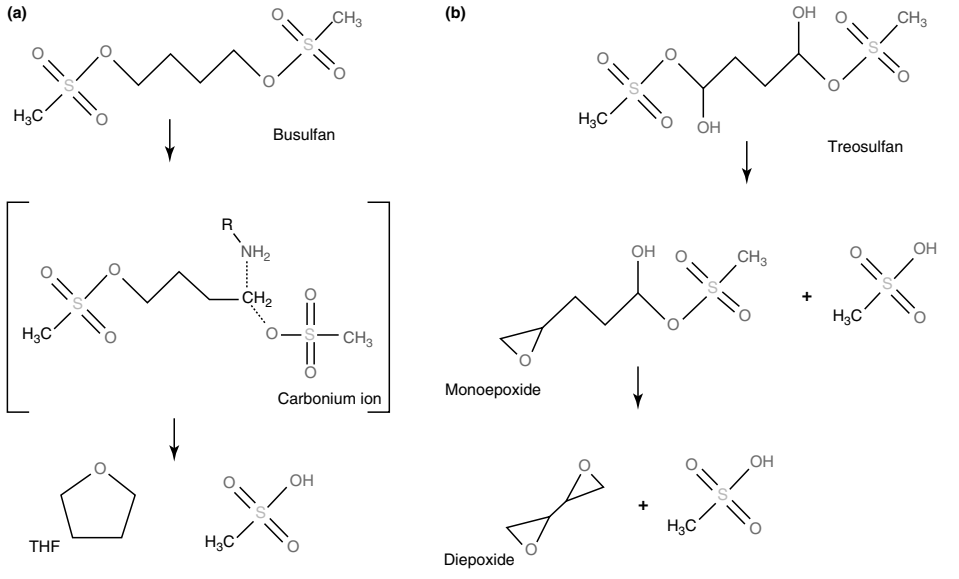

Figure 2.1 Activation of busulfan and treosulfan. a) Busulfan is hydrolysed and releases one methanesulfonate group. Through the reaction with a thiol group (NH_2-R) an instable carbonium ion is formed. When releasing the second methanesulphonate group, tetrahydrofuran (THF) is formed. b) Treosulfan is non-enzymatically, pH-dependently converted by intramolecular nucleophilic substitution into a monoepoxide ((2S,3S)-1,2-epoxy-3,4 butanediol 4-methanesulphonate) and a diepoxide (L-diepoxybutane).

Mechanism of action of treosulfan

Treosulfan has a strong myeloablative potential and is considered less toxic than busulfan and, therefore, an interesting alternative for busulfan in conditioning before HSCT. The first clinical application of treosulfan in pediatric patients prior to HSCT was in 2002.[6] Treosulfan is a prodrug and a water-soluble alkylating agent. It is non-enzymatically, pH-dependently converted by intramolecular nucleophilic substitution into a monoepoxide ((2S,3S)-1,2-epoxy-3,4 butanediol 4-methanesulphonate) and a diepoxide (L-diepoxybutane) (Figure 2.1).[7] Conversion occurs at a pH > 6.0 and the conversion to the monoepoxide is necessary for DNA alkylation, DNA crosslinking occurs via the diepoxide only.[8] Treosulfan gives a rapid and sustained myeloablation, which is comparable to that of busulfan. This was demonstrated by a fast reduction in colony-forming unit granulocyte macrophages in mice; aplasia was reached on day 1 and was maintained after completion of treatment.[9] Furthermore, the immunosuppressive profile of treosulfan was demonstrated by a strong and durable splenic B and T cell depletion and low pro-inflammatory cytokines release. *In vitro* and *in vivo* data suggest a stronger immunosuppressive and cytotoxic effect against leukemic cells compared with busulfan.[9,10]

Busulfan and treosulfan in malignant disease

In malignant disease, myeloablative conditioning (MAC) is usually used, aimed at myeloablation and maximum reduction of leukemic cells. High-dose busulfan or total-body irradiations are the cornerstones of MAC. Initially, busulfan was mainly combined with cyclophosphamide, which is an effective therapy, but is accompanied by severe toxicities. Recently, several attempts have been made to maintain the efficacy of a MAC, but reduce the toxicity; for example, by targeting busulfan exposure and the replacement of cyclophosphamide with fludarabine.

In malignant disease, treosulfan-based conditioning was only recently introduced. It is usually used in patients who are not eligible for the standard preparative regimen because of pre-existent morbidity and in cases of second HSCT after initial traditional myeloablative conditioning. Casper *et al*.[11] initially investigated treosulfan in malignant disease in adult patients, but studies on treosulfan in malignant disease in pediatric patients are limited. A large retrospective study on behalf of the European Society for Blood and Marrow Transplant (EBMT) Pediatric Diseases Working Party on the effectiveness and safety of treosulfan-based conditioning was performed in pediatric patients with high risk or advanced hematologic malignancies.[12] The treosulfan-based conditioning demonstrated efficacy rates similar to rates found in busulfan-based studies and the toxicity profile was comparable to that of reduced intensity conditioning (RIC). Although treosulfan seems to be a promising candidate in HSCT for malignant disease, there is no direct comparison of busulfan- and treosulfan-based conditioning. Therefore, a prospective study directly investigating both agents in malignant diseases in pediatric patients is warranted.

An overview of all recent studies (2008–2013) using busulfan- and treosulfan-based conditioning in allo-HSCT in pediatric patients is provided in Tables 2.1 and 2.2, respectively. This period was selected as an update of the review by Glowka *et al*. on treosulfan.[13]

Busulfan and treosulfan in non-malignant disease

An increasing number of non-malignant disorders in pediatric patients are suitable for allo-HSCT. These patients often have severe comorbidities or are very young. In these types of disease, the main goal of HSCT is to establish normal donor hematopoiesis and to reverse or halt disease progression. The level of donor engraftment that is needed for cure is dependent on the disease and the extent of engraftment of a certain lineage of hematopoietic cells. The main risk of allo-HSCT in non-hematologic disease is graft rejection and toxicities related to the HSCT, such as acute GvHD (aGvHD), infections and organ toxicity. Busulfan-cyclophosphamide conditioning was the standard conditioning in non-malignant diseases. To reduce the toxicity

Table 2.1 Overview of recent studies and outcomes in busulfan-based conditioning before allogeneic HSCT in pediatric patients

Patients (n) and median age (range)	Underlying disease[a]	Donor matching and source[b]	Conditioning regimen[c]	Engraftment and chimerism[d]	GvHD[e]	Toxicities[f]	Survival[g]	Ref
114 4.8 yr FluBu 2.6 yr BuCy (0.2–19 yr)	Non-malign.: 39% Malignant: 61%	MSD: 18% UCB: 66% UBM: 16%	Bu/Flu: 56% Bu IV dosing on BW[i] AUC: 80–95 mg*h/L Flu: 40 mg/m^2/d 4d Bu/Cy/(Mel): 44% Bu IV: 80 mg/m^2 <1 yr or 120 mg/m^2 > 1yr AUC: 74–82 mg*h/L Cy: Malignant: 60 mg/kg/d 2d Non-malign.: 50 mg/kg/d 4d Mel: 140 mg/m^2/d 2d (in malignant diseases) ATG: 10 mg/kg 4d in UD	BuFlu: ANC(0.5): 17d Platelet(50): 40d BuCy: ANC(0.5): 21d Platelet(50): 56d	cGvHD: BuFlu: 9% BuCy: 26%	Lung injury: BuFlu: 16% BuCy: 36% SOS: BuFlu: 3% BuCy: 28%	OS 2yr: BuFlu: 82% BuCy: 78% EFS 2yr: BuFlu: 78% BuCy(Mel): 72%	75
18 14 yr (10–18)	Thal M	MFD: 61% MUD: 17% MMFD: 11% MMUD: 11%	Bu/Flu/ATG Bu: 130 mg/m^2/d 4d Flu: 35 mg/m^2/d 4d ATG: 1.5 mg/kg/d 3d + sequential pretransplant immunosuppression Flu: 40 mg/m^2/d 5d Dex: 25 mg/m^2/d 5d 1 or 2 cycles	Primary engraftment: 100% ANC(0.5): 12d Plts(20): 18d 2nd mixed chim: 11%	aGvHD: gr 2–4: 22% gr 3–4: 11% lim cGvHD: 28%	Mucositis gr 1–2: 22% Mild SOS: 16%	OS: 89% DFS: 89%	91

44 8 yr (1–21)	Thal M Cl 1: 16% (MAC) Cl 2: 55% (MAC) Cl 3: 30% (RIC)	MSD: 91% MFD: 9% BM: 73% (MAC) PBSC: 27% (RIC)	Bu/Cy/ATG (MAC): 70% Bu oral: 5 mg/kg/d 4d <3 yr or 4 mg/kg/d 4d >3 yr Cy: 50 mg/kg/d 4d ATG: 30 mg/kd/d 5d Bu/Flu/ATG/TLI (RIC): 30% Bu oral: 4 mg/kg/d 2d Flu: 35 mg/m^2/d 5d ATG: 30 mg/kd/d 5d	Primary engraftment: 100% MAC: ANC(0.5): 15 d Plts(20): 21 d RIC: ANC(0.5): 14.5 d Plts(20): 18 d 2nd Graft failure: 11.3% MAC: 6% RIC: 23%	aGvHD≥2: 21% MAC: 16% RIC: 31% lim cGvHD: 7% MAC: 6% RIC: 8% ext cGvHD: 5% MAC: 6% RIC: 0%	Mild SOS: 20% MAC: 23% RIC: 15% Gonadal failure: MAC: 13% RIC: 31% Growth failure:[iii] MAC: 13% RIC: 8%	OS 5yr: 98% MAC: 97% RIC: 100% DFS 5yr: 86% MAC: 90% RIC: 77% TRM: 2% MAC: 6% RIC: 0%	92
44 12.5 yr (4–14)	ALL CR1: 41% CR2: 32% CR3: 16%	MSD: 95% MMFD: 2% MMUD, CB: 2% BM: 9% PBSC: 91%	Bu/Cy Bu oral: 4 mg/kg/d 4d Cy: 60/mg/kg/d 2d ST: Mismatched	Primary engraftment: 100% ANC(0.5): 12d Plts(20): 15d	aGvHD: gr 1–2: 82% gr 3–4: 25% lim cGvHD: 23% ext cGvHD: 5%	No data	OS: 80% OS 3yr: 68% DFS 3yr: 50% Relapse: 34%	93
35 7 yr (0.4–20)	Non-malign.: 86% Malignant: 14% (all myeloid)	MFD: 34% MUD: 46 % MMUD 9/10: 20% BM: 57% PBSC: 37% BM+CB: 3% BM+PBSC: 3%	Bu/Flu/Amab Bu IV: 3.6–4.8 mg/kg/d 4d C_{ss}: 900 ng/ml malignant C_{ss}: 600 ng/ml non-malign. Amab: 0.5mg/kg/d 3d Flu: 1.3 mg/kg/d 4d (<4yr) or 40 mg/m^2/d 4d (>4yr)	Non-engraftment: 9% ANC (0.5): 17d Median donor chimerism: 1 mnt: 99% 2 yr: 92%	aGvHD: 17 % gr 1–2: 14% gr 4: 3% cGvHD 8%	Infusion reaction to Amab gr 1: 20% gr 2: 34% Mucositis gr 3–4: 43% Moderate SOS: 3% (+ prophylaxis)	OS: 78% TRM: 9% EFS: 61% Non-malign.: 66% Malignant: 40%	94

Table 2.1 continues on next page

Table 2.1 *Continued*

Patients (n) and median age (range)	Underlying disease[a]	Donor matching and source[b]	Conditioning regimen[c]	Engraftment and chimerism[d]	GvHD[e]	Toxicities[f]	Survival[g]	Ref
12 16 yr (2–20)	Malignant: 58% APML, CML, NHL, Other Non-malign.: 42%	PBSC: 75% RD: 17% UD: 58% UCB: 17% UBM: 17%	Bu/Flu/Amab Bu IV: 4 mg/kg/d 4d (≤4 yr) or >4yr 3.2mg/kg/d 4d C_{ss}: 600–900 ng/ml or AUC: 1800–2400 mmol*min/L Flu: 30mg/m^2/d 6d Amab: 54 mg/m^2 in 5 d (max 83 mg)	ANC(0.5): 16d Platelet(20): 31d Chimerism: 1 mnt: 93% donor chim 3 mnt: 88.6% donor chim Graft failure: 8%	aGvHD: gr 2–4: 42% gr 3–4: 25% lim cGvHD: 8%	Any gr 3–4: 8% Liver gr 4: 8% Infections: Fungal: 25% Line: 50%	OS 3yr: 91%	[95]
27 8.6 yr (3.3–17.4)	SCA	BM or MSD: 100%	Bu/Cy/ATG Bu oral or IV: 3.5 mg/kg/d 4d < 1500 µmol*min/L: 40% 900–1100 µmol*min/L: 40% 575–877 µmol*min/L: 20% Cy: 50 mg/kg/d 4d ATG: 30 mg/kg/d 3d	Chimerism: Full donor: 84% Stable mixed: 16%	aGvHD: gr 1–2: 12% gr 4: 4%	SOS: 30% Pneumonitis: 4% Seizures: 16%	OS 5 yr: 96%	[96]
71 9 yr (1.6–27)	Thalassamia: 96% SCA: 4%	MFD: 100%	Bu/Cy/TT Bu IV dosing: EMA nomogram[ii] + TDM Cy 90–200 mg/kg: 100% TT 10 mg/kg: 38% ST: SCA or MFD	Primary engraftment: 97% ANC(0.5): 20d Platelet(20): 24d Chimerism: 3 mnt: 89% full donor chim 6 mnt: 94% full donor chim Graft failure: 6%	aGvHD: gr 2–3: 30% gr 3–4: 6% ext cGvHD: 12%	Any gr 3–4: 14% Moderate SOS: 1% Liver: 38% Stomatitis: 17% Diarrhea: 10% Hem. cystitis: 7%	OS 3yr: 91% DFS 3yr: 87%	[97]

61 7.9 yr (0.8–21.6)	AML CR2: 97% CR3+: 2% Refractory: 2%	BM: 35% MD: 71% MMD: 29% PBSC: 20% MD: 67% MMD: 33% CB: 45% MD: 8% MMD: 92%	Bu /Cy or /Mel or /Flu Bu Oral: 26% or IV: 74% +Cy: 57% +Mel: 31% +Flu: 12% (no dosages available)	ANC(0.5): 17 d Non-engraftment 2% Relapse 2yr: 27% Relapse 4–6yr: 31%	aGvHD: gr 2–4: 45% gr 3–4: 18% lim cGvHD: 13% ext cGvHD: 13%	No exact data Death due to Pulmonary tox: 5%	OS 2yr: 66% OS 4–6yr: 56% TRM 2yr: 16% EFS 2yr: 63% EFS 4yr: 49% EFS 6yr: 37%	[1]

[a] ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, APML: acute promyelocytic leukemia, NHL: non-hodgkin lymphoma, SAA: severe aplastic anemia, SCA: sickle cell anemia, Thalas M: β-thalassemia major, Cl: class, CR: complete remission.

[b] MSD: matched sibling donor, (M)MFD: (mis)matched family donor, (M)MUD: (mis)matched unrelated donor, MD: matched donor, MMD: mismatched donor, PBSC: peripheral blood stem cells, (U)BM: (unrelated) bone marrow, (U)CB: (unrelated)cord blood.

[c] Bu: busulfan, Flu: fludarabine, ATG: anti-thymocyteglobuline, Dex: dexamethasone, CY: cyclophosphamide, TT: thiotepa, Amab: alemtuzumab, ST: serotherapy, Mel: melphalan, Hu: hydroxyurea, Aza: azathioprine, IV: intravenous, ibusulfan dosing based on BW described in [89], iibusulfan dosing based on EMA nomogram: patient <9 kg: 1.0 mg/kg, 9–16 kg: 1.2 mg/kg, 16–23 kg: 1.1 mg/kg, 23–34 kg: 0.95 mg/kg and >34 kg: 0.8 mg/kg.

[d] ANC(0.5): first of 3 consecutive days with an absolute neutrophil count ≥ 0.5 x 10^9/L, Plts (20) or (50): platelet count ≥ 20 x 10^9/L or ≥ 50 x 10^9/L unsupported for 7 days.

[e] aGvHD or cGvHD: acute or chronic GvHD, lim: limited, ext: extensive, gr: grade.

[f] Hem. cystitis: hemorrhagic cystitis, tox.: toxicity, iiigrowth failure requiring growth hormone replacement.

[g] DFS: disease-free survival, EFS: event free survival.

Table 2.2 Overview of recent studies and outcomes in treosulfan-based conditioning before allogeneic HSCT in pediatric patients

Patients (n) and median age (range)	Underlying disease[a]	Donor matching and source[b]	Conditioning regimen[c]	Engraftment and chimerism[d]	GvHD[e]	Toxicities[f]	Survival[g]	Ref
19 3.9 yr (0.3–22)	HLH	MSD: 5% MFD: 26% MUD: 32% MMD: 37% (9/10)	Treo/Flu/TT Treo 42 or 36 g/m^2 (<12kg) Flu: 150–180 mg/m^2 or 5–6mg/kg TT: 10 mg /kg or 7 mg/kg (<12kg) ST: dose depending on matching	Primary engraftment: 100% Rejection: 11% Chimerism: Full donor: 47% Mixed: 53%	aGvHD: 30% gr 1–2: 23% gr 3: 6%	SOS: 6% (+prophylaxis) Hemorrhage sev: 12% Mucositis gr 3–4: 12% Skin gr 3: 18%	OS: 100% DFS: 100%	98
70 0.7 yr (0.1–14.6)	PID	MSD: 11% MFD: 19% UD (7–10/10): 64% HaploID: 6% BM: 57% PBSC: 19% CB: 24%	Treo/Cy or Flu Treo: 36–42 g/m^2 Cy 200 mg/kg; 43% Fu 150 mg/m^2: 57% ST: All except MSD, URD-CB 9/10 (4/6), MSD (3/5), MFD, and 2nd Tx	Rejection: 3% Mixed, boost: 4% Chimerism 1 yr: Full donor: 57% Stable mixed: 43%	aGvHD: 26% gr 3–4: 10% lim cGvHD: 6%	Seizures: 6% Sev SOS: 3% (Cy) Virus infection: 26% Skin: common Mucositis: mild (no numbers)	OS: 81% TreoFlu: 85% TreoCy: 77%	14
51 8 yr (0.7–17)	HR or advanced hematologic malignancy: AML, ALL, MDS, NHL, CML, LCH	MSD: 47% UD (9–10/10): 53% BM: 59% PBSC: 37% CB: 4%	Treo/various Treo: 30–42 g/m^2 Cy±VP-16/Mel: 59% Flu±Mel: 35% Mel: 6% Flu: 150–180 mg/m^2 Cy: 120 mg/kg Mel: 140 mg/m^2 VP-16: 30–40 mg/kg ST: UD recipients	Primary engraftment: 94% ANC(0.5): 17 d Plts(20): 20 d Chimerism: Full donor: 90%	aGvHD: gr 3–4: 18% lim cGvHD: 9% ext cGVHD: 9%	Mucositis: 35% GI: 14% Hepatic: 18% Pulmonary: 2%	NRM: 10% DFS: 67% Relapse: 22% MUD: 8% MSD: 39%	12

6 10 mnt (9–11)	ALL infants CR1: 83% CR2: 17%	UCB: 83% 4/6: 17% 5/6: 33% 6/6: 50% MSD, BM: 17%	Treo/Cy Treo: 36–42 g/m^2 CY: 60 mg/kg/d 2d ST: 67%	Primary engraftment: 100% ANC(0.5): 18 d Chimerism: Full donor: 83% Mixed >80% donor: 17%	aGvHD≥2: 33%	SOS mild: 17% Mucositis sev: 17% Infections: 67% Microangiopathy: 17%	OS: 83% Relapse: 17%	99
50 11 yr (2–21)	Thal M Cl 3 +	MRD: 97% BM: 26% PBSC: 74%	Treo/Flu/TT Treo: 42 g/m2 Flu: 30 mg/m2/d 4d TT: 8 mg/kg ST: none	Primary engraftment: 94% ANC(0.5): 16 d Plts(20): 21 d Rejection: 8%	aGvHD: 35% gr 2–4: 26% cGvHD: 11%	SOS: 22% (other toxicities not reported)	OS (3yrs): 87% TRM: 12%	16
28 9.6 yr (2–18)	Thal M Cl 1–3	BM: 75% BM+CB: 18% PBSC: 7% Blood group: mismatch: major: 11% bidirectional: 7% minor: 14%	Treo/Flu/TT Treo: 42 g/m^2 Flu: 30 mg/m^2/d 4d TT: 8 mg/kg ST: None	ANC(0.5): 15 d Plts(20): 21 d Chimerism: Full donor: 64% Mixed: 18% Rejection: 7%	aGvHD: gr 2–4: 14% lim cGvHD: 10%	Mucositis: gr 1–3: 68% gr 3: 7% SOS sev: 11%	OS (±1yr): 79% TRM: 21%	17

Table 2.2 continues on next page

Table 2.2 *Continued*

Patients (n) and median age (range)	Underlying disease[a]	Donor matching and source[b]	Conditioning regimen[c]	Engraftment and chimerism[d]	GvHD[e]	Toxicities[f]	Survival[g]	Ref
109 8 yr (0.1–20.1)	Malignant and non-malign. disease and also solid tumors	MSD: 22% MFD: 45 % MMFD: 15% MUD: 56% MMUD: 2% BM: 49% PBSC: 45% CB: 2% CB+PBSC: 2%	Treo + various Treo: 21–42 g/m^2 Flu: 30 mg/m^2/d 5d or 6d 67% TT: 40% Mel: 295% Radiotherapy: 1% ST: 77%	Primary engraftment: 100% WBC (1.0): 20 d Plts(20): 17.5 d Chimerism: Mixed chimera + boost: 3% Rejection: 3%	aGvHD: gr 2–4: 26%	SOS: 3% Skin gr 4: 4% Pulmonary gr 4: 9% Neuro: 9% Seizures: 4% Cardiac: 6.4% Mucositis: mild GI: mild Nappy rash/skin: common	OS (2.5yr): Non-mailgn: 88% Malignant: 49% TRM: 12%	[37]

[a] ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, DBA: Diamond-blackfan anemia, HLH: hemophagocytic lymphohistiocytosis, JMML: juvenile myelomonocytic leukemia, MDS: myelodysplastic syndrome, Non-malign.: non-malignant, PID: primary immune deficiency, Thalas M: β-thalassemia major, HR: high risk, CR: complete remission, CI: class.

[b] MSD: matched sibling donor, (M)MFD: (mis)matched family donor, UD: unrelated donor, (M)MUD: (mis)matched unrelated donor, HaploID: haploidentical donor, PBSC: peripheral blood stem cells, BM: bone marrow, CB: cord blood.

[c] TREO: treosulfan, CY: Cyclophosphamide, VP-16: etoposide, Flu:fludarabine, Mel:melphalan, TT: thiotepa, ST: serotherapy (anti-thymocyteglobuline or alemtuzumab).

[d] ANC(0.5)or ANC (1.0): first of 3 consecutive days with an absolute neutrophil count ≥ 0.5 x 10^9/L or 1.0 x 10^9/L, WBC(1.0): White blood cell count of 1000/μL, Plts (20) or (50): platelet count ≥ 20 x 10^9/L or ≥ 50 x 10^9/L unsupported for 7 days.

[e] aGvHD or cGvHD: acute or chronic GvHD, ext: extensive, lim: limited.

[f] GI: gastrointestinal tract toxicity, Neuro: neurological toxicity, gr: grade, sev: severe,

[g] RRM: regimen-related mortality, NRM: non-relapse mortality, DFS: disease-free survival.

of the conditioning, RIC was developed to minimize the toxic effects before HSCT. A busulfan-based conditioning with an intermediate dose of 8 mg/kg in total was applied in RIC and treosulfan was introduced as an alkylating agent.[12] In non-malignant disease, busulfan and treosulfan are applied in the same group of patients, in line with current Working Party Inborn Errors/EBMT guidelines. Most experience with treosulfan-based conditioning is gained in primary immune deficiencies (PID) and β-thalassemia. In a large study of 70 pediatric patients with a PID, treosulfan was combined with fludarabine or cyclophosphamide. In these children, a generally mild toxicity profile was observed combined with an overall survival (OS) of more than 80%.[14] The largest study of treosulfan in pediatric patients and young adults with β-thalassemia demonstrated a 5-year OS of 93%, a β-thalassemia-free survival of 84% and no cases of hepatic sinusoidal obstruction syndrome (SOS).[15] The authors suggest that treosulfan-based conditioning is as effective as busulfan-cyclophosphamide-based conditioning and is probably accompanied with less toxicity. However, the long-term effects of treosulfan-based conditioning are not known yet.

Again, there is no study available directly comparing treosulfan- and busulfan-based conditioning in pediatric patients with non-malignant disease. Two studies compared treosulfan, combined with fludarabine and thiotepa, with a retrospective cohort of busulfan (Bu/Cy) in patients with high-risk β-thalassemia.[16,17] In the first study, TRM and OS were more beneficial in the treosulfan-treated group; OS was 87% in the treosulfan group versus 64% in the busulfan group. The main toxicity was SOS, and the incidence was significantly higher in the Bu/Cy group (66%) compared with the treosulfan-treated group (22%). However, the second study found a higher TRM in the treosulfan group (21%), compared with the busulfan-treated group (0%) and 11% of the treosulfan-treated patients died because of severe SOS. Remarkably, in both studies, especially in the treosulfan group, the incidence of SOS was unusually high.

Therefore, treosulfan is a potentially effective agent with a relatively mild toxicity profile. However, there is a need for a prospective study directly comparing busulfan- and treosulfan-based conditioning in non-malignant diseases. Furthermore, data on the long-term effects of treosulfan in pediatric patients are warranted.

Toxicity of busulfan

Early toxicity

The main early toxicities of busulfan are liver toxicity, pulmonary toxicity, hemorrhagic cystitis, seizures, skin toxicity, diarrhea and mucositis.[18,19] Busulfan-based regimens are

known to cause liver toxicity, ranging from elevated liver enzymes to SOS. Together with GvHD and infections, SOS is one of the most common early complications after HSCT, occurring in 5–40% of pediatric patients with potentially fatal outcomes.[20,21] The syndrome is characterized by hepatomegaly, elevated serum bilirubin levels and fluid retention resulting in weight gain. The variability of incidence of SOS can be influenced by the conditioning regimen, patient characteristics, age, underlying disease and existing liver damage.[22] High busulfan exposure and busulfan combined with cyclophosphamide is related to an increased risk of SOS.[23–25] Sufficient time between busulfan and cyclophosphamide administration to allow recovery of glutathione (GSH) depletion or replacement of cyclophosphamide by fludarabine could reduce SOS incidence.[26] Furthermore, orally administered busulfan is associated with a higher rates of SOS, because of high variability in exposure and possibly the first-pass effect after oral administration leading to high busulfan concentrations in the small hepatic venules, resulting in damage.[27]

The incidence of seizures in pediatric patients receiving busulfan has been reported to be between 2% and 10%.[28,29] It is common practice to administer seizure prophylaxes during busulfan-based conditioning and a variety of antiepileptic drugs has been applied with success in clinical practice.

Late toxicity

Many pediatric allo-HSCT recipients develop long-term complications. Given their improved life expectancy as a result of HSCT, long-term effects are of major concern. In contrast to total-body irradiation, busulfan itself does not cause growth retardation. Growth retardation after busulfan-based conditioning is probably caused by factors such as prior cranial irradiation, underlying disease or long-term use of high doses of glucocorticoids for chronic GvHD.[30,31] Gonadal dysfunction is a prominent adverse effect after busulfan-based conditioning, especially in young girls, where up to 70% of patients have ovarian failure following busulfan-based HSCT.[32,33] Furthermore, both hypothyroidism and hyperparathyroidism frequently occur after Bu/Cy-based conditioning for HSCT.[33] Busulfan causes permanent alopecia in up to 50% of the patients and is related to busulfan exposure.[34]

Toxicity of treosulfan

The main toxicities of treosulfan are mucositis, skin toxicity, diarrhea and hepatic toxicity.[12,14,35] Mucositis and hepatic toxicity are generally mild compared with busulfan-based conditioning.[36] Only rarely does hepatic toxicity develop into SOS and occurrence of SOS is dependent on

pre-HSCT comorbidities and the combination of treosulfan with other alkylating agents (e.g. cyclophosphamide or melphalan).[12,14] In a recent report, 513 children received treosulfan-based conditioning for their allo-HSCT. The overall SOS incidence was 5%. However, the incidence was higher in patients younger than 6 months (12%).[35] In other reports, nappy rash and skin toxicities were reported regularly in infants.[37] It is suggested that nappy rashes are probably caused by secondary excretion of the active metabolite l-epoxybutane in the urine.[38] Slatter *et al*.[14] reported seizures in treosulfan treated infants after cessation of treosulfan; however, other studies did not report any seizures in such children when patients had pre-HSCT central nervous system (CNS) injury. In general, the use of anticonvulsant prophylaxes before treosulfan is not recommended. However, prophylaxes might be useful in infants.

Given that treosulfan treatment has only recently been introduced for HSCT, only limited data on the long-term effects of treosulfan treatment are available. To date, no long-term toxicities have been reported. Therefore, common long-term toxicities in busulfan-treated patients, such as gonadotoxicity, are to be evaluated in pediatric patients undergoing treosulfan-based conditioning.

PK OF BUSULFAN

Busulfan PK is best described as a one-compartment model. When busulfan is administered orally, absorption is a determinant of high inter- and intrapatient variability in exposure. C_{max} generally occurs within 1.5–2.5 h and bioavailability is approximately 70–90%, but highly variable.[39] Different factors can cause reduced and variable bioavailability in pediatric patients; the administration of numerous tablets can cause nausea and vomiting and different methods of administration are applied. Crushed tablets are suspended in water, mixed with food or encapsulated to promote swallowing, and tablets can be administered directly or through a gastric tube. Furthermore, a higher gastric pH, differences in transit time and, potentially, a higher first-pass clearance in children can affect bioavailability.[40] A higher intestinal clearance in children is probably caused by upregulation of glutathione-S-transferase (GST) activity in younger children (0–4 years) compared with older children.[41] Intrapatient variability in exposure was reduced after introduction of the intravenous formulation and is generally <15%.[42] Interpatient variability with an oral formulation can be up to 50% and, when using the intravenous formulation, approximately 20–30% interpatient variability is observed.[42,43]

Busulfan clearance is age dependent; clearance in pediatric patients is enhanced compared with adults and can be two to four times higher.[40] In Table 2.3, different PK models are described and an overview of key PK parameters of busulfan in the pediatric population is

Table 2.3 Overview of population pharmacokinetic models recently developed in pediatric patients

Author	Model	Age range (y)	Clearance (Cl)	Volume of distribution(V)	Dosing accuracy and IIV/IOV	Ref
Trame	One-compartment model with 1st order absorption 2 models 1. Cl: corrected for BSA 2. Cl allometric scaled for BW Scaling exponent: 0.75 V in both models as function of BW	0.4–18.8	4.16 L/h/m^2 4.11 L/h/kg$^{0.75}$	15.2 L/kg	Dose =19.6 x BSA Dose = 19.4 x (BW/27.2)$^{0.75}$ 75–80% of patients within target AUC (900–1500 μM*min)	51
Bartelink	Two-compartment model Cl and V allometric scaled BW Variable scaling exponent Cl, based on BW: L1*BW^M = 1.2 - 0.55 $Cl = Cl_{15.3kg} * (BW/15.3)^{L1*BW^M}$ Scaling exponent V = 0.89(fixed)	0.1–26	3.47 L/h L1 = 1.56 M = -0.226	11.1 L	Model based nomogram: Clearance: IOV 15% IIV 27%	89
Paci	One-compartment model Cl and V allometric scaled for BW Scaling exponent for Cl: < 9kg = 1.25 ≥ 9 kg = 0.76 Scaling exponent for V: 0.86	0.03–15	2.18 L/h	nd	IIV Cl 23% V 22% IOV Cl 11%	100
Savic	One-compartment model Cl and V allometric scaled for BW Scaling exponent Vd = 1.0 Cl = 0.75 Cl also corrected for maturation effect: $Cl_i = Cl_{pop}\left(m + (1-m) * \left[1 - e^{-age*K_{mat}}\right]\right) * (BW/8kg)^{0.75}$ m = maturation magnitude effect for age K_{mat} =maturation rate constant	0.08–3.3	2.3 L/h m = 0.46 K_{mat} = 1.4	6.4 L/kg	IIV Cl 25% V 25%	53

BSA: body surface area (m^2), IIV: interindividual variability, IOV: interoccasion variability. Allometric scaling based on body weight occurs according to the following formula: BW_i^x / BW_{pop} in which BW_i is the body weight of the individual, BW_{pop} is the body weight of a typical individual set as such in the model and x the scaling exponent.

provided. Busulfan is metabolized by conjugation with glutathione, resulting in formation of a glutathione conjugate. This reaction is catalyzed by GSTA1, GSTM1 and GSTP1, and occurs in the liver and intestine. GSTA1 is the predominant GST enzyme involved in busulfan metabolism; GSTM1 and GSTP1 have 46% and 18% of the activity of GSTA1 in busulfan metabolism, respectively.[44] The glutathione-conjugate dissociates into γ-glutamyldehydroalanylglycine and tetrahydrothiophene (THT). THT is oxidized into sulfolane and subsequently into 3-hydroxy sulfolane (Figure 2.2).[45,46] It is suggested that cytochrome P450 enzymes are involved in the oxidation of THT and sulfolane.[47] Furthermore, it is suggested that transporters are involved in active transport of the glutathione conjugate out of the cell (Figure 2.2).[48,49]

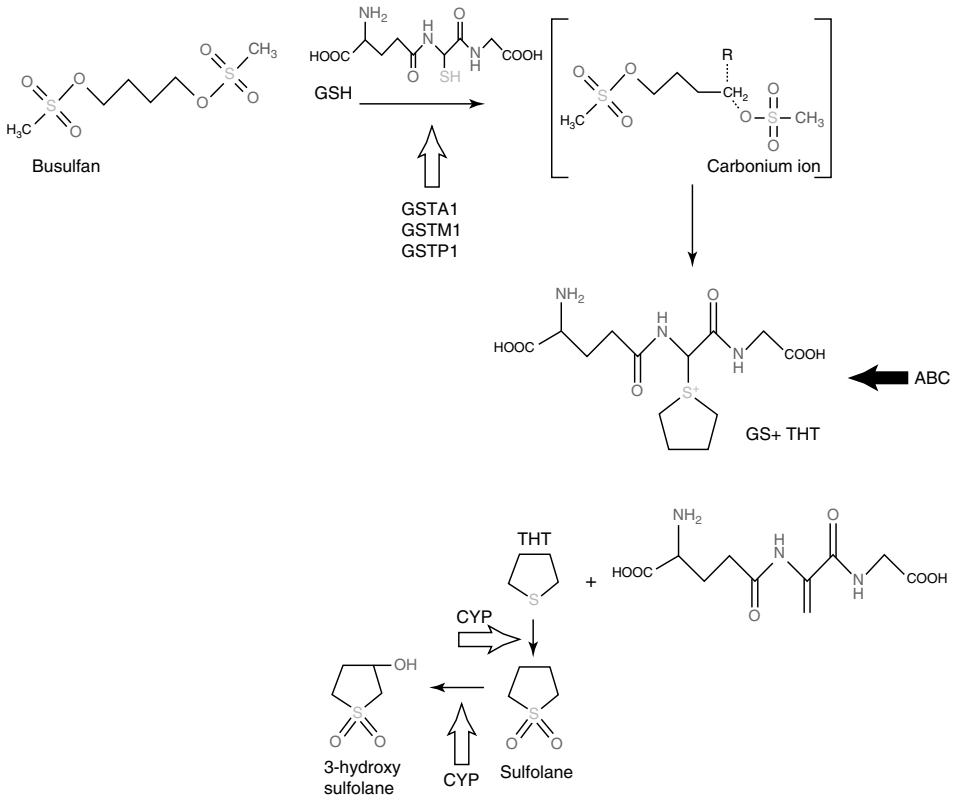

Figure 2.2 Metabolism of busulfan and potential involvement of enzymes and transporters. Busulfan conjugation with glutathione (GSH) is catalyzed by glutathione-S-transferases (GST). The conjugate dissociates into γ-glutamyldehydroalanylglycine and tetrahydrothiophene (THT). THT is oxidized into sulfolane and subsequently into 3-hydroxy sulfolane. GSTs are involved in the conjugation of busulfan with GSH. It is suggested cytochrome P450 enzymes (CYPs) are involved in the oxidation of THT and sulfolane and transporters are involved in active transport of the glutathione conjugate out of the cell. ABC: ATP-binding cassette transporters.

Factors influencing busulfan PK

Children have an increased busulfan clearance compared with adults, which is partially caused by an increased liver size to body weight (BW) ratio.[50] Therefore, clearance should be expressed by body surface area or allometrically scaled BW to account for this age-related variability in hepatic function.[47,51] These measures do not account for all the differences in busulfan clearance between adults and pediatric patients. It is demonstrated that younger children (2–4 year) have an elevated ratio THT+ (the metabolite of busulfan) to busulfan, because of enhanced conjugation of busulfan compared with older children and adults. No elevated GSH levels were measured and it was suggested that children have an increased metabolism due to higher expression or activity of GST rather than through more available GSH.[52] These processes have been incorporated in population PK models (Table 2.3). Furthermore, Savic *et al*.[53] developed a population PK model for busulfan in infants (<12 kg). In the model, BW was allometrically scaled as a measure of physiological growth. Age was also incorporated into the model to account for maturation of enzyme function during the first 2 years of life. In infants, clearance increased 1.7-fold between 6 weeks and 2 years of life. Overall, maturation in the GST expression or activity during the first 2 years of age is likely to have an important role and GST activity reaches a maximum by the age of 2 years.

Several investigators studied the effect of polymorphisms in the genes encoding GSTs involved in busulfan metabolism. The first study in pediatric patients demonstrated that children who were heterozygous or homozygous for the *GSTA1*B* haplotype (regardless of age) exhibited a 30% decrease in busulfan clearance after intravenous administration.[54] However, other groups did not find an association of *GSTA1* genotype with busulfan clearance, or only in patients receiving busulfan orally.[23,55,56] There are also conflicting results on the effect of the *GSTM1* genotype in relation to busulfan PK; one study demonstrated a lower busulfan clearance in patients with the *GSTM1*-null genotype,[23] which is conflicting with the findings of Srivastava *et al*.[57], who demonstrated a higher clearance and risk of SOS in *GSTM1*-null patients. The authors suggest that toxicity is caused by the metabolite rather than by busulfan itself. Several factors could account for these conflicting results, including the route of administration, the age range of patients and the way clearance is expressed. If clearance is not adjusted for body size, the interpatient variability in clearance is larger and the effect of body size dominates the probably smaller effect of the genetic variants.

Apart from GSTs, the involvement of other enzymes and transporters has been suggested.[48,49] Polymorphisms in transporters and other enzymes in relation to busulfan PK have been studied; Krivoy *et al*.[58] found a combined association of the *GSTM1* and ATP-binding cassette, sub-family B

(MDR/TAP), member 1 (*ABCB1*) genotype and oral busulfan clearance in adults and another study demonstrated the involvement of *CYP2C9* and *CYP2B6* in busulfan metabolism.[48] Furthermore, in a recent study, 40 genetic polymorphisms, including several genes encoding drug transporters and CYP enzymes, in relation to HSCT outcome were studied.[59] Only one polymorphism in GSTA2 was related to OS and TRM. In most of the PG studies on busulfan PK, a small set of polymorphisms was investigated that were likely to be involved in busulfan metabolism, according to the candidate gene approach. To further investigate the PG contribution to interpatient variability in busulfan PK, a shift from the candidate gene approach to a more broad PG analysis should be made.

In several studies, clearances between different disease groups were compared. It is suggested that patients with β-thalassemia have an increased and highly variable busulfan clearance caused by an iron overload inducing GST activation and pre-HSCT liver injury.[60] Pediatric patients with inherited diseases were associated with a low and highly variable busulfan clearance.[61] This could have been caused by the young age of the patients (mean age < 1 year) and co-medication. Hence, the role of underlying disease on busulfan PK is not clear, given that different studies demonstrate conflicting results.

Of the regularly administered co-medication, theoretically phenytoin, itraconazole, acetaminophen and metronidazole are associated with altered busulfan clearance.[62–64] Itraconazole is a strong inhibitor and phenytoin a strong inducer of cytochrome P450 enzymes, but its effect on busulfan metabolism is not clear. Phenytoin is often replaced with a different anticonvulsant, such as levetiracetam or clonazepam. The effect of itraconazole on busulfan exposure in clinical practice is not always evident; in one study, no influence of itraconazole on busulfan exposure was observed.[65] The effect of metronidazole on busulfan PK is probably caused by GSH depletion by the metronidazole reactive metabolites.[64] However, clinical evidence is missing, and metronidazole is rarely combined with busulfan. Hence, several interacting drugs for busulfan have been identified, although their effects in clinical practice are limited.

Analytical methods

To relate a PK profile of a drug to outcome and to guide dosing based on exposure, it is essential to have a proper bioanalytical method. Any method applied should be accurate and precise over the whole concentration range; the lower limit of quantification (LOQ) and concentration range should be adequate for busulfan determination in clinical practice and the method should be selective. Furthermore, relative short run times and sample preparations are important, because of the short course of busulfan. An overview of bioanalytical methods for determination of busulfan, cited in recent literature on busulfan, is given in Table 2.4. When

Table 2.4 Overview of bioanalytical methods for determination of busulfan, cited in recent literature on busulfan

Author	Method	Concentration range	Samples quantity	Run time	Derivatization time	Estimated total time	Ref
Kellog	LC-MSMS	123–2463 µg/L	50 µL plasma	3 min	NA	2 hour for 10 samples	[101]
Mürdter	LS-MS	10–2000 µg/L	200 µL plasma	10 min	NA	Unknown	[88]
Quernin	GC-MS	20–2000 µg/L	1000 µL plasma	14 min	2 hour	Unknown	[102]
Lai	GC-MS	40–4000 µL/L	1000 µL blood	12 min	1 hour	24h for 40 samples 3 h manual labour	[66]
Bleyzac	HPLC-UV	100–2000 µg/L	200 µL plasma	10 min	Direct	32 minutes per samples	[103]
Cremers	HPLC-UV	30–8000 µg/L	200 µL serum	10 min	Direct	2 hour for 10 samples	[67]

busulfan is administered daily, it is completely eliminated and, therefore, the LOQ of a method should be as low as possible, but at least 40 µg/L. In HPLC-UV and GC-MS analytical assays, derivatization of busulfan is conducted to make the compound detectable. This procedure is selective; only derivatized compounds are detected.[66,67] However, it can be a time consuming step; for example, in GC-MS, derivatization steps can take up to 2 hours. Another essential part is the required volume of patient material (plasma or serum), especially in young children. A limited sampling model is a helpful method to reduce the required total blood volume and, with that, patient burden. In modern LC-MSMS methods, 50–100 µl of serum or plasma can be sufficient. Older methods require up to 1000 µl. Overall, LC–MSMS methods are the preferred methods because of the smaller volumes required, shorter run times and simple sample preparation. However, disadvantages of these methods include the complexity and high costs of equipment.

PK and clinical outcome

For oral busulfan, several investigators demonstrated a relation between busulfan exposure and clinical outcome. In adults, high exposure was related to toxicity, especially SOS, and lower exposure was related to rejection of the transplant.[68]

However, in children, this relation was not that evident, which is likely to be the result of a lower overall exposure in pediatric patients because of an enhanced clearance, resulting in less toxicity and lower SOS incidence. Furthermore, soon after the relation between exposure and clinical outcome was established, therapeutic drug monitoring (TDM) was introduced to

optimize exposure, thereby reducing interpatient variability.[69] However, there are several studies demonstrating an association between exposure and clinical outcome in pediatric patients. Ljungman *et al*.[70] investigated the busulfan concentration at steady state (C_{ss}) in a cohort of adults and pediatric patients and demonstrated that a C_{ss}>721 ng/mL was associated with increased TRM and OS. Furthermore, in two pediatric cohorts, C_{ss}>600 ng/mL was associated with a higher probability of engraftment.[71,72] Unfortunately, these studies provided no target for busulfan exposure. Finding the optimal target for busulfan is hampered by the application of different measures of exposure: C_{ss} in ng/mL, area under curve (AUC) in μM*min or mg*h/L. Furthermore, these measures can be expressed per dose or cumulative over a 4-day course. In addition, busulfan can be administered one, two or four times daily, which results in different exposure levels per dose. Therefore, we advocate that busulfan exposure is expressed as a cumulative exposure over a 4-day course to make exposures more easily comparable.

Bartelink *et al*.[73] attempted to find the optimal busulfan AUC in pediatric patients receiving Bu/Cy-based conditioning. Exposure proved related to event free survival (EFS), graft failure and relapse, and the authors advocated targeting busulfan to a narrow therapeutic range of 74–82 mg*h/L. Many studies applied an AUC target of 900–1350 μM*min/dose, which is equivalent to a total AUC of 57.6–86.4 mg h/L which is a broad target compared with that defined by Bartelink *et al*.[73]

Using TDM, the total exposure can be optimally guided toward a narrow range. In a recent study, the cumulative exposure over a four day course was not related to clinical outcome and 90% of the patients were within the target range.[74] However, in this study, a C_{ss} after the first dose below 600 ng/ml was associated with increased OS and EFS and decreased non-relapse mortality. According to the authors, a higher first dose C_{ss} causes increased depletion of GSH, resulting in decreased metabolism of cyclophosphamide and increased toxicity.

Overall, busulfan exposure is related to clinical outcome and tight control of exposure could improve HSCT outcome after busulfan-based conditioning. Furthermore, the target exposure of busulfan depends on the regimen it is applied in. Optimal targets have mainly been investigated in Bu/Cy regimens. The optimal range for busulfan exposure in more recent applied combinations, such as busulfan and fludarabine, is more difficult to define, because of the up-front targeting of busulfan. In a recent study on busulfan–fludarabine-based conditioning in pediatric patients, the target AUC for busulfan was 80–95 mg h/L. This conditioning regimen proved to have a favorable toxicity profile compared with Bu/Cy (Mel) conditioning and was equally effective.[75] Moreover, more individualized target exposures should be defined, depending on the total conditioning regimen and the disease type of the patient.

PK OF TREOSULFAN

Treosulfan is non-enzymatically converted into its active metabolites; a mono- and diepoxide.[7] The two metabolites are excreted in the urine and no enzyme or transporter is known to be involved in treosulfan metabolism. There are several studies investigating the PK of treosulfan in patients, most of which were performed in adults. In almost all the studies, a linear relation between dose and exposure of treosulfan was found. Yet, the exposures demonstrated in the different studies are not in accordance with each other. The PK of treosulfan was first analyzed in adult patients with solid tumors.[76,77] Patients received 8 or 10 g/m^2, which is a typical dose in solid tumors,[76] or 20–56 g/m^2 with stem cell support.[77] The half-life of treosulfan was 1.8 hour[76] and 2.0 hour[77] and urinary excretion of the parent compound was approximately 25%. Half-life and AUC were somewhat increased in patients receiving the highest dose of 56 g/m^2, possibly because of acidotic changes in plasma leading to the decreased formation of active metabolites. Both studies demonstrated a linear relation between dose and exposure. After 10 g/m^2, a mean $AUC_{0-24\,h}$ of 977 ± 182 µg/ml*h was observed and a dose of 20 g/m^2 resulted in a mean AUC of 2325 µg/ml*h. In a third study in adult patients receiving 12 or 14 g/m^2 of treosulfan before SCT, a mean treosulfan exposure of 898 ± 104 µg/ml*h and 1104 ± 173 µg/ml*h, respectively, was observed.[78] Renal excretion of treosulfan was approximately 39%, a half-life that was comparable to earlier studies. However, exposures after 12 and 14 g/m^2 were comparable to the earlier observed AUC after a dose of 10 g/m^2. In another study with HSCT recipients, pediatric patients were also included. The mean AUC levels were higher than in the studies with solely adult patients, although the AUC levels after two different doses were similar: 1365 ± 293 µg/ml*h and 1309 ± 262 µg/ml*h, for 12 g/m^2 and 14 g/m^2, respectively.[79] Therefore, the authors suggested that increased doses would be unlikely to lead to increased efficacy.

PK studies of treosulfan in pediatric populations are limited. Glowka *et al*.[80] described the PK of a set of seven pediatric patients. In this study, three different dosages were administered: 10 g/m^2 (n = 1), 12 g/m^2 (n = 5) and 14 g/m^2 (n = 1) daily. Treosulfan PK was best described by a two-compartment disposition model with first-order elimination. Interpatient variability in patients receiving 12 g/m^2 was large, with a coefficient of variation of 70%. The exposure in the single patient receiving 14 g/m^2 was relatively high (1960 µg/ml*h) when considering a linear relation between dose and exposure. This high AUC could result from metabolic acidosis as a result of methanesulfonic acid formation resulting in a reduction of treosulfan conversion into the active derivatives.

There are some conflicting results between the different PK studies in adults and pediatric patients and, therefore, larger pediatric cohorts should be investigated. At this point, dose optimization-based PK of treosulfan is of interest, especially in pediatric patients.

Analytical methods

The PK data regarding treosulfan are scarce, which could be because of the limited availability of an analytical method for determination of treosulfan. Although treosulfan is a prodrug, most bioanalytical methods are aimed at analyzing treosulfan itself. Treosulfan is non-enzymatically converted into its active metabolites. Therefore, it is suggested that the concentration of treosulfan itself is a good representation of the alkylating activity.[76] Most analytical methods are based on reversed phase-HPLC methods (rp-HPLC) with refractometric detection.[77,81] To make treosulfan (and its metabolites) detectable via UV detection, treosulfan can be derivatized.[82] UV-detection is a more selective and sensitive detection method, although derivatization can be time consuming. The conversion of treosulfan is pH dependent; therefore, in most studies, blood samples were acidified directly after collection by adding citric acid. This could be considered a complicated logistics step that might hamper sample collection in clinical practice.

PK and clinical outcome

Data of treosulfan exposure in relation to clinical outcome are scarce. Moreover, most of the studies on treosulfan PK have only limited patient numbers and insufficient power to assess an association with clinical outcome, although attempts have been made to relate clinical outcome to treosulfan dose. Scheulen *et al*.[77] performed a dose escalation study to assess the maximum tolerated dose. Doses higher than 47 g/m^2 were associated with severe toxicities. Normally in treosulfan-based conditioning, treosulfan doses up to 42 g/m^2 are divided over 3 days. It is unknown whether toxicities are comparable when the cumulative dose is administered as one dose or when it is divided over multiple days.

In a small adult patient population (n = 18) with malignant disease, the PK and clinical outcome of two different doses of treosulfan (36 and 42 g/m^2, cumulative dose) were studied. There were no differences in clinical outcome (engraftment, non-relapse mortality and OS) between the two doses.[78] Different doses of treosulfan in pediatric patients undergoing HSCT because of malignant diseases (myeloid and lymphoid, n = 51) have been studied.[12] In this study, the higher dose of treosulfan (total 42 g/m^2) was more beneficial in relation to donor engraftment and hematopoietic chimerism. Nevertheless, there was no significant difference in DFS in patients receiving different doses of treosulfan.

The cumulative dose of 42 g/m^2 is a common dose regimen for treosulfan-based conditioning; toxicity is mild and engraftment might be enhanced at this dosing level. However, the outcome of HSCT is based on many factors and the most appropriate dose remains to be elucidated.

Whether exposure is a better marker for treosulfan efficacy and safety than the cumulative dose, as observed in busulfan, needs to be further investigated. Furthermore, in clinical practice, children under 1 year of age frequently receive a reduced dose of 30–36 g/m^2. However, there is currently no evidence supporting this practice.

DOSE OPTIMIZATION OF BUSULFAN

Therapeutic drug monitoring

Therapeutic drug monitoring (TDM) is of clinical value for drugs with a narrow therapeutic index, a clear relation between exposure and clinical outcome, substantial interpatient variability and small intrapatient variability, repeated administration, absence of alternative laboratory parameter and an appropriate analytical method.[83]

Busulfan meets all the criteria for applying TDM, except for intrapatient variability in oral busulfan, which is relatively large. Lindley *et al*. applied a test dose to predict busulfan exposure after oral administration and, in only 46% of the patients, the apparent oral clearance predicted the appropriate dose to achieve the target AUC.[84] After intravenous administration, the intrapatient variability was significantly reduced and, in a higher percentage of the patients, the target exposure was reached when TDM was applied.[85] Therefore, TDM-guided dosing is more useful in an intravenous regimen of busulfan. Furthermore, because of the short treatment course with a maximum of 4 days, it is necessary to have sufficient expertise and a proper logistic process in place for TDM-guided dosing of busulfan.

Especially in pediatric patients, it is essential to limit the burden of TDM caused by serial blood sampling. Therefore, the application of a limited sampling model can help to achieve this goal.

Based on two to four plasma-level measurements, the exposure to busulfan can be adequately estimated. Many published limited sampling models are based on a regression algorithm. Regression algorithms are capable of predicting the busulfan exposure accurately and precisely using concentrations measurements in three samples.[86] However, the disadvantage of these algorithms is the necessity of sampling at the exact time points included in the algorithm. Small deviations in the sampling time point can results in significant deviations in predicted AUC. In addition, Bayesian PK procedures have been used to determine busulfan exposure. These procedures provide more flexibility in sampling times and, with that, higher accuracy in exposure estimation.[67]

Dosing nomograms and population PK modelling

A population PK model (pop-PK model) can be constructed to describe the PK profile of a drug in the population, incorporating patient characteristics that contribute to the interpatient variability. From these PK models, dosing nomograms can be extracted, ultimately leading to target exposure in individual patients without requiring TDM or at least rapid achievement of target AUC. Nguyen *et al*. developed a five-step nomogram for intravenous busulfan with different doses based on body weight of the patient (<9 kg, 1.0 mg/ kg; 9–16 kg, 1.2 mg/kg; 16–23 kg, 1.1 mg/kg; 23–34 kg, 0.95 mg/ kg; and >34 kg, 0.8 mg/kg).[87] This nomogram is the official dosing recommendation of the European Medical Agency (EMA), although studies have demonstrated that, with this dosing nomogram, a large portion of the patients do not reach the target AUC and TDM remains necessary to individualize the dose.[88] Recent models apply allometric scaling of the PK parameters clearance and volume of distribution. For clearance, the scaling component can be either fixed at 0.75 or the exponent can be varied based on the age or weight of the individual. The latter approach results in a higher exponent in infants and neonates, which accounts for a faster increase in clearance with growth and maturation. Not only body size, but also an increase in GST activity causes this phenomenon.[53,89] Dosing simulations based on the recently developed pop-PK models (Table 2.3) demonstrate that a large proportion of patients theoretically reach the target exposure. However, the proposed regimens have not been validated prospectively in independent cohorts. Furthermore, approximately 25% of the interpatient variability remains unexplained, suggesting that TDM is still a necessity to further optimize busulfan dosing.

DOSE OPTIMIZATION OF TREOSULFAN

In a phase I dose escalation study in adults, it was demonstrated that a dose of 47 g/m^2 is the maximum tolerated dose of treosulfan.[77] Furthermore, in a few studies, different doses of treosulfan in patients undergoing HSCT were applied, and relations with clinical outcome were assessed. However, most of these studies were limited by sample size[78] or highly variable patient characteristics[12] and no definite conclusion about the optimal dose can be made based on them.

It is essential to further study the PK and PD profile of treosulfan in pediatric patients. It seems more rational to choose the treosulfan dose based on the underlying disease and condition of the patient. In malignant diseases and, for example, β-thalassemia, a myeloablative regimen is required to give a maximum reduction of the malignancy and autologous hematopoiesis and to allow adequate donor engraftment. Treosulfan was first applied in allo-HSCT as a reduced intensity regimen. However, aplasia induced by treosulfan is rapid and sustained, and engraftment after high doses of treosulfan is prompt, resulting in early achievement of full donor

chimerism.[90] To date, it is not clear which dose of treosulfan is more likely to be a RIC or MAC, and which other factors are involved. For example, other drugs in the conditioning regimen and age of the patient and, with that, the level of treosulfan exposure could contribute to the myeloablative potency and toxicity profile of treosulfan.

CONCLUDING REMARKS

Busulfan and treosulfan are both effective in conditioning before HSCT in pediatric patients. The experience with busulfan is broader than with treosulfan, and many studies have demonstrated that TDM-based dosing is pivotal to achieve optimal busulfan exposure and, thus, clinical outcome. Furthermore, improved understanding of maturation processes in children, the development of pop-PK models and PG studies has revealed factors accounting for interpatient variability in busulfan exposure. The role of PG markers in the efficacy of busulfan should be further elucidated by broader PG analyses and in larger cohorts. Additionally, the effect of genetic markers could be incorporated in pop-PK models and could explain a portion of the remaining interpatient variability.

Knowledge of the treosulfan PK profile in pediatric patients is limited and PK-PD studies are necessary to optimize the dose. The first steps to increase this knowledge have been made. It is essential to have a proper bioanalytical method and population PK methods should be developed. At this point, PG analysis on treosulfan metabolism is probably not relevant because no metabolic enzymes or transporters appear to be involved in treosulfan PK.

The two agents were not prospectively compared in children with either malignant or non-malignant disease. Therefore, trials comparing both agents in well-defined patient groups are warranted. The development of a new study in pediatric patients with acute lymphoblastic leukemia in which treosulfan and busulfan are randomized versus total-body irradiation based conditioning is important. In this study, primary outcome will be mainly focused on relapse risk.

In addition, the usefulness of TDM and PG to achieve optimal clinical outcome is mainly based on short-term outcome parameters, such as relapse, engraftment and GvHD, whereas, for both alkylating agents, long-term outcome parameters (such as longterm survival and late toxicities) are also important. In the long run, given the potential long-term toxicity of chemotherapeutic agents, one would like to define the lowest dose level at which these drugs are effective in the various disease situations, thus limiting adverse effects as much as possible. In conclusion, better understanding of the PK and PG of busulfan and treosulfan has the potential to further improve the outcome of pediatric allo-HSCT.

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Chapter 3

Effect of genetic polymorphisms in genes encoding GST isoenzymes on busulfan pharmacokinetics in adult patients undergoing hematopoietic stem cell transplantation

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ABSTRACT

Busulfan is used in conditioning regimens before allogeneic hematopoietic stem cell transplantation (allo-HSCT). High busulfan exposure is associated with toxicity, whereas low busulfan exposure leads to higher rates of therapy failure. The pharmacokinetics of busulfan show large interpatient variability, hypothesized to be caused by variability in busulfan metabolism. In this report, the effect of genetic polymorphisms in three glutathione-S-transferase genes involved in busulfan metabolism *GSTA1* (-69C/T), *GSTM1* (deletion–mutation) and *GSTP1* (rs1695) on the pharmacokinetics of busulfan in Caucasian adult patients was investigated. In all, 66 adult patients received busulfan as part of their conditioning regimen. After the first infusion, two serum samples were collected and measured using a HPLC assay. A one-compartment population model was used to estimate individual pharmacokinetic parameters. The genetic variants of the three glutathione S-transferase (GST) genes were determined by pyrosequencing and PCR. A reduction of 14% in busulfan clearance was seen for the *GSTA1**B allele and an increase in busulfan exposure was found. No relationship was found between polymorphisms in *GSTM1* and *GSTP1* and busulfan pharmacokinetics. This study shows that an increasing number of copies of *GSTA1**B allele results in a significant decrease of busulfan clearance.

INTRODUCTION

Busulfan is frequently used in high-dose conditioning regimens before allogeneic hematopoietic stem cell transplantation (allo-HSCT) in malignant and non-malignant diseases. Busulfan has a narrow therapeutic index; high busulfan exposure is associated with toxicity, such as veno-occlusive disease (VOD) and mucositis, whereas low busulfan exposure leads to higher rates of graft failure and rejection.[1,2]

Initially, busulfan was available as an oral formulation only, which resulted in high variability in exposure, probably as a result of inter- and intraindividual variation in bioavailability. However, the introduction of an intravenous formulation did not completely eliminate the variability in pharmacokinetics.[3] Therefore, treatment with busulfan in clinical practice is often individualized by therapeutic drug monitoring.

It is presumed that the variability in busulfan exposure could be assigned to busulfan metabolism. Conjugation with glutathione is the primary route of metabolism of busulfan, catalyzed by isoenzymes of the glutathione S-transferase (GST) protein superfamily. GSTA1 is the predominant GST isoenzyme involved; GSTM1 and GSTP1 have 46 and 18% of the activity of GSTA1 in busulfan metabolism, respectively.[4] Therefore, it is hypothesized that single nucleotide polymorphisms (SNPs) in the genes encoding for the isoenzymes of GST could influence busulfan clearance and that interindividual variability might be explained by SNPs in these genes.

Expression of *GSTA1* is variable due to four SNPs in linkage disequilibrium in the promoter region of the gene; -631T or G, -567T, -69C, -52G, designated as *hGSTA1**A; and -631G, -567G, -69T, -52A, designated as *hGSTA1**B.[5] *GSTM1* is highly polymorphic; a deletion mutation resulted in no expression of the gene,[6] which is the case in 50% of the Caucasian population.[7] The SNP 313A/G in *GSTP1* leading to amino acid replacement in the active binding site of GSTP1, decreases the catalytic activity of the enzyme.[8,9] We hypothesize that SNPs in the genes encoding for the three GST isoenzymes influence the pharmacokinetics of busulfan. Furthermore, this effect may be dependent on race or age of the patients. We reported earlier on a study in which the effect of polymorphisms in the GSTs on busulfan pharmacokinetics in children was investigated.[10] No overall effect was found, however, a small effect was found in the older children suggesting an age effect on the expression of the GSTs. For this reason, we investigated the association between polymorphisms in genes encoding for the GSTs GSTA1, GSTM1 and GSTP1 and busulfan exposure in Caucasian adult patients undergoing HSCT.

PATIENTS AND METHODS

Patient and disease characteristic

Patients receiving allo-HSCT between 2004 and 2008 in the Leiden University Medical Center were included using the following selection criteria: age between 18 and 70 years, Caucasian ethnicity, receiving busulfan as conditioning before their allo-HSCT with measured busulfan blood samples. Diagnoses that required HSCT were malignant; acute myeloid leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, chronic myelomonocytic leukemia, non-Hodgkin's lymphoma or multiple myeloma or non-malignant; aplastic anemia or sickle cell anemia.

Conditioning regimes and transplantation details

Patients received either myeloablative conditioning or non-myeloablative conditioning. The myeloablative conditioning consisted of intravenous busulfan 0.8 mg/kg four times daily from day -9 to -6,intravenous alemtuzumab 15 mg once daily on days -6 and -5 and intravenous cyclophosphamide 60 mg/kg once daily on days -4 and -3. The non-myeloablative conditioning consisted of intravenous busulfan 0.8 mg/kg four times daily on days -6 and -5, oral fludarabine 50 mg/m^2 once daily from day -10 to -5 and anti-thymocyte globulin from day -4 to -1. Patients with chronic myelomonocytic leukemia received the same non-myeloablative conditioning plus 2 days of cyclophosphamide 750 mg/m^2 once daily on days -4 and -3. The graft was incubated with 20 mg of alemtuzumab just before infusion in all patients. Busulfan dosing was based on actual body weight. All patients received 92 mg phenytoin three times daily for seizure prophylaxis, starting 1 day before busulfan administration.

Blood sampling

Busulfan was administered intravenously in a 2 h infusion, starting in the morning at 0900 hours. Serum drug level measurements were carried out at 2.5 and 4.0 h after the start of the first infusion on the first day of treatment as part of routine patient care. Blood samples were stored at -20°C before processing, when processing took place on the same day, the samples were stored at 4°C.

A validated limited sampling model was used in order to minimize the number of blood samples necessary to calculate the busulfan clearance and area under curve (AUC).[11] Busulfan was analyzed in serum by a validated HPLC involving precolumn derivatization, liquid/liquid

extraction, and UV detection according to Chow *et al*.[12] The assay was linear between 0.03 and 8 mg/L. The limit of quantification was 30 mg/L. Precision at 0.2 and 1.5 mg/L was 3.5 and 0.8%, respectively. The pharmacokinetic model used was developed in MW/Pharm version 3.6 (Mediware, Groningen, The Netherlands).[13] A one-compartment population model with linear elimination as formulated, based on a busulfan model developed for children by this group first in NONMEM[11] and after that in MW Pharm.[14] Using the KinPop module of MW Pharm, in which an iterative two- stage Bayesian procedure based on busulfan serum concentration values of 34 adult patients was carried out, the new means, medians and standard deviations of the pharmacokinetic parameters were calculated. During the iterative two-stage Bayesian procedure, pharmacokinetic parameters were set to be distributed normally. The mean final pharmacokinetic parameters of the population pharmacokinetic model used are clearance: 13.3 L/h, volume of distribution: 50.8 L and busulfan half-life: 2.65 h. The calculated mean population pharmacokinetic parameters, clearance and half-life, were individualized according to the maximum a posteriori Bayesian fitting method.[15] AUC was calculated by dividing the administered dose by clearance. No dose adjustments were made on the basis of the individual calculated AUC.

DNA extraction and GST analysis

Residual blood samples taken for routine patient care were used for genotyping. All blood samples were anonymized according to the instructions stated in the Codes for Proper Use and Proper Conduct (http://www.federa.org). A crude DNA extract, prepared by lysing WBCs in SDS and proteinase k containing buffer, was used for PCR. The *GSTA1* haplotype was determined by investigating the SNP at -69C/T. The following genetic variants for *GSTP1* (313A/G, rs1695) and *GSTM1* (deletion–mutation) were determined.[6] The SNPs in the *GSTA1* and *GSTP1* genes were separately genotyped by pyrosequencing[10] (Isogen, Maarssen, The Netherlands) and presence of the *GSTM1* was determined by PCR amplification (215 bp) together with an internal control (albumin gene (350 bp). PCR products were analyzed on 2% agarose gels, as previously described by Zwaveling *et al*.[10]

Clinical outcomes

Next to pharmacokinetic parameters, the association of GST SNPs and HSCT clinical outcome was assessed. VOD was diagnosed according to the Seattle criteria: the occurrence of at least two of the following symptoms; painful hepatomegaly, unexplained weight gain of ≥ 2% from baseline and hyperbilirubinaemia; bilirubin 34 mmol/L or greater, present before

day 21 after HSCT and after exclusion of other possible causes.[16] The diagnoses and severity of acute graft-versus-host disease (aGvHD) was defined according to Glucksberg *et al*.[17] Rejection of the graft was defined as absence of donor cells. Engrafment was defined as stable donor chimerism.

Statistical analysis

All pharmacokinetic parameters are shown as mean ± s.d. Associations with busulfan pharmacokinetic parameters (clearance and AUC) and polymorphisms were tested with analysis of variance for *GSTA1* and *GSTP1* or Student's t-test for *GSTM1*. The effect of the number of copies of the T-allele in *GSTA1* genotype was tested by linear regression analysis. The effect of the polymorphism on the defined clinical outcome was explored by χ^2-test. P-values < 0.05 were considered significant. All statistical analyses were carried out in PASW statistics, version 17.0.01 (SPSS Inc., Chicago, IL, USA).

RESULTS

Description of the cohort

A total of 66 patients that received busulfan in the period between 2004 and 2008 were included. Baseline patient characteristics are shown in Table 3.1. The average age of the patients was 52.1 years and 59% were male. Most of the patients (n = 64) received busulfan in the non-myeloablative regimen. Patients included in this study received an average dose of 59.3 mg busulfan, which resulted in a mean busulfan clearance of 0.18 ± 0.05 L/h/kg and a mean AUC of 1153 ± 331 mmol*min/L.

GST polymorphisms

The overall success rate of genotyping the *GSTA1*, *GSTM1* and *GSTP1* polymorphisms was 97, 98 and 88%, respectively. An overview of genotype frequencies for all three SNPs is given in Table 3.2. Missing *GSTP1* genotypes were due to lack of patient material (n = 8) and assay genotyping failure (n = 2 for *GSTA1* and n = 1 for *GSTM1*). However, all variants showed Hardy–Weinberg equilibrium and were comparable with distributions of the genotypes found in other studies.[10,18,19]

Table 3.1 Baseline characteristics of HSCT patients

Characteristic	Mean (range)
Age in years	52.1 (25–70)
Weight	74 (51–108)
Body surface area	1.89 (1.51–2.36)
Body mass index	24 (19–34)
Sex (%(N))	
Male	56 (37)
Female	43 (29)
HLA matching	
Matched	99 (65)
Mismatched	1 (1)
Donor	
Matched family	46 (30)
Mismatched family	1 (1)
Matched unrelated	53 (35)
Disease*	
Malignant	88 (58)
Nonmalignant	12 (8)
Conditioning	
Myeloablative	3 (2)
Non-myeloablative	97 (64)
Conditioning regime**	
Flu Busulfan ATG	94 (62)
Flu Busulfan ATG CY	3 (2)
Busulfan AL CY	3 (2)

*Presented underlying diseases in the category malignant diseases are , acute myeloid leukemia (n = 14), chronic myeloid leukemia (n = 3), chronic lymphocytic leukemia (n = 6), non-Hodgkin lymphoma (n = 12), multiple myeloma (n = 21), chronic myelomonocytic leukaemia (n = 2) and non-malignant diseases aplastic anaemia (n = 7) and sickle-cell anemia (n = 1).
**Dosing schemes refer to Flu busulfan ATG: busulfan 0.8 mg/kg four times daily on days -6 and -5, oral Flu 50 mg/m2 once daily from day -10 to -5 and ATG from day -4 to -1. Flu busulfan ATG CY: busulfan 0.8 mg/kg four times daily on days -6 and -5, oral Flu 50 mg/m^2 once daily from day -10 to -5, ATG from day -4 to -1 and CY 750 mg/m^2 once daily on days -4 and -3. Busulfan AL CY: busulfan 0.8 mg/kg four times daily from day -9 to -6, AL 15 mg once daily on days -6 and -5 and CY 60 mg/kg once daily on days -4 and -3.
ATG: anti-thymocyte globusulfanlin, AL: alemtuzumab, Flu: fludarabine, CY: cyclophosphamide.

Effect of GST on busulfan pharmacokinetic parameters

After the first dose of 0.8 mg/kg, a higher clearance was observed in the *GSTA1**A/*A genotype group (0.21 ± 0.055 L/h/kg) compared with *GSTA1**B heterozygous patients (0.18 ± 0.041 L/h/kg) and *GSTA1**B homozygous patients (0.15 ± 0.039 L/h/kg; Table 3.3). Analyzing using linear

Table 3.2 Distribution of *GSTA1*, *GSTM1* and *GSTP1* genotype in allo-HSCT patients

Gene	Genotype	Patients % (n)
GSTA1	CC	30 (20)
	CT	49 (32)
	TT	18 (12)
GSTM1	Positive	58 (38)
	Deletion	41 (27)
GSTP1	AA	32 (21)
	AG	45 (30)
	GG	11 (7)

GSTA1 genotype could not be determined in two patients (3%) and *GSTM1* genotype could not be determined in one patient (1.5%) because of assay failure. *GTSP1* genotype could not be determined in eight patients (12%) because of the lack of material.

Table 3.3 Effect of *GSTA1* genotypes on busulfan pharmacokinetic parameters and HSCT patient parameters

Parameter	Overall (n = 64)	CC (n = 20)	CT (n = 32)	TT (n = 12)	P-value
Age (years)	53.3 (10.3)	53.8 (11.7)	53.3 (9.1)	52.6 (11.6)	0.95
Weight (kg)	73.7 (12.6)	71.5 (12.8)	74.6 (11.5)	75.0 (15.6)	0.63
Dose (mg)	59.3 (9.9)	57.9 (10.1)	59.9 (9.2)	60.1 (12.0)	0.74
Cl (L/h/kg)	0.18 (0.05)	0.21 (0.055)	0.18 (0.041)	0.15 0.039)	**0.002**
AUC (μmol*min/L)	1149 (336)	996 (311)	1172 (301)	1346 (368)	**0.013**
Sex (% of male gender)	55	60	59	33	0.26**

All numbers presented are mean (s.d.) unless otherwise indicated and statistical tests were carried out with analysis of variance unless otherwise indicated.
**χ^2-test.
P value < 0.05 is regarded as significant and indicated in bold font.
AUC: area under curve.

regression a reduction of 14% in busulfan clearance was found per *GSTA1**B allele. The *GSTA1* polymorphism could explain 18% of the variability in clearance. An inverse effect was observed for the AUC, as shown in Figure 3.1, AUC increases with 18% in *GSTA1**B heterozygous patients and 35% for *GSTA1**B homozygous patients. No significant differences in other parameters and *GSTA1* genotypes were detected (Table 3.3). No relationship was found between the investigated polymorphisms in *GSTM1* and *GSTP1* and busulfan clearance. In *GSTM1*-positive individuals, a mean busulfan clearance of 0.18 ± 0.049 L/h/kg was found, whereas in patients with a deletion mutation busulfan clearance was 0.19 ± 0.049 L/h/kg. Busulfan clearance in patients with different *GSTP1* genotypes were similar: 0.19 ± 0.050 L/h/kg for the AA genotype, 0.18 ± 0.041 L/h/kg for the AG genotype and 0.21 ± 0.067 L/h/kg for the GG genotype (p = 0.162; Figure 3.1).

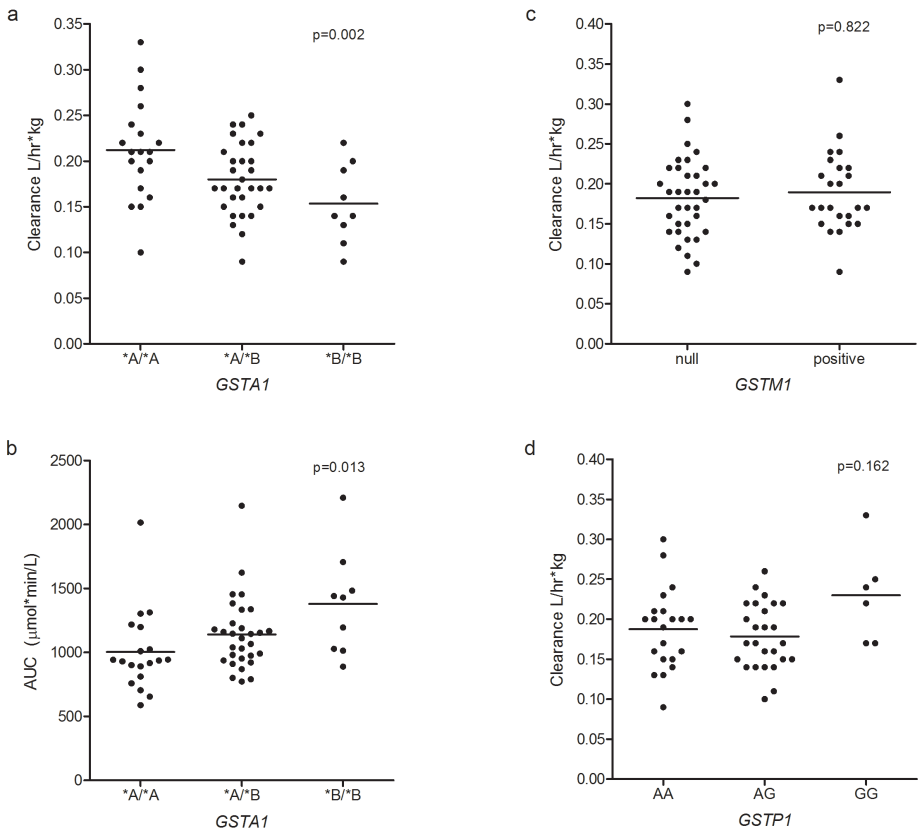

Figure 3.1 The relationship between *GSTA1*, *GSTM1* and *GSTP1* genotype and busulfan pharmacokinetic parameters. a) Relationship between *GSTA1* and clearance per kg of busulfan. b) Relationship between *GSTA1* AUC of busulfan mmol*min/L). c) Relationship between *GSTM1* and clearance per kg of busulfan. d) Relationship between *GSTP1* and clearance per kg of busulfan. Bars indicate mean values for each category.

Effect of GSTA1 on clinical outcome

VOD did not occur in any of the patients in this study and eight patients were diagnosed with mild aGVHD (grade one). In one patient engraftment did not occur and three patients experienced a secondary rejection of their transplant several months after transplantation. Of the HSCT patients with mild aGvHD, one patient was *GSTA1**A/*A genotyped, five patients *GSTA1**A/*B and two patients were *GSTA1**B/*B ($p = 0.2$). The three patients with rejection were *GSTA1**B/*B genotyped ($p = 0.001$), whereas the patient with non-engraftment was a *GSTA1**B heterozygote. After a follow-up of 3 years 36 of 66 patients died, the OS was 45%. No effect of *GSTA1* on OS was seen.

DISCUSSION

In our adult Caucasian population, polymorphism in the *GSTA1* gene resulted in a significantly lower busulfan clearance, hence a significantly higher exposure to busulfan. However, the effect of *GSTA1* genotype on busulfan pharmacokinetics did not seem to influence busulfan toxicity in this study. However, all patients who experienced rejection were *GSTA1**B homozygotes. In addition, no relation was found between polymorphisms in *GSTM1* and *GSTP1* genes and busulfan pharmacokinetic parameters. Therefore, interpatient variability in busulfan pharmacokinetics could be partly assigned to variances in the gene encoding the *GSTA1* isoenzyme. Busulfan shows large interpatient variability in clearance, which causes unpredictable exposure of busulfan resulting in either toxicity or therapy failure. It is hypothesized that differences in activity of the enzymes involved in busulfan metabolism (GSTA1, GSTM1 and GSTP1), caused by genetic polymorphisms, could explain the large interpatient variability. This is the first study investigating the effect of polymorphisms in the genes encoding for these three GST isoenzymes on busulfan pharmacokinetics in Caucasian adults.

The effect of polymorphisms in *GSTA1* and *GSTM1* on busulfan pharmacokinetics in adults has been studied before by Kusama *et al*.,[20] Kim *et al*.[21] and Abassi *et al*.[18] The first two studies were carried out on Asian patients. In both studies no *GSTA1**B homozygotes were observed. Kusuma *et al*. investigated the effect of the polymorphisms in 12 adult Japanese patients receiving busulfan orally and showed a reduction in clearance (Cl/F) of 40% in *GSTA1**A/*B patients at steady state. Kim *et al*. showed a decrease in clearance of 12% after intravenous busulfan administration for *GSTA1**A/*B patients. These results are in line with our results, though the reduction in clearance per *GSTA1**B allele is somewhat less pronounced in comparison with the Japenese study in which busulfan is administered orally: 14% for *GSTA1**A/*B genotype and 29% for *GSTA1**B/*B genotype as measured after the first dose. Clearance after oral administration (CL/F) might be influenced differently by GST polymorphisms compared with clearance after intravenous administration, as busulfan is conjugated locally in the small intestine with glutathione and genetic variances could alter the first-pass effect. Furthermore, our patient population is Caucasian, which might result in a different effect of polymorphisms in the GSTs and busulfan pharmacokinetics than in Japanese patients.

Abassi *et al*. investigated the effect of genetic variances in *GSTA1* and *GSTM1* in a combined pediatric and adult population. No significant association between the genetic variances and intravenous busulfan clearance was found. Oral busulfan clearance was associated with the *GSTA1* haplotype, busulfan this was not considered clinically relevant.

In our previous study,[10] the effect of *GSTA1* genotype on busulfan pharmacokinetic parameters in children was investigated; no significant effect of the polymorphism on busulfan clearance was detected. However, a small association of genetic variances in *GSTA1* and busulfan clearance in older children was seen. This observation was the main reason for this study; to investigate the effect of the genetic variances in GSTs on busulfan clearance in adults.

However, the clinical relevance of these findings is not clear, as in our study no clear effect of *GSTA1* genotype on clinical outcome was observed. Ultimately, pharmacogenetics aims for prevention of aberrant drug response. We presume that the effect of polymorphisms in *GSTA1* is probably relevant for the more toxic regimes only, since overall, a low incidence of toxicity and therapy failure was seen in our patient population. Nevertheless, we demonstrated that part of the interpatient variability in busulfan exposure can be explained by genetic variances in *GSTA1* (18%). Busulfan therapy is often individualized by therapeutic drug monitoring. Eventually, the aim is to optimize individual therapy by determining the pharmacogenetic profile of the patient and predict pharmacokinetics of the drug before administering busulfan. Additional studies are needed to investigate the effect of *GSTA1* genotype on busulfan pharmacokinetics and outcome in more toxic regimes and to optimize genotype-based dosing of busulfan.

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Chapter 4

Exploratory analysis of 1936 SNPs in ADME genes for association with busulfan clearance in adulthematopoietic stem cell recipients

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ABSTRACT

Busulfan is used in preparative regimens before stem cell transplantation. There is significant interpatient variability in busulfan pharmacokinetics (PK) and exposure is related to outcome. Polymorphisms in genes encoding glutathione-S-transferases have been associated with busulfan PK but only explain a limited portion of the observed variability. The aim of this study is to identify additional genetic variants associated with busulfan PK by interrogating 1,936 variants in 225 genes involved in drug absorption, distribution, metabolism, and excretion (ADME). In an exploratory cohort (n = 65), patients who received busulfan were genotyped with the DMET array. Top SNPs and haplotypes associated with busulfan clearance were validated in an independent validation cohort (n = 78). In the exploratory cohort, seven variants were identified to be associated with busulfan clearance ($p < 0.001$). In the validation cohort, only *GSTA5* (rs4715354 and rs7746993) remained significantly associated with busulfan clearance ($p = 0.025$). This is the first study using an exploratory pharmacogenetic approach to explain the interindividual variability in busulfan PK. The role of glutathione-Stransferases was confirmed, but no additional genetic markers involved in drug ADME appear to be associated with busulfan PK.

INTRODUCTION

Busulfan is an alkylating agent used widely in high doses in conditioning regimens beforeallogenic hematopoietic stem cell transplantation (allo-HSCT) in adult and pediatric patients. Interpatient variability is significant in busulfan pharmacokinetics (PK); even when busulfan is administered intravenously and body weight adjusted dosing is applied, interpatient variability in exposure or clearance remains between 20 and 30%.[1,2] Furthermore, busulfan exposure is related to outcome. High busulfan exposure appears to result in a higher incidence of toxicities such as sinusoidal obstruction syndrome and mucositis, whereas low busulfan exposure apparently leads to higher rates of graft failure and rejection.[3–5] Therefore, often, therapeutic drug monitoring is applied to individualize busulfan therapy and optimize clinical outcome. Many efforts have been made to develop a population PK model to further characterize busulfan PK.[6,7] The facilities needed for applying therapeutic drug monitoring in a clinical setting are, to date, a barrier to universal acceptance. Furthermore, up-front-dose adaption is not possible. In addition to targeting busulfan doses on the basis of PK-models, pharmacogenetic profiling of patients could play a more important role.

Multiple studies have been carried out to search for a pharmacogenetic explanation for the high variability in busulfan PK. To date, mainly polymorphisms in genes encoding for glutathione-S-transferases (GSTs) have been studied.[8–12] Busulfan is metabolized by conjugation to glutathione. This reaction is catalyzed by different GSTs enzymes, of which GSTA1 is the predominant enzyme responsible for 46% of busulfan conjugation.8 After conjugation, the complex is oxidized in the liver and metabolites are mainly excreted in the urine. In several studies, a single nucleotide polymorphism (SNP) in GSTA1 (rs3957357) was shown to be related to busulfan PK in adult patients receiving busulfan before their HSCT.[9,10] We confirmed this in an independent cohort of adult patients.[11] In this study, three SNPs in three different GST genes were tested for association with busulfan clearance. Only rs3957357 in *GSTA1* was found to be associated with busulfan clearance; however, this SNP could only explain 14% of its variability. Most of the available studies investigating variability in busulfan PK have applied the candidate gene method. Although this method has proven to be successful in the past, its major limitation is that it cannot identify genetic markers in genes not previously associated with busulfan PK. We hypothesized that other absorption, distribution, metabolism, and excretion (ADME) genes involved in drug metabolism or transport of busulfan could add to the variability of PK, meaning that a set of genetic variants may be more important to explain interindividual differences in busulfan exposure than a single gene. Genetic profiling with the Affymetrix drug metabolizing enzymes and transporters genotyping array offers the ability to determine 1,936 variants in 225 genes involved in drug metabolism and disposition.[12] In this

report, we present the results of the exploration of genetic factors associated with busulfan PK using this genotyping array.

PATIENTS AND METHODS

Patient and disease characteristic

Patients receiving allo-HSCT between 2004 and 2011 in the Leiden University Medical Center were included on the basis of the following selection criteria: age between 18 and 70 years, receiving busulfan as conditioning before their allo-HSCT with measured busulfan serum samples, and presence of DNA for genotyping. The Institutional Ethics Committee approved the study protocol. Written informed consent was obtained from all study participants according to the Helsinki Declaration. Diagnoses that required allo-HSCT were malignant: acute myeloid leukemia, chronic myeloid leukemia, chronic lymphoid leukemia, acute lymphoblastic leukemia, chronic myelomonocytic leukemia, non-Hodgkin's lymphoma or multiple myeloma or non-malignant: aplastic anemia, β-thalassemia or sickle cell anemia. Patients receiving busulfan between 2004 and 2008 were included in the exploratory cohort. This cohort was genotyped using de DMET plus arrays (Affymetrix UK Ltd, High Wycombe, UK). Patients receiving busulfan between 2008 and 2011 were included in the validation cohort. In this cohort, the positive findings of the exploratory cohort were tested.

Treatment regimens

Patients received either myeloablative conditioning or non-myeloablative conditioning. The non-myeloablative conditioning consisted of intravenous busulfan 0.8 mg/kg four times daily on days -6 and -5 before allo-HSCT, oral fludarabine 50 mg/m^2 on days -10 to -5, and alemtuzumab 15 mg for 2 days. In case of a matched unrelated donor, antithymocyte globulin 1–3 mg/kg was added. Alternatively, busulfan on days -6 and -5 before allo-HSCT was combined with oral fludarabine 50 mg/m^2 on day -10 to -5, intravenous cyclophosphamide 750 mg/m^2 on days -4 and -3, and alemtuzumab 15 mg during 2 days. The myeloablative conditioning consisted of intravenous busulfan 0.8 mg/kg four times daily on days -9 to -6 before allo-HSCT, intravenous cyclophosphamide 60 mg/kg on days -4 and -3, and alemtuzumab 15 mg during 2 days. Dosing of the different drugs, including busulfan, is based on the actual body weight. All patients received 92 mg phenytoin three times daily for seizure prophylaxis starting one day before busulfan administration. Busulfan was administered intravenously in a 2-hour infusion.

Serum drug level measurements were performed at 2.5 and 4.0 h after the start of the first infusion on the first day of treatment. Busulfan was analyzed in 200 ml serum using a validated high-performance liquid chromatographic assay involving precolumnderivatization with diethyldithiocarbamate, liquid/liquid extraction, and UV detection, described previously by our group[13] and on the basis of the method of Chow *et al*.[14] The assay is linear between 30 and 8000 mg/L and the limit of quantification is 30 mg/L. Accuracy at 200 and 1500 mg/L was 94 and 98%, respectively. Intraday variability in precision is 3.5 and 0.8% and interday variability in precision is 4.4 and 2.4% at 200 and 1500 mg/L, respectively. A validated limited sampling model was used to minimize the number of blood samples necessary to calculate the busulfanclearance.[13] A one-compartment population PK model for busulfan with linear elimination, developed and validated in MW/Pharm version 3.6 (Mediware, Groningen, the Netherlands), was used.[11,13,15,16] The calculated mean population PK parameters, clearance, volume of distribution, and half-life, were individualized according to the maximum a posteriori Bayesian fitting method.[17] No dose adjustments were made on the basis of the individual PK parameters.

Genotyping methods

DNA was isolated from EDTA blood using Maxwell (Promega, Leiden, the Netherlands) or Magnapure compact (Roche, Almere, the Netherlands). All samples were anonymized according to the instructions in the Codes for Proper Use and Proper Conduct (http:// www.federa.org).

DNA samples of the exploratory cohort were analyzed using DMET plus arrays (Affymetrix UK Ltd) according to the manufacturers' prescription and described in detail by Caldwell *et al*.[18] and Dumaual *et al*.[19]

Genotypes were calculated with DMET console software version 1.1 using the Dynamic Genotype Boundaries algorithm (Affymetrix UK Ltd). Patients with a call rate of less than 90% were excluded from the analyses. All individual SNPs were tested for Hardy–Weinberg equilibrium in gPlink 2.050[20]; SNPs that were not in equilibrium ($p < 0.001$) were excluded from further analysis.

In the validation cohort, SNPs were determined by high-resolution melting (HRM) of small amplicons with the LightScanner (HR-96; Idaho Technology, Salt Lake City, Utah, USA). Oligonucleotides used for small amplicon (40–60 bp) genotyping were chosen adjacent to the SNP. Melting curves were analyzed with LightScanner Software using Call-IT 2.0 (Idaho Technology). As a quality control measure, 10% of samples were genotyped in duplicate. Traditional Sanger sequencing was used to confirm HRM results for each SNPs. An overview of primers used in HRM and sequencing is presented in Table 4.1.

Table 4.1 Patient characteristics

	Exploratory cohort	Validation cohort	p-value
Number of patients	62	78	
Age in years (mean ± s.d.)	53 ± 11	57 ± 10	0.04
Sex			
Male	34	51	0.20
Female	28	27	
Disease			
AML	11	28	
CML	3	2	
CLL	5	7	
ALL	0	1	
MM	22	18	
NHL	10	18	
CMML	2	1	
AA	7	2	
β-thl	0	1	
SCA	2	0	
Busulfan clearance (L/hr/kg)	0.19 ± 0.05	0.17 ± 0.05	0.16
Volume of distribution	47.6 ± 16.6	51.0 ± 15.4	0.21
Conditioning regime			
Flu Bu (ATG or Alemtuzumab)	59	74	
Flu Bu CY	2	3	0.55
Bu Cy Alemtuzumab	1	1	

AML: acute myeloid leukemia, CML: chronic myeloid leukemia, CLL: Chronic lymphocytic leukemia, ALL: acute lymphoblastic leukemia , MM: Multiple Myeloma , NHL: Non-Hodgkin lymphoma , CMML: chronic myelomonocytic leukemia, AA: aplastic anemia, β-thl: β-thalassemia , SCA: sickle cell anemia, Flu: fludarabine, Bu: busulfan, Cy: cyclofosfamide, ATG: anti-thymocyteglobuline.

In both cohorts, more than 93% of the patients were of White ethnicity. Ethnicity was declared by the physician of the patient, but ethnicity was not studied as such in this study. To evaluate the ethnicity and to exclude the possibility of population stratification of the samples in the exploratory cohort, multidimensional scaling (MDS) was used, as implemented in software package plink. Four MDS coordinates were used and plots were created for adjacent MDS components.

Haplotype estimation

In the exploratory cohort, haploblocks were composed of SNPs associated with busulfan clearance within one gene. The haploblocks were tested to detect linkage disequilibrium.

If linkage disequilibrium between SNPs was present, haploblocks (with several haplotypes) were determined. A haplotype was set if the haplotype uncertainty parameter Rh^2 was greater than 0.95. Haplotypes with an Rh^2 of 0.95 or less were not considered for further analysis. Rare haplotypes (frequency < 10%) were pooled into one group in the association analysis. The following SNPs were analyzed in haploblocks: *ABCB1* rs2032588 and rs2235015; *ABCB4* rs45595532, rs2109505, and rs1202283; *ABCC2* rs7899457 and rs8187706; *ABCC6* rs8058694 and rs8058696; *CYP2B6* rs8058694 and rs8058696; *CYP39A1* haploblock 1 rs2277119 and rs59926524; *CYP39A1* haploblock 2 rs9381468 and rs953062; *CYP3A7* rs45467892 and rs45494802; *CYP4F2* rs2108622 and rs3093106; *FMO1* rs742350 and rs1126692, *GSTA1* rs4715332 and rs4715333; *GSTA5* rs4715354 and rs7746993; *NR3C1* rs6195, rs6190 and rs6189; *PPARD* rs7746988, rs6901410, rs6457815, rs7757196, rs7754530, rs7739752, rs6913026, rs6922548, rs6940722, rs6915115, rs6457816, rs6906237, and rs1053046; and *UGT2B15* rs4148269, rs3100, and rs4148269.

In the validation cohort, a tagging SNP was identified in each haploblock to determine haplotypes. The tagging SNP was selected on the basis of the frequencies of the different haplotypes in the exploratory cohort. In case it was not possible to determine haplotypes in the exploratory cohort on the basis of one tagging SNP, two SNPs were selected for determination of the haplotype. Care was taken such that the same SNPs could be analyzed in the exploratory and validation cohorts. This resulted in the following analysis in the validation cohort: Three haploblocks contained two SNPs: *GSTA5*: two tagging SNPs (rs4715354 and rs7746993), *CYP39A1* block 1: one tagging SNP (rs2277119) and block 2: two tagging SNPs (rs9381468 and rs953062). One haploblock (*ABCB4*) contained three SNPs, of which two were determined (rs2109505 and rs1202283). For each individual patient, haplotypes were estimated and haplotype Rh^2 was calculated using gPlink haplotypes; with Rh^2 more than 0.95 haplotypes were considered present.

In Table 4.2, an overview is presented of the number of SNPs and also the frequencies of the different haplotypes in both the exploratory and the validation cohort.

Statistical analysis

PK parameters are presented as means (± SD) (normal distribution) or geometric mean in case of log-normal distribution (± SD) of the parameter. In the exploratory cohort, associations with busulfan clearance and SNPs were tested initially by linear regression analysis with the SNPs in the additive model using gPlink. The effect of the number of copies of the haplotypes was tested in the same manner and the effect of gene copy number variation on busulfan clearance was tested using Student's t-test, both in PASW statistics, version 17.0.01 (SPSS Inc., Chicago,

Table 4.2 Statistical data of univariate and multivariate analysis in the exploratory and validation cohort

		Exploratory, univariate				Multivariate	Validation, univariate			
Gene	Haplotype / SNP	MAF or haplotype frequency	Explained variance (R^2, %)	p-value	Log OR	Log OR	MAF or haplotype frequency	Explained variance (R^2, %)	p-value	Log OR
GSTA5	CG	0.54	24.7	0.00004	0.038	0.016	0.52	0.065	0.026	0.017
SLC22A4	46011C>T	0.43	13.5	0.004	-0.026	-0.021	0.22	0.005	0.529	0.005
ABCB4	GAT	0.58	11.8	0.006	-0.024	-0.025	0.59	0.002	0.727	-0.0001
SLC7A8	-1065T>G	0.40	14.7	0.002	0.027	0.013	0.37	0.004	0.594	-0.004
CYP39A1	TC	0.27	21.3	0.00018	-0.036	-0.018	0.32	0.001	0.769	-0.02
CYP2C19	-806C/T	0.23	6.6	0.046	0.023	0.023	0.22	0.031	0.125	0.015
CYP39A1	GC	0.80	15.0	0.002	0.033	0.018	0.74	0.018	0.245	0.01

Seven markers in six genes identified in the multivariate analysis in the exploratory cohort.
MAF: minor allele frequency, OR: odds ratio.

Illinois, USA). Candidate markers with a p-value less than 0.05 (SNPs or haplotypes) and SNPs with a minor allele frequency more than 10%, to maintain a high enough power, were included in a multivariate linear regression analysis using a stepwise forward conditional approach with busulfan clearance as a dependent variable. The combination of SNPs and haplotypes explaining the largest portion of total variability in clearance was selected (highest R^2). The selection of these most informative SNPs and haplotypes was used to create a pharmacogenetic model predicting busulfan clearance (Cl_{PG}). This model was tested in the validation cohort.

All results from the multivariate analyses with a p-value less than 0.05 were considered significant. As this was an exploratory study, no correction for multiple testing was performed. In addition, this exploratory study used an uninformative approach, meaning that no adjustments for confounding factors and assumptions of polymorphism effects were made.

In the validation cohort, a predicted busulfan clearance was calculated for each patient with the regression model developed in the exploratory cohort (Cl_{PG}). The algorithm to calculate Cl_{PG} was based on the βs of each genetic marker from the multivariate analyses (Table 4.2) and a constant of 0.211; an additive model was assumed, according to Eq 4.1.

$$Cl_{PG} = 0.016*GSTA5 - 0.021*SLC22A4 - 0.025*ABCB4 + 0.013*SLC7A8 - 0.018*CYP39A1\_TC + 0.023*CYP2C19 + 0.018*CYP39A1\_GC \quad \text{(Eq 4.1)}$$

The value of each genetic marker is 0, 1, or 2 depending on the number of variant alleles. The busulfan clearances calculated using the pharmacogenetic model (Cl_{PG}) and the busulfan clearances calculated using the population pharmacokinetic model (Cl_{PK}) of each patient were compared on a case-by-case base with Pearson correlation. Furthermore, the association of the seven genetic markers with busulfan clearance was tested individually by linear regression analysis with the marker in the additive model in gPlink.

RESULTS

Description of the patient population

We carried out a two-stage approach, an exploratory analysis, and an independent validation study. Sixty-five adult patients were included in the exploratory cohort. Three patients were excluded because of a genotype call rate of less than 90%. The validation cohort included 78 adult patients. Patient characteristics of both cohorts are shown in Table 4.3. In the exploratory and validation cohort, 55 and 65% of patients were men, respectively (p = 0.20). The mean age

Table 4.3 Primers for high-resolution melting and Sanger sequencing of the 10 SNPs

Gene	SNP	Orientation	HRM primers 5'->3'	Sequence primers 5'->3'
GSTA5	rs4715354	Forward	GAGCTTTGTTCAGTCAACAC	TTCAGCAGAAAGAAAGGGAG
		Reverse	TCCTTGAGCTCTCAGGTTC	AACAACATAATCAGACAGGAG
GSTA5	rs7746993	Forward	TGTGGCATCTACACCACCC	GGTTCTCCATCACATGTGC
		Reverse	ATGTGGAAGAAGCAAGCTGG	GGGAATGAGGAGAGCAGAG
SLC22A4	rs1050152	Forward	TCTGACTGTCCTGATTGGAAT	CAATTACCTCCACCTTAAGAG
		Reverse	CTTTCAGGGAAAAAAAGGGTG	CTGATAGAGCTAGTCTCTAC
ABCB4	rs2109505	Forward	CTTTGTCACTAAATGCCGAG	AGCTACCCTCCTGCCTTG
		Reverse	CTGTTTCTTTTCTGTCCAG	CAGTAGATGTGGAACTTGAC
ABCB4	rs1202283	Forward	GTATTGAGTTCAGTGGTGTC	ATGGCATAGGCTATAGATGC
		Reverse	GAAATAGGATGGTTTGACATC	GATGAATAAAGGATGGTAGGG
SLC7A8	rs7141505	Forward	ATAAATCAGGGAACAGTTGTG	CTCCATCCTCAGTCCCGTC
		Reverse	TTCTGATGGCATTAAAAGTATC	GCGATTTTGTTTCCTCTGTAC
CYP39A1	rs9381468	Forward	TGGCAAAAATGTTAGCAGTT	TGTCATGAAATCTGTAAACCAC
		Reverse	CTCTATCTCTACCATTGAGA	GTGTTTAAAGTTCTTAAGTGAAG
CYP39A1	rs953062	Forward	GCTCATGGTCTGATGGAAAG	GCTCTCTATACAAATACCATGG
		Reverse	ATCATATTGATTAGAAATTAACTC	CAACCTCTCGGAGATGTTC
CYP2C19	rs12248560	Forward	TGTGTCTTCTGTTCTCAAAG	TGTCTGGAGGAGACCAGG
		Reverse	GCATTATCTCTTACATCAGAG	ATCCTATATCGAAGATTAGGAG
CYP39A1	rs2277119	Forward	GCAGTGCAAAATATCGTTTATC	TTTCTACTTATTTCATAGTACAAG
		Reverse	CTATCTGAAAATATTCTTTACCTG	CTGAGTAACATGTAACCAGTC

of the patient population was 53 ± 10 and 57 ± 11 years for the respective cohorts (p = 0.04). The mean busulfan clearance (Cl_{PK}) was similar in the exploratory cohort (0.19 ± 0.05 L/h/kg) and in the validation cohort (0.17 ± 0.04 L/h/kg) (p = 0.16).

The two different cohorts were created sequentially on the basis of the date of HSCT and busulfan administration. The two cohorts were similar in terms of sex distribution, average busulfan clearance, indication, and conditioning regimen. There was a significant difference in the average age between the two cohorts. However, age is not associated with busulfan clearance in adult patients.

Genotyping

In the exploratory cohort, of the 1936 SNPs determined, 46 SNPs were X-chromosomal. As there was no sex difference in busulfan clearance levels (p = 0.19), these SNPs were excluded from the analysis. All 62 patients passed QC metrics and produced useable genotypes.

The average call rate of the remaining 1890 SNP assays was more than 98%. One SNP (RS7436963/*UGT2B17*) failed to meet Hardy–Weinberg equilibrium (p < 0.001) and was excluded from further analysis. Of the remaining 1889 SNPs, 851 SNPs were polymorphic in the study population and were used in the association study. An overview is presented in Figure 4.1.

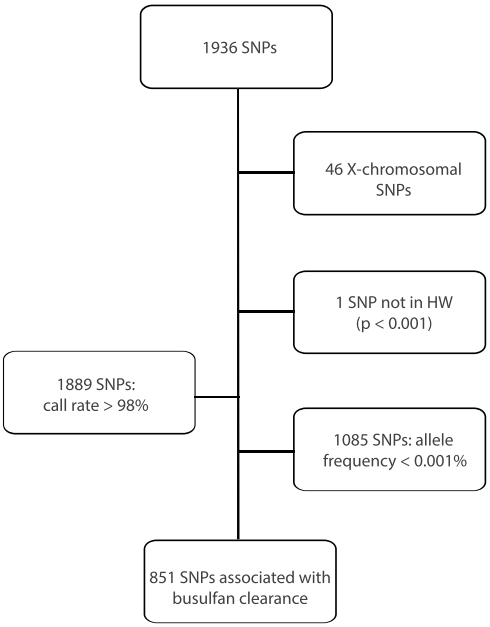

Figure 4.1 Flow charts of genotyping results in the exploratory cohort. HW: Hardy-Weinberg, SNP: single nucleotide polymorphism.

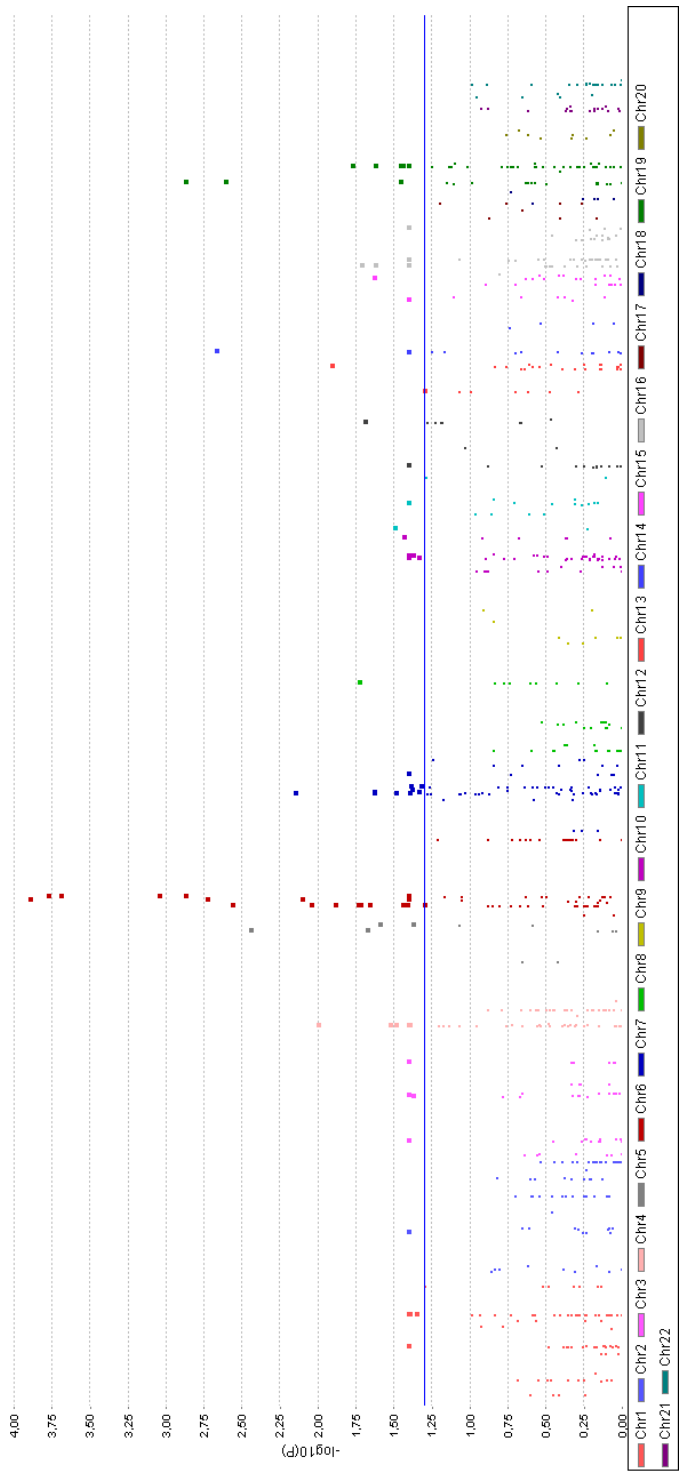

Figure 4.2 Manhattan plot of –Log (p-values) from linear regression analysis of 851 individual genetic variants and busulfan clearance. Genetic variants are organized per chromosome. Blue line indicating a p-value of 0.05.

To exclude population stratification, MDS plots were created (Supplemental Figure 4.1). The plots do not indicate the presence of strong population stratification. If population stratification would be present, clear separated clusters would be observed in the graphs, indicating a difference in allele frequencies between subpopulations.

In the validation cohort, 10 SNPs, which were selected in the exploratory cohort, were determined. The average call rate was more than 99%; all variants showed Hardy–Weinberg equilibrium ($p > 0.05$).

Association of genetic variants with busulfan clearance in the exploratory cohort

Of the 851 SNPs, 86 SNPs in 52 different genes showed a significant association with busulfan clearance ($p < 0.05$; see also Figure 4.2). Of the 45 SNPs, 15 haploblocks, containing 29 haplotypes, located in 14 different genes, were formed. In the next step of association analysis, SNPs and haplotypes that fulfilled the selection criteria ($p < 0.05$, minor allele frequency > 10%) were applied in the multivariate analyses. Seven variants (three SNPs and four haplotypes) in six genes were selected as the top genetic markers, explaining 64% (adjusted R^2) of variance in busulfan clearance in the exploratory cohort ($p < 0.001$) (Table 4.2). To be more specific, three genes of the model are involved in drug metabolism processes in general: *GSTA5* (rs4715354 and rs7746993) and two cytochrome P450 genes: *CYP2C19* (rs12248560) and *CYP39A1* (rs2277119 and rs9381468 and rs953062) and three genes were found to be involved in drug transport: *ABCB4* (rs2109505 and rs1202283), *SLC22A4* (rs1050152), and *SLC7A8* (rs7141505). The effects of each individual genetic marker on busulfan clearance are presented in individual graphs in Figure 4.3.

Predication of busulfan clearance in validation cohort

In the validation cohort, busulfan clearance was calculated using the prediction algorithm and the individual genotypes of the seven markers. The average predicted clearance (Cl_{PG}) was 0.19 ± 0.04 L/h/kg. A correlation graph and a Bland–Altman plot are provided in Supplemental Figure 4.2 showing a poor correlation between Cl_{PG} and clearance calculated using the PK model (Cl_{PK}), $R^2 = 0.024$, but no systematic bias between the two methods.

When the seven genetic markers were tested separately, one haplotype in *GSTA5* (rs4715354 and rs7746993) remained statistically significant ($p = 0.025$) for correlation with busulfan clearance. Busulfan clearance of patients with one variant allele was decreased by 11% and 18% in patients with two variant alleles as compared with *GSTA5* wild-type patients. In the validation cohort, the *GSTA5* haplotype could explain 6.5% of variability in busulfan clearance.

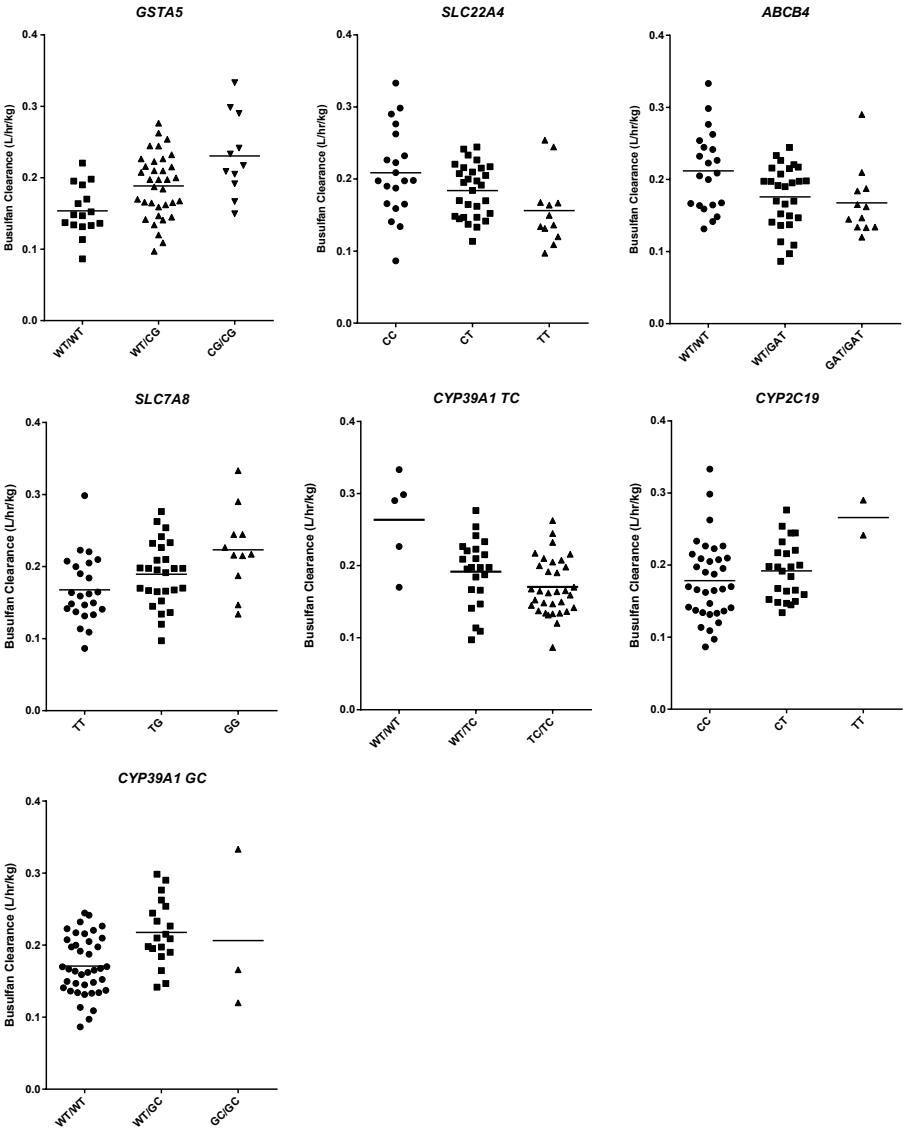

Figure 4.3 Effects of individual genotypes associated with busulfan clearance in the exploratory cohort.

DISCUSSION

This is the first study using an exploratory pharmacogenetic approach including a wide range of genes involved in drug ADME to investigate the interindividual variability in busulfan clearance in adults. The *GSTA5* haplotype was found to be associated significantly with busulfan

clearance both in the exploratory and in the validation cohort. No additional genetic markers involved in drug metabolism and transport appeared to be associated with busulfan clearance. Busulfan is metabolized primarily by conjugation with glutathione, catalyzed by GSTs. Different GST enzymes are involved in busulfan metabolism, GSTA1 being the predominant enzyme.[8] The effect of SNPs in genes encoding for three GST enzymes (GSTA1, GSTM1, and GSTP1) on busulfan PK has been studied before, leading to ambiguous results. Our group[11] and Kim *et al*.[10] showed an effect of a *GSTA1* SNP (rs3957357) on busulfan clearance in adult patients receiving busulfan intravenously; the other two SNPs showed no effects. Our group[21] and Ansari *et al*.[22] studied the same SNPs in a pediatric population. Both studies showed no association between the *GSTA1* SNP and busulfan PK.

Moreover, Hassan *et al*.[23] suggested the involvement of transporter enzymes in busulfan metabolism, transporting the glutathione–busulfan conjugate out of the cell. Only one study investigated the effect of a transporter on busulfan PK. In this study, the effect of three *GST* SNPs and two *ABCB1* SNPs (rs1045642 and rs2032582) was investigated and it was found that combined polymorphisms in *GSTM1* and *ABCB1* were associated with busulfan PK.[24]

In the exploratory cohort, genetic markers in different transporters (two solute carriers and one ABC transporter) were identified to be potentially related to variance in busulfan PK. These findings could not be confirmed in the validation cohort, thus suggesting that the involvement of transporters in busulfan PK is not likely.

In our study, genetic variants were tested in relation to busulfan clearance. Unfortunately, our study does not have sufficient statistical power to relate genetic variations to clinical outcome parameters such as toxicity, efficacy (engraftment), or relapse. Instead, we explored relationships with busulfan clearance. However, for busulfan, a clear relationship between PK and clinical outcome has been described.[3–5]

The *GSTA5* haplotype was found to be associated significantly with busulfan clearance. The *GSTA5* gene is a member of the same family as *GSTA1* and *GSTA5* protein is absent in human tissue.[25] However, the previously studied *GSTA1* SNP (rs3957357, -69C/T), a tagging SNP in a haploblock with three other SNPs in the promoter of *GSTA1* (-631T/G, -567T/G, -69C/T, and -52G/A), and the tagging SNP of *GSTA5* (rs4715354) are linked; haploview linkage disequilibrium of these SNPs is $D' = 1$ and $r^2 = 0.69$. Thus, the association with the *GSTA5* SNP is in fact a positive control for earlier findings for the association of *GSTA1* SNP and busulfan PK. The *GSTA1* SNP (rs3957357) is not present on the DMET array and was therefore analyzed separately. The absence of the tagging SNP of the *GSTA1* gene on the DMET array and the relatively small sample size resulted in a slightly larger effect size of *GSTA5* in comparison with other *GSTA1* SNPs on the

DMET array in the exploratory cohort. Separate analyses of *GSTA1* (rs3957357) showed an association with busulfan clearance in the exploratory cohort ($p < 0.01$) and in the validation cohort ($p = 0.02$). Linkage of the two SNPs could be confirmed in both cohorts, resulting in $D'=1$ and $r^2 = 0.57$ in the exploratory cohort and $D'= 1$ and $r^2 = 0.71$ in the validation cohort.

Previous studies indicate that only a small proportion of variability in busulfan PK can be attributed to genetic variants in *GST*s. We hypothesized that several yet unknown metabolic enzymes and drug transporters could be involved in busulfan PK, each with a small influence. Therefore, in this study, we used an unsupervised genetic association approach. The advantage of such an approach is the possibility of discovering new pathways and genetic variants that might contribute toward variability in busulfan exposure. One of the first examples of the possibility of finding a novel unexpected relationship using the DMET array is given by Ross *et al*.[26], showing an association between cisplatin ototoxicity and genetic variants in *TPMT* and *COMT* using the same genotyping platform.

A disadvantage of this exploratory approach is the difficult interpretation of its results as the effects of the identified variants on gene function or expression are not always clear. Hence, the effect of variants found in the exploratory cohort on busulfan metabolism can only be hypothesis-generating and should be confirmed in a validation study, as we performed in the current study.

An overview of the function and possible effects on busulfan clearance of the seven genes that were found in the exploratory study is shown in Supplemental Table 4.1.

Concomitant medications have been associated with busulfan PK. Especially antifungals, but also antiepileptic drugs are known for drug–drug interactions with busulfan. All patients included in our study received 92 mg phenytoin three times daily for seizure prophylaxis, starting one day before busulfan administration. Phenytoin could induce the metabolism of busulfan. However, the effect of phenytoin is not very strong. Furthermore, if this interaction would affect busulfan PK, it will be the same in all patients included in this study. A more significant interaction is the effect of antifungals on busulfan PK. Antifungals such as ketoconazole can increase busulfan exposure, probably because of inhibition of busulfan metabolism in the liver. In our study population, the only antifungal administered to the patients concomitantly with busulfan was oral amphotericin B. This drug is not absorbed and will therefore not affect busulfan metabolism in the liver.

In our study, a large number of SNPs were evaluated in the exploratory cohort, which introduces the potential problem of multiple testing and an increase in the risk of false-positive findings. To enrich our SNP set with predictive SNPs to be used by a prediction model for busulfan clearance,

we applied a mild statistical threshold of p less than 0.05 in the exploratory cohort. P-value thresholds in an exploratory set are meant to enrich for associated SNPs not strictly controlling type I error (following Purcell *et al*.[27]). Evaluation of the association of scores in the exploratory set has descriptive merits and formal evaluation of significance is carried out in the validation set. It is possible to use alternative strategies for the construction of a prediction model by using different model selection techniques (such as penalized regression or ensemble methods); however, by testing external validity in an independent data set, we offer a conservative and robust approach to model evaluation. Construction of a simple model has the advantage of overfitting and making our results more comparable with similar studies (compare Purcell *et al*.[27]).

Evaluation of the associations in the validation cohort resulted in confirming one of the seven genetic markers (e.g. *GSTA5* haplotype). However, the explained variability of *GSTA5* in the validation cohort was relatively small in comparison with the findings in the exploratory cohort. This decrease in explained variability could be because of unmeasured confounders that are different between the two different cohorts. Furthermore, the explained variability of 64% in the exploratory cohort might be an overestimate because of the so-called winners curse. To assess the magnitude of this phenomenon, we performed a data-split by fitting the model in the first half and computing the R^2 of the resulting predictor in the second half. With this analysis, the R^2 decreased from 75 to 37%, indicating the presence of overfitting (aka winner's curse). Still, this analysis confirms that the model has considerable explanatory power. The findings of involvement of *GSTA5* on busulfan PK will not directly have an impact on clinical practice.

This is the first study using an exploratory pharmacogenetic approach in 225 genes involved in ADME to find an explanation for the interindividual variability in busulfan clearance. The *GSTA5* haplotype, and thus *GSTA1*, was significantly associated with busulfan clearance both in the exploratory and in the validation cohort. No additional genetic markers involved in drug metabolism and transport were found to be associated with busulfan clearance.

Acknowledgments

The Central Clinical Hematology Laboratory of the Leiden University Medical Center is acknowledged for providing DNA samples.

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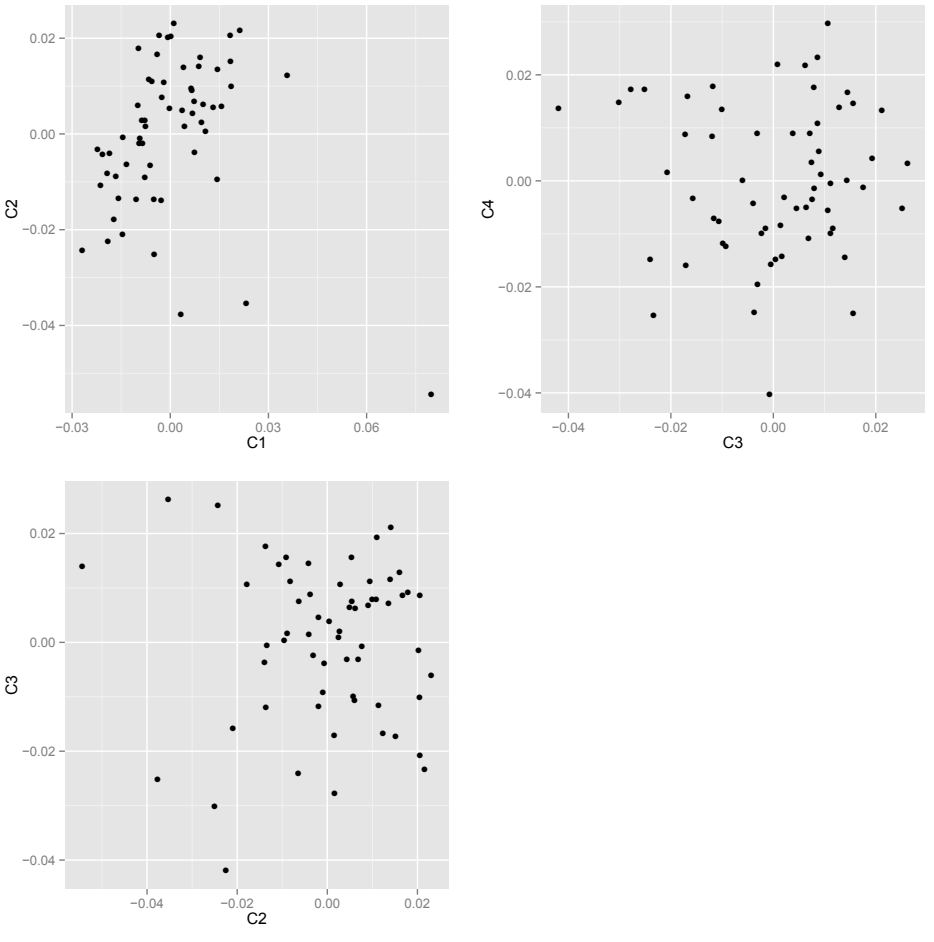
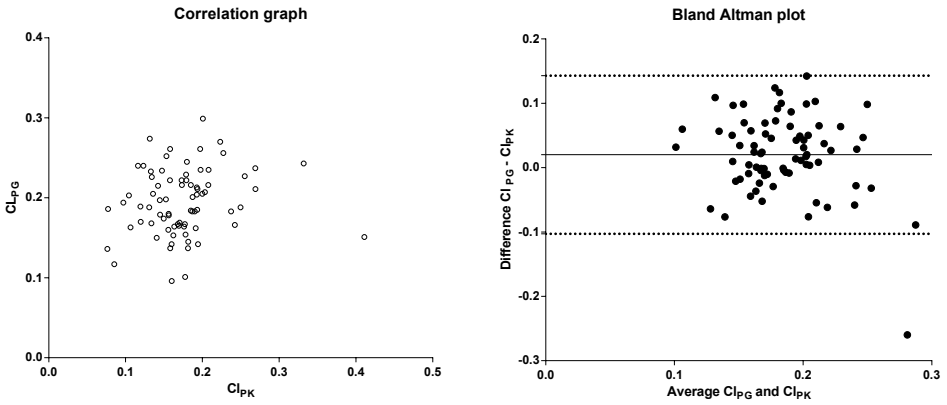
SUPPLEMENTAL MATERIAL



Supplemental Figure 4.1 Multidimensional scaling (MDS) plots of adjacent MDS components The plots do not indicate the presence of strong population stratification. If stratification would be present, clear clusters would be depicted in the graphs, demonstrating a difference in allele frequencies between subpopulations.



Supplemental Figure 4.2 Correlation graph and Bland Altman Plot. Correlation graph showing busulfan clearance based on PK data (Cl_{PK}) on the x-axis and clearance predicted with genetic markers (Cl_{PG}) on the y-axis, $R^2 = 0.024$. Bland Altman plot: on the x-axis: average of Cl_{PG} and Cl_{PK} per individual and on the y-axis the difference between Cl_{PG} and Cl_{PK} per individual. Middle line: the average difference and the two dashed lines: upper and lower limits of agreement (-2SD and +2SD).

Supplemental Table 4.1 An overview of the function and possible effects on busulfan clearance of the 7 genes that were found in the exploratory cohort

Gene	Role gene[1]	Postulated effect
GSTA5	Part of the alpha class of gluthationetransferases, catalyze conjugation with gluthation.	No functional protein identified in human.[2]
SLC22A4	Influx transporter also known as carnitine/organic cation transporter (OCTN1), present in liver, kidney, intestine and other organs. Critical for elimination of many small organic cations.[3]	
ABCB4	ATP-binding cassette, sub-family B (MDR/TAP). This gene encodes a full transporter and member of the p-glycoprotein family; it may involve transport of phospholipids from liver hepatocytes into bile.	p-GP is a transporter of gluthatione conjugates out of the cell and may transport busulfan conjugate.[4]
SLC7A8	Cationic amino acid transporter, also known as *LAT2* gene.	SNP located in haploblock in promoter region of gene; no evidence for effect of SNP on expression of gene.[5]
CYP39A1 Block A & B	A member of the cytochrome P450 superfamily of enzymes. This endoplasmic reticulum protein is involved in the conversion of cholesterol to bile acids.	
CYP2C19	This protein localizes to the endoplasmic reticulum and is known to metabolize many xenobiotics including omeprazol and anticonvulsive drugs.	*17 allele: increased transcription and ultrarapid allele.[6] Busulfan conjugate is oxidized in the liver, which may be facilitated by *CYP2C19*.

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Chapter 5

Effect of genetic variants *GSTA1* and *CYP39A1* and age on busulfan clearance in pediatric patients undergoing hematopoietic stem cell transplantation

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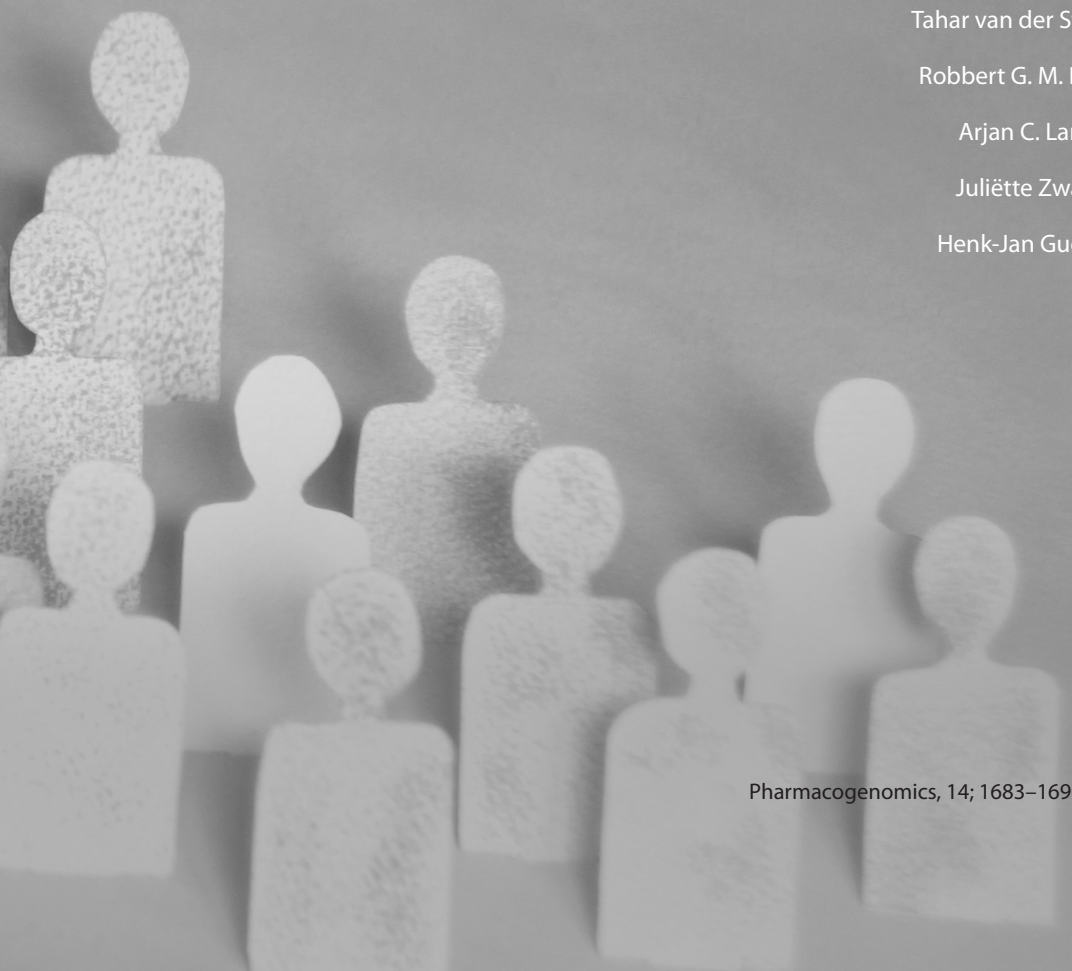

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ABSTRACT

Busulfanis used in preparative regimens prior to hematopoietic stem cell transplantation in pediatric patients. There is significant interpatient variability in busulfan pharmacokinetics (PK) and exposure is related to outcome. To date, only polymorphisms in genes encoding for glutathione-S-transferases were studied, but could only explain a small portion of the variability in PK. In this study we investigate the effect of seven genetic markers on busulfan clearance and the effect of ontogenesis on these genetic variants in a pediatric population. In an earlier study of our group seven genetic markers in *GSTA1*, *CYP2C19*, *CYP39A1*, *ABCB4*, *SLC22A4* and *SLC7A8* were associated with busulfan clearance in adult patients. Eighty four pediatric patients were genotyped for these markers and genotype was associated with busulfan clearance. *GSTA1* and *CYP39A1* were found to be associated with busulfan clearance. When combined, the two haplotypes explained 17% of the variability in busulfan clearance. Furthermore, the effect of *GSTA1* haplotype on clearance was dependent on age.

INTRODUCTION

Busulfan is an alkylating agent commonly used in high doses in conditioning regimens prior to allogeneic hematopoietic stem cell transplantation (allo-HSCT) in adult and pediatric patients. Busulfan has a narrow therapeutic index and its exposure is related to clinical outcome. Indeed, an increased risk for busulfan toxicity such as (hepatic) sinusoidal obstruction syndrome and mucositis are related to high busulfan exposure whereas rejection of the graft and disease recurrence is related to low busulfan exposure.[1–4] Furthermore, interpatient variability in busulfan pharmacokinetics (PK) is considerable. Even when busulfan is administered intravenously, and thus excluding potential variation in absorption, interpatient variability in exposure remains between 20–30%.[5,6] To limit variation in busulfan exposure and to optimize clinical outcome,[7] therapeutic drug monitoring is often applied in the clinical setting and population PK models have been developed.[5,8] Furthermore, pharmacodynamics of busulfan differs based on the conditioning regimen prior to stem cell transplantation, the age of the patient and the recipients underlying disease.[9]

Variability in busulfan clearance could possibly be explained by genetic variation in genes involved in the PK of busulfan. A tagging SNP (rs3957357, -69C/T) in a haplotype of the gene coding for GSTA1, an enzyme predominantly involved in busulfan metabolism, was found to be associated with busulfan clearance in earlier studies in adult populations.[10,11] The percentage of variability in busulfan PK explained by genetic variation in *GSTA1* differs per study, possibly owing to differences in ethnicity of the studied population or administration route in the different studies (oral versus intravenous administration of busulfan).

In pediatric patients the contribution of rs3957357 on busulfan PK is not as apparent as in adults: studies showed conflicting results for the effect of the *GSTA1* haplotype on busulfan PK.[12–15] This could be owing to other factors having a more profound effect on busulfan clearance in pediatric patients, such as bodyweight. In the pediatric population variation in bodyweight is much larger than in adults and it is known that busulfan clearance is significantly related to bodyweight in children. Furthermore, developmental changes affect PK pathways in young children.[16] Also the role of pharmacogenetics could be influenced by ontogenesis in young children. To date, studies looking for pharmacogenetic biomarkers related to busulfan PK have been limited by a candidate gene approach. In a recent study of ten Brink *et al*.[17], the Drug Metabolizing Enzymes and Transporters (DMET) genotyping array (Affymetrix) was used to interrogate 1936 genetic markers in 225 genes involved in drug transport and metabolism in an adult population. Through systematic screening, seven potential genetic markers in six genes were identified. The identified markers were located in *GSTA5*, *CYP2C19*, *CYP39A1*, ABCB4, *SLC22A4* and *SLC7A8*.

However, these results cannot be directly extrapolated to a pediatric population, since other factors such as ontogenesis or bodyweight can have a pronounced effect on busulfan PK. Therefore, the aim of the current study is to investigate the effect of seven genetic markers on busulfan clearance and the effect of ontogenesis on these genetic variants in the pediatric population.

MATERIALS AND METHODS

Patient characteristics

In this retrospective study, pediatric patients (≤ 18 years) receiving busulfan conditioning prior to their allo-HSCT from March 2006 to March 2012 at the Leiden University Medical Center in The Netherlands were included. Other criteria for inclusion were the availability of DNA and the availability of busulfan blood concentration measurements. The institutional ethics committee approved the study protocol. Written informed consent was obtained from all study participants according to the Helsinki Declaration.

Treatment regimens

Patients received busulfan (Busulfex®; Pierre Fabre Oncology, CastresCedex, France) intravenously (iv.) once daily in a 4-day course. The starting dose was 120 mg/m^2 or 80 mg/m^2 and the second to fourth dose was calculated targeting a cumulative area under the curve (AUC) over 4 days of 80–90 mg*h/L or 60–80 mg*h/L.

All conditioning regimens were according to The European Group of Blood and Marrow Transplantation (EBMT) protocols. An overview of number of patients per regimen is given in Table 5.1. Busulfan was combined with cyclophosphamide alone: busulfan: day -9 until day -6 and cyclophosphamide iv. 50 mg/kg/day at day -5 until day -2. When etoposide iv. was added to this regimen, it was administered at day -12 and -11, 350 mg/m^2/day. Or busulfan (day -9 until -6) was combined with a 2-day course of cyclophosphamide iv. 60 mg/kg/day at day -4 until -3 and melphalan iv. 140 mg/m^2 at day -1. When busulfan was combined with fludarabine both drugs were always administered on the same days: day -7 until day -4. The fludarabine iv. dose was 40 mg/m^2/day. Both drugs could be combined with thiotepa iv. 8 mg/kg (day -8), melphalan iv. 140 mg/m^2 (day -1). When busulfan and fludarabine were combined with clofarabine iv. (30 mg/m^2/day, also on day -7 until day -4), the fludarabine dose was 10 mg/m^2/day. Serotherapy consisted of antithymocyte globulin or alemtuzumab. All patients received clonazepam 25 µg/kg (four-times daily) as seizure prophylaxis, 1 day before start of busulfan.

Table 5.1 Patient characteristics and conditioning regimens

Characteristic	Mean	(s.d.)
Age (years)	6.14	(5.4)
Weight (kg)	25.4	(17.3)
Length (cm)	113	(32.9)
Sex (n and % male)	58	(69%)
Conditioning regimen	**Patients (n), (serotherapy)***	
Bu Cy	15 (10)	
Bu Cy Melphalan	13 (6)	
Bu Cy Etoposide	8 (7)	
Bu Flu	29 (24)	
Bu Flu Thiotepa	14 (13)	
Bu Flu Melphalan	4 (3)	
Bu Flu Clofarabine	1 (1)	
Diagnosis for HSCT	**Patients (n)**	
Immune deficiency	28	
Hematological malignancy	31	
Other non-malignant / hematological disease	4	
Thalassemia	21	
Graft type	**Patients (n)**	
Bone marrow	62	
Peripheral blood stem cells	11	
Cord blood	11	
Donor matching		
Matched unrelated donor	53	
Identical related donor	24	
Other related donor	7	

*Serotherapy: antithymocyte globulin/Campath.
Bu: Busulfan, Cy: Cyclophosphamide, Flu: Fludarabine, HSCT: Hematopoietic stem cell transplantation, SD: Standard deviation.

Busulfan pharmacokinetics

Busulfan was administered in a 3 h infusion. Serum drug level measurements were collected at 4, 5 and 7 h after the start of busulfan infusion on the first day of treatment as part of routine patient care. A validated limited sampling model was used to limit the amount of samples necessary to calculate busulfan clearance and AUC.[8]

Busulfan concentrations were analyzed in serum by a validated high-performance liquid chromatographic assay involving precolumnderivatization, liquid/liquid extraction and UV detection, previously described by our group.[18] Briefly, the limit of quantification was 30 µg/L

and precision at 200 and 1500 µg/L was 3.5 and 0.8%, respectively. Individual PK parameters were calculated using a one-compartment population PK model for busulfan with linear elimination, developed in MW/Pharm version 3.6 (Mediware, Groningen, The Netherlands).[18,19] The calculated mean population PK parameters, clearance and half-life, were individualized according to the maximum a posteriori Bayesian fitting method.[20]

Exposure (AUC) was calculated by dividing the busulfan dose by estimated busulfan clearance and was used to individualize busulfan dosing after the first dose. Clearance was adjusted to body surface area (BSA), since BSA describes busulfan clearance in children well.[21,22]

Genotyping and haplotype estimation

Patients were genotyped for the seven potential genetic markers, in *GSTA5*, *ABCB4*, *CYP39A1*, *CYP2C219*, *SLC7A8* and *SLC22A4*, previously identified by our group.[17] However, post hoc analysis revealed that *GSTA5* is in linkage with *GSTA1*, which is the functionally active gene in humans. Therefore, we included *GSTA1* (rs3957357) instead of *GSTA5*. Nine SNPs were analyzed in *GSTA1* (rs3957357), *ABCB4* (rs2109505 and rs45595532), *CYP39A1* (rs9381468, rs953062 and rs2277119), *CYP2C19* (rs12248560), *SLC7A8* (rs7141505) and *SLC22A4* (rs1050152). DNA was extracted from available patient material including blood, peripheral blood mononuclear cells and bone marrow cells. Patient material was acquired 1–2 weeks before the HSCT procedure was performed. Samples were stored in liquid nitrogen at approximately -180°C for preservation until DNA extraction. DNA was extracted using the MagNAPure (Roche, Basel, Switzerland) system or Maxwell 16 (Promega, Madison, WI, USA) system.

Genotypes were determined with high-resolution melting (HRM) curve analysis of small amplicons with the LightScanner® (HR-96, Idaho Technology, UT, USA). Oligonucleotides used for small amplicon genotyping (40–60 bp) were chosen adjacent to the SNP. Melting curves were analyzed with LightScanner® Software using Call-IT 2.0. The *GSTA1* SNP (rs3957357) was genotyped by pyrosequencing[13] (Qiagen, Venlo, The Netherlands).

As a quality control 10% of samples were genotyped in duplicate. SNPs with a call rate < 0.95 were removed from the analysis. Traditional Sanger sequencing was used to confirm HRM results for each SNP.

SNPs in *CYP39A1* (rs9381468 and rs953062) and ABCB4 (rs2109505 and rs45595532) were included in the analysis as haplotypes. For each patient, haplotypes were estimated and haplotype Rh^2 was calculated using gPlink.[23] Haplotypes with $Rh^2 > 0.95$ were considered present.

Statistical analysis

The primary end point of the study was the associations of each of the genetic markers with busulfan clearance. This was tested by univariate linear regression analysis with the SNP or haplotype in the additive model in PASW statistics, version 17.0.01 (SPSS Inc., IL, USA). Next, the significantly associated genetic markers were tested in a multivariate analysis. To explore the effect of ontogenesis in *GSTA1*, the effect of both haplotypes on busulfan clearance were studied in two different age groups (patients younger and older than 2 years). P-values less than 0.05 were considered statistically significant.

RESULTS

Description of the patient population

In this study, 84 patients receiving busulfan therapy were included. The mean age of patients was 6.1 years (± 5.4) and 69% of the patients were male. The mean busulfan clearance was 6.6 ± 1.8 L/h/m^2 and mean AUC after the first dose was 19.9 ± 5.8 mg*h/L. Twenty of the patients were younger than 2 years of age. The busulfan clearance in the group of patients younger than 2 years was lower (5.8 ± 2.2 L/h/m^2) than in the patient older than 2 years (6.8 ± 1.7 L/h/m^2; p = 0.031). Detailed patient characteristics are shown in Table 5.1.

Indications for HSCT were hematologic malignancies (acute lymphoblastic leukemia, chronic and acute myeloid leukemia, juvenile myelomonocytic leukemia and myelodysplastic syndrome (n = 31), β-thalassemia (n = 21), immune deficiencies (n = 28)) and other non-malignant hematologic diseases (n = 4; Diamond–Blackfan anemia, Glanzmann and congenital amegakaryocytic thrombocytopenia). Patients with immune deficiencies had a significantly lower clearance (5.7 ± 1.8 L/h/m^2) compared with the other indications; hematologic malignancies: 7.1 ± 1.6 L/h/m^2, β-thalassemia: 6.9 ± 2.0 L/h/m^2 and other non-malignant hematologic diseases: 6.3 ± 1.3 L/h/m^2 (p = 0.02).

Genotyping & association of genetic variants with busulfan clearance

The call rate of all eight SNPs determined with HRM was at least 98%. The call rate of the *GSTA1* SNP with pyrosequencing was 96.4%. All SNPs showed Hardy–Weinberg equilibrium (p > 0.05).

Univariate regression analysis of the seven markers identified two markers being associated with busulfan clearance: *GSTA1* (rs3957357; p = 0.004) and *CYP39A1* (rs9381468 and rs953062;

p = 0.011), see also Table 5.2. Patients who were heterozygous for *GSTA1*A/*B* had an 8% lower busulfan clearance compared with wild-type *GSTA1* patients and homozygous **B/*B* patients had a 26% lower clearance. Patients who were carriers of one of the variant *CYP39A1* alleles had a 13% lower clearance and homozygous patients had a 17% lower clearance, compared with *CYP39A1* wild-type patients.

The genetic markers in *GSTA1* and *CYP39A1* combined could explain 17% of variability in busulfan clearance in this pediatric patient population. Patients who are homozygous carriers for both haplotypes of *GSTA1* and *CYP39A1* had a 39% lower busulfan clearance in comparison to patients who were wild-type for both haplotypes (7.8 ± 2.1 L/h/m^2 vs 4.8 ± 1.1 L/h/m^2). In patients with increasing numbers of variant alleles, the busulfan clearance was lower in comparison with patients with more wild-type alleles, see also Figure 5.1.

Table 5.2 Effect of genotypes on busulfan clearance

SNP/Haplotype	Genotype	n (%)		Busulfan clearance (L/h/m^2) (mean ± sd)	P-value
GSTA1	*A/*A	32	(39.5%)	7.2 ± 1.9	
	*A/*B	38	(46.9%)	6.6 ± 1.7	0.004
	*B/*B	11	(13.6%)	5.2 ± 1.6	
CYP39A1_TC	WT/WT	21	(25.3%)	7.2 ± 2.0	
	WT/TC	32	(38.6%)	6.3 ± 1.6	0.011
	TC/TC	30	(36.1%)	6.0 ± 1.6	
CYP39A1	G/G	42	(50.0%)	6.7 ± 2.0	
	G/A	33	(39.3%)	6.7 ± 1.7	0.30
	A/A	9	(10.7%)	5.7 ± 1.5	
CYP2C19	C/C	52	(61.9%)	6.6 ± 2.1	
	C/T	27	(32.1%)	6.6 ± 1.5	0.81
	T/T	5	(6.0%)	6.3 ± 1.1	
ABCB4_GAT	WT/WT	28	(34.1%)	6.4 ± 1.8	
	WT/GAT	33	(40.2%)	6.6 ± 1.8	0.4
	GAT/GAT	21	(25.6%)	6.8 ± 2.0	
SLC7A8	A/A	35	(41.7%)	6.7 ± 1.7	
	A/C	35	(41.7%)	6.6 ± 2.0	0.35
	C/C	14	(16.6%)	6.1 ± 1.8	
SLC22A4	C/C	33	(39.8%)	6.4 ± 1.9	
	C/T	39	(47.0%)	6.7 ± 1.8	0.47
	T/T	11	(13.3%)	6.8 ± 2.0	

Distribution of genotypes of each of the seven genetic markers and mean busulfan clearance per genotype.
SD: Standard deviation, WT: wild-type.

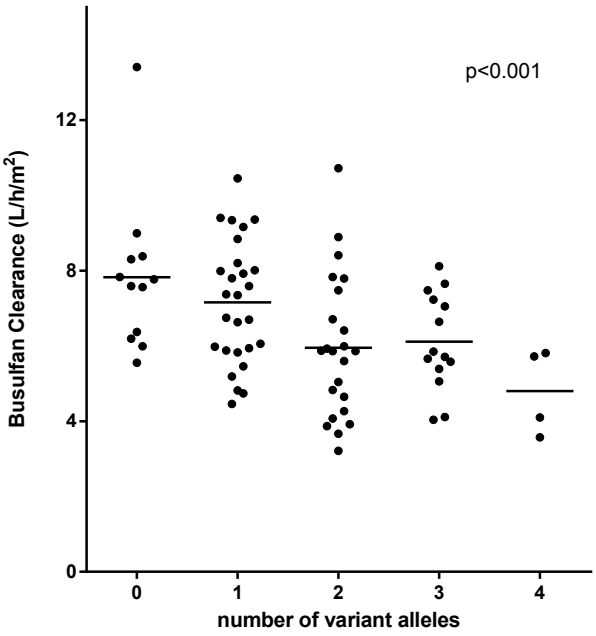

Figure 5.1 Association of the number of variant alleles in *GSTA1* and *CYP39A1* and busulfan clearance.

Effect of ontogenesis on *GSTA1*

Twenty children were younger than 2 years and 64 children were older than 2 years. In younger children it was noted that *GSTA1* had a larger effect; explaining 20% of variability in clearance ($p = 0.046$) than in older children, explaining only 5.2% ($p = 0.078$), see also Figure 5.2.

In the children younger than 2 years, the mean clearance was 6.9 ± 2.3 L/h/m^2 in the *GSTA1* wild-type group, 5.9 ± 2.2 L/h/m^2 in the heterozygous group (**A/*B*) and 4.1 ± 0.5 L/h/m^2 in the homozygous **B/*B* group. In the group of children older than 2 years **A/*A* carriers had a mean clearance of 7.2 ± 1.9 L/h/m^2, this was 6.8 ± 1.4 L/h/m^2 in heterozygous patients and 5.9 ± 1.6 L/h/m^2 in the **B/*B* carriers.

We performed a multivariate analysis taking *GSTA1* genotype, *CYP39A1* genotype, age (older or younger than 2 years) and underlying disease as covariates. Underlying disease was excluded as a covariate from the model and the two genotypes and age resulted in an explained variance of 21% (adjusted R^2) and $p = 0.004$.

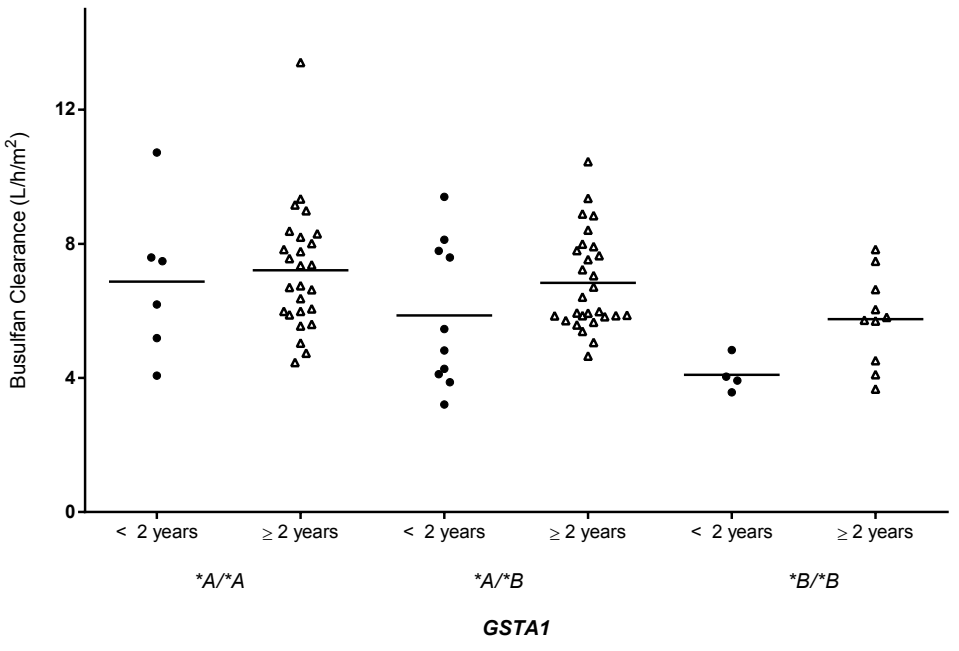

Figure 5.2 Relationship between *GSTA1* genotype and clearance per m^2 per age group. Closed circles: children younger than 2 years. Open triangles: children older than 2 years. Bars indicate mean values for each category.

DISCUSSION

In this study, *GSTA1* and *CYP39A1* were found to be associated with busulfan clearance. The involvement of *GSTA1* in busulfan PK confirms earlier findings in pediatric patients; the involvement of a haplotype in *CYP39A1* in busulfan PK is new. When combined, the two haplotypes explain 20% of the variability in busulfan clearance.

Busulfan is primarily metabolized by conjugation with glutathione, catalyzed by glutathione-*S*-transferases (GSTs). GSTA1 is the predominant GST isoenzyme involved; GSTM1 and GSTP1 have 46% and 18% of the activity of GSTA1 in busulfan metabolism, respectively.[24] The effect of SNPs in genes encoding for GST enzymes on busulfan PK have been studied previously, both in adults and pediatric patients, leading to unclear results. The amount of variability in busulfan clearance explained by the most important SNP in *GSTA1* (rs395735) differs per study, possibly due to ethnicity, busulfan route of administration (oral versus iv.) and age differences in the studied populations. Especially in the pediatric population, the role of the GSTA1 haplotype in busulfan PK is unclear.

The first study in pediatric patients, carried out by Johnson *et al*., showed that children who were heterozygous or homozygous for the *GSTA1*B* haplotype (regardless of age) exhibited a 30% decrease in busulfan clearance.[12] We also investigated the effect of SNPs in different GST genes (*GSTA1*, *GSTM1*, *GSTP1* and *GSTT1*) and we did not find an association of the SNPs with busulfan clearance.[13] Also Ansari et al. did not find an effect of *GSTA1* on busulfan clearance in pediatric patients, but showed lower busulfan clearances in patients with the *GSTM1*-null genotype.[14] Gaziev *et al*. studied the effect of the *GSTA1* SNP in pediatric thalassemia patients showing 10% lower busulfan clearance in patients with the *GSTA1*B* variant.[15]

An important difference between the positive and negative studies towards the effect of genetic variation in *GSTA1* on busulfan clearance is the way in which clearance was expressed. In the positive studies clearance was normalized for weight, which was not the case in the negative studies.

The effect of body size on busulfan clearance in pediatric patients has been studied extensively and could explain a major part of the variability.[25] In this study busulfan clearance was adjusted for BSA, as suggested by Trame *et al*.[21] and McCune *et al*.[22] We hypothesize that, when clearance is not normalized for body size (expressed as BSA, bodyweight or allometric scaled bodyweight), variability is much larger and the effect of body size surpasses the probably smaller effect of the genetic variants.

Several studies have identified factors affecting the PK of busulfan; recipients underlying disease, age and concomitant administration of fludarabine.[22,26,27] McCune *et al*. demonstrated a larger variability in clearance in infants (i.e., ≤ 12 kg bodyweight) in comparison to older children, also when normalized for BSA.[22] This was also observed in our data; a variability in clearance of 36% was observed in younger children versus 25% in older children.

In our population, patients with different underlying diseases, age and cyclophosphamide- and fludarabine-based conditioning regimens were included. We did not see a difference in clearance in patients with cyclophosphamide-based conditioning versus patients with fludarabine-based conditioning ($p = 0.9$).

Patients with immune deficiencies had an 18% lower clearance compared with patients with other underlying diseases ($p = 0.02$). Age also appeared to be related to clearance and clearance was 15% lower in patients younger than 2 years ($p = 0.31$). However, the proportion of patients with immune deficiencies was larger in patients younger than 2 years ($p = 0.02$) and in the multivariate analysis only age remained as an independent predictor of clearance.

Apart from inherited differences in enzyme function, developmental changes in enzyme function also play an important role in the metabolic capacity of pediatric patients. Ontogenesis

in young children could influence the explanatory power of pharmacogenetic biomarkers affecting busulfan PK. Unfortunately there are no specific data on ontogenesis of GSTAs available. However, for most enzymes developmental changes in expression is complete in the first 2 years after birth. Therefore, we explored the potential role of ontogenesis on pharmacogenetic differences in *GSTA1* by studying the effect of *GSTA1* genotype in two different age groups (younger and older than 2 years of age). Our study shows for the first time an age-dependent effect of genetic differences in *GSTA1*. The effect of *GSTA1* on busulfan clearance was much stronger in younger children. This might be owing to an incomplete development of enzyme capacity in young children, resulting in a more pronounced effect of genetic variation on busulfan clearance.

This study revealed a potential role of the *CYP39A1* haplotype in busulfan PK. The functional effects of the SNPs in the *CYP39A1* haplotype are currently unknown and warrant further study. Also, the role in busulfan metabolism needs clarification. Interestingly, the software of the DMET platform classifies both SNPs as being part of the *CYP39A1* gene, but according to the NCBI SNP database, both SNPs are located in the *SLC25A27* gene. In fact, the promoter region of *CYP39A1* and the gene of *SLC25A27* are overlapping.

CYP39A1 encodes for a member of the CYP450 superfamily of enzymes, and it is involved in the conversion of cholesterol to bile acids. The synthesis and excretion of bile acids comprise the major pathway of cholesterol catabolism in humans. The *SLC25A27* gene, which encodes member 27 of the solute carrier 25 family, is also known as UPC4. These proteins are part of the family of mitochondrial anion carrier proteins and are involved in the transfer of anions from the inner to the outer mitochondrial membrane.[28] The effect of this identified haplotype should be further investigated.

The combined haplotypes in *GSTA1* and *CYP39A1* could explain 17% of the remaining variability in busulfan clearance. The clearance in patients who are homozygous for both variant haplotypes was 39% lower in comparison with wild-type patients. The effect of these two SNPs on busulfan clearance calls for further research into their relationship with clinical outcomes such as engraftment and toxicity.

Two haplotypes in *CYP3391* and *GSTA1* were found to be associated with busulfan clearance in pediatric patients. The *CYP39A1* haplotype was not previously related with busulfan PK. The role of the haplotype in *GSTA1* is in line with earlier findings. Furthermore, the effect of *GSTA1* haplotype on clearance was dependent on age. In young children this haplotype explained 20% of the variability in busulfan clearance, in the older children GSTA1 explained 5.2% of the variability.

Busulfan is one of the cornerstones of conditioning regimens in HSCT in pediatric patients. Busulfan dosing can be optimized by increasing our knowledge of the variables affecting busulfan PK, including the effect of pharmacogenetic markers. The effect of *GSTA1* and *CYP39A1* polymorphisms on busulfan-related treatment outcomes such as engraftment and toxicity should be investigated. Busulfan pharmacogenetics ultimately holds the potential to optimize conditioning of pediatric HSCT patients by decreasing toxicity and increasing efficacy.

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Chapter 6

Pharmacokinetics of treosulfan in pediatric patients undergoing hematopoietic stem cell transplantation

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ABSTRACT

High-dose treosulfan is used in conditioning regimens before hematopoietic stem cell transplantation in children. Pharmacokinetic data to optimize treosulfan dosing are scarce in this patient population. The aims of this study were the development and validation of an analytical method for treosulfan in human serum and the development of a pharmacokinetic model for treosulfan in pediatric patients. Furthermore, we aimed to develop a limited sampling strategy to estimate treosulfan systemic exposure with a minimum of inconvenience and risk for the patient.

A reversed phase high-performance liquid chromatography method using ultraviolet detection to determine treosulfan in human serum samples was developed and validated according to food and drug administration guidelines. Serum pharmacokinetics after the first treosulfan administration was investigated in 20 children using nonlinear mixed-effect modeling, and a limited sampling strategy was developed and validated.

The assay was validated in a 10–500 mg/L concentration range with a lower limit of quantification of 10 mg/L. Accuracies were within the 90%–110% limit. The coefficients of variation of the within-day imprecision and between-days imprecision were less than 5%. Pharmacokinetics was adequately described with a 1-compartment model. The population estimates for clearance (CL) and volume of distribution were 6.85 L/h and 13.2 L for a typical patient of 20 kg, respectively. Treosulfan exposure could be adequately quantified with 2 samples, at 4 and 7 hours after the start of a 3-hour treosulfan infusion, with a mean deviation of 3% of individual CL and area under the curve based on limited sampling in comparison with the full data set in a total cohort.

In conclusion, in this study a bioanalytical method, PK model, and limited sampling model were developed and validated. Furthermore, PK parameters of 20 pediatric patients were analyzed, demonstrating an interpatient variability in area under the curve of 14.5%. This study demonstrates the essential developments in the optimization of treosulfan therapy based on PK data.

INTRODUCTION

For a broad spectrum of malignant and non-malignant disorders in pediatric patients, allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only curative option. However, allo-HSCT is accompanied with considerable acute and long-term toxicity, in which the chemotherapeutic agents given in the conditioning regimen play an important role.[1–3] Various conditioning regimens have been developed which differ in immunosuppressive and myeloablative potential. Myeloablative conditioning aims at maximum elimination of host hematopoiesis and malignant cells and is therefore associated with a relatively high risk of severe early and late toxicity. Non-myeloablative conditioning and reduced intensity conditioning have been developed to reduce treatment-related toxicities in patients with compromised organ function and for patients in whom full myeloablation is not required for cure of the underlying disease. However, the relapse rates and risk of graft failures in the latter conditioning regimens are higher.[4] Therefore, conditioning regimens could still be improved by combining myeloablative and immunosuppressive capacities with a low toxicity. Treosulfan, a bifunctional alkylating drug, has a suitable profile for use in myeloablative regimens; it gives a rapid and stable myeloablation and exhibits strong immunosuppressive activity to allow donor engraftment.[5] Furthermore, treosulfan seems to have a favorable toxicity profile; diarrhea, mucositis, stomatitis, skin toxicity, and metabolic acidosis (due to the formation of methanesulfonic acid during treosulfan activation) are the most often reported toxicities and treosulfan rarely causes hepatotoxicity.[6–8] Treosulfan is a structural analogue of busulfan. However, its mechanism of action is different; treosulfanis a prodrug and the mono- and diepoxybutane derivates are responsible for alkylation of DNA and DNA cross-linking.[9] These active derivates are formed by a non-enzymatic intramolecular nucleophilic substitution which is pH and temperature dependent; no conversion of treosulfan occurs at a pH <6.0.[7] Pharmacokinetic data of treosulfan are scarce; studies available in adult patients show a linear relationship between area under the curve (AUC) and dose.[6] Only 2 reports describe the pharmacokinetics of treosulfan in pediatric patients.[10,11] In these studies, the interpatient variability in exposure AUC of 5 children seems to be very large (CV = 70%), and therapeutic drug monitoring could therefore be of great importance to optimize treosulfan therapy for the individual patient. We developed and validated a reversed phase high performance liquid chromatography (RP-HPLC) method using ultraviolet (UV) detection for quantification of treosulfan inhuman serum. Furthermore, we developed and validated in an independent cohort a population pharmacokinetic model of treosulfan and a limited sampling strategy (LSS).

MATERIALS AND METHODS

Patient characteristics

Pediatric patients (≤ 18 years) receiving treosulfan-based conditioning before their allo-HSCT in the Leiden University Medical Center in the Netherlands were included. The institutional ethics committee approved the study protocol. Written informed consent was obtained from all parents of the study patients and the patients themselves if they were older than 12 years according to the Helsinski Declaration. Indications for HSCT were hematologic malignancies, hemoglobinopathies, or immune deficiencies. All patients received treosulfan 14 g/m^2 intravenously from day 7 to day 5 before HSCT and combined with fludarabine 30 mg/m^2 intravenously from day 7 to day 3 and in some cases with additional thiotepa 8 mg/kg on day 8. Serotherapy consisted of antithymocyte globuline 2.5 mg/kg from day 6 to day 2 or alemtuzumab 0.2 mg/kg from day 5 to day 2. In one patient, treosulfan was combined with thoracoabdominal irradiation and alemtuzumab.

Bioanalytical method development

The bioanaltyical method for treosulfan presented in this article is based on the method to determine busulfan developed in our department[12] and the method for treosulfan presented by Glowka *et al*.[11] and Hilger *et al*.[13] Because treosulfan is non-enzymatically converted into its active metabolites, it is suggested that the concentration of treosulfan itself is a good representation of the alkylating activity.[13]

Chromatographic conditions

An RP-HPLC method using UV detection to determine treosulfan in human serum samples was developed. Busulfan was used as an internal standard. Gradient elution with a mobile phase consisting of phosphate buffer (pH 4.0), acetonitrile, and methanol was used. The acetonitrile concentration in the eluant was kept constant throughout the analytical run. The flow rate was 0.8 mL/min, and the eluent was monitored at 275 nm. For all types of samples, the injection volume was 10 mL and separation was performed on an ODS Hypersil column (3 mm, 100 x 4.6 mm; Thermo Fischer Scientific, Waltham, MA) with a Prevail C18 precolumn (5 mm, 7.5 x 4.6 mm; Alltech Associations Inc, Deerfield,IL). The runtime per sample was 15 minutes.

Serum sample preparation

To all 200 mL serum of patient, calibration (10, 20, 50, 100, 250, and 500 mg/L) and quality control (QC) samples (15, 80, and 400 mg/L), 30 mL of 1 mol/L citrate buffer (pH 5.5), and 200 mL acetonitrile was added and the mixture was shaken vigorously; next 200 mL of 0.44 mol/L diethyldithiocarbamate (DDTC) was added to derivatize treosulfan by incubating the mixture for 15 minutes at 50°C. After cooling down to room temperature, 10 mL of busulfan 3 mg/mL, the internal standard, was added and the solution was vortex mixed during 30 seconds. To make treosulfan and busulfan detectable with UV, derivatization with DDTC was performed. Treosulfan and busulfan are derivatized separately; busulfan is added at the end of the treosulfan derivatization. In this way, complete derivatization of both compounds can be achieved. When busulfan is derivatized at the same conditions as treosulfan, the busulfan derivate is partly degraded, causing disturbance of the chromatograms. Two milliliters of ethyl acetate was added, vortexed for 30 seconds, and after centrifugation at 3220 *g* during 3 minutes, the organic layer was transferred into a new glass tube and evaporated to dryness at 50°C under nitrogen gas. The residue was dissolved in 300 mL of methanol of which 200 mL was transferred into a glass autoinjector vial.

Validation

The analytical assay was validated according to food and drug administration guidelines.[14] Serum QC samples were prepared with a concentration of 15, 80, and 400 mg/L. Accuracy, within-day imprecision and between-day imprecision were determined by analyzing each QC sample 5 times on 1 day and on 5 different days. Accuracy was defined as the mean measured concentration and should be between 90% and 110% of the nominal concentration. The imprecision was expressed as % CV and should be less than 10% for within-day imprecision and less than 15% for between-day imprecision. The lower limit of quantification was calculated from the residual standard deviation (using 99.9% confidence interval) and the slope of the calibration line. The recovery of treosulfan extraction was determined in triplicate by comparing processed serum samples of 15, 80, and 400 mg/L with reference samples in citrate buffer (pH 5.5) of the same concentration. Selectivity and matrix interference was investigated by measuring 2 different concentrations prepared in 6 different blank serum samples. When CV was less than 10%, the interferences of the matrix were considered insignificant. Short-term and long-term stability at 2 concentrations (20 and 400 mg/L) were studied at room temperature and 4°C (short term) and at -20°C and -80°C (long term). Furthermore, postprocessing stability and freeze–thaw stability were also studied at the same 2 concentrations. All stability measurements

were performed in triplicate. When concentrations measured are within 100% ± 10% of the initial concentration, the samples were considered stable.

Pharmacokinetics

Blood sampling

All patients received 14 g/m^2 treosulfan in a 3-hour infusion on 3 consecutive days. On day 1, patient blood samples were collected at 1.5, 3.5, 4, 5, 7, and 9 hours after the start of a 3-hour treosulfan infusion. Blood samples were collected in serum tubes without gel through the lumen of the catheter that was not used for intravenous treosulfan administration. Samples were centrifuged as quickly as possible after the collection of the patient sample, at least within 5 hours. Serum was transferred into a polystyrene tube and stored directly at -80°C until analysis.

Pharmacokinetic model development

The treosulfan population pharmacokinetic model was developed by using nonlinear mixed-effect modeling as implemented in the NONMEM software package (version 7 level 2; Icon Development Solutions, Ellicott City, MD). Diagnostic graphics, exploratory analyses, and post-processing of NONMEM output were performed using S-Plus (version 8.0 Professional; Insightful Corp, Seattle, WA). In the first step, the PK model was developed using the data from an initial cohort of 12 patients. The final PK model was validated using the data from a second independent cohort of 8 patients. Different linear compartmental models (1-, 2-, or 3-compartmental models) were explored to describe the PK of treosulfan. The parameters (clearance (CL) and volume of distribution (V)) among individuals were assumed to be log-normally distributed. Consequently, the random effects at the individual level were implemented as exponential terms (Equation 1):

$$\theta_i = \theta \exp(\eta_i) \qquad (1)$$

With η_i as the random effect describing the deviation of the individual parameter estimate (θ_i) of the parameter for the ith individual from the typical population parameter estimate θ_i. η_i is assumed to follow a normal distribution with mean zero and variance ω^2. The magnitude of residual variability between observed and predicted values was modeled using a proportional model (Equation 2):

$$C_{ij} = C_{pred,ij}(1 + \varepsilon_{ij}) \qquad (2)$$

where C_{ij} is the jth observation for the ith individual; $C_{pred,ij}$ is the corresponding model predicted value and ε_{ij} represents the residual deviation of the observed concentration from the predicted concentration. ε_{ij} is assumed to follow a normal distribution with mean zero and variance σ^2.

Improvement in the fit of the model was assessed using the likelihood ratio test. This statistical test compares the fit of 2 models by evaluating the difference in the minimum value of objective function (MVOF), which is equal to minus twice the log-likelihood of the data.[15] A difference of 10.8 points in MVOF was considered statistically significant ($p < 0.001$, given 1 degree of freedom, assuming χ^2 distribution). In addition, standard goodness-of-fit plots were inspected visually to evaluate the model fit and the predictive performance of the developed model was evaluated using simulation-based diagnostics such as visual predictive check (VPC). In the VPC, it was investigated visually if the identified model predicts the median concentration and variability from the population that was used for model identification adequately. To this end, the PK of each individual was simulated 500 times by means of a Monte Carlo simulation. The distribution (median and 10th and 90th percentiles) of the simulated concentration–time profiles was compared with the distribution of the observed concentration–time profiles.

Limited sampling strategy

The aim of developing a LSS was to make adequate estimates of treosulfan exposure AUC with a minimal number of serum samples and thus with a minimal patient burden. A hundred data sets were simulated using the final population PK model. These 100 data sets were back fitted with the final model, resulting in 100 population estimates of CL and V. Different strategies of 2, 3, and 4 time points were investigated. Per strategy, the resulting population estimates for CL and V (test CL and V) were compared with original population estimates for CL and V (reference CL and V). Predictive performance of the different strategies was assessed. The prediction bias was calculated as the mean prediction error (MPE); average difference between test and reference CL and V. The prediction precision was assessed as the mean absolute percentage error (MAPE); average absolute difference between test and reference CL and V.

External validation

The external validation of the population PK model to describe the concentration–time profiles of treosulfan was performed using a second independent cohort of 8 patients. It was investigated if the model could adequately predict individual concentrations and CLs in this external data set. Again, visual exploration of the distribution of the observed and predicted values in standard goodness-of-fit plots was used to evaluate the model fit. In addition, the LSS was validated. The individual CL and corresponding AUCs based on the full data set (6 samples) were compared with the individual CL and AUC calculated based on a data set with a reduced number of samples.

RESULTS

Description of the patient populations

The population PK model for treosulfan was developed using a first cohort of 12 pediatric patients. Details on the patient characteristics, conditioning regimens, diagnosis for HSCT, and donor information are presented in Table 6.1 for the first and second cohort separately and the total of 20 patients. Briefly, the mean age and weight of the patients in this first cohort were 6.9 (± 5.5) years and 21.5 (± 14.2) kg, respectively, and 7 patients were male. In the second cohort, the mean age was 5.4 (± 5.5) years and the mean weight was 21.0 (± 14.3) kg and 6 patients were male. When looking at the combination of both cohorts, for 4 patients, the indication for HSCT was a hematologic malignancy: 3 patients were diagnosed with secondary (n = 1) or relapsed (n = 2) acute myelogenous leukemia and 1 patient with juvenile myelomonocytic leukemia. Twelve patients had a hemoglobinopathy; 11 patients with a homozygous β-thalassemia and 1 patient with sickle cell disease. Three patients had a (severe) combined immunodeficiency and

Table 6.1 Patient characteristics and conditioning regimens

	Total (n = 20)	1st cohort (n = 12)	2nd cohort (n = 8)
Characteristic		Mean (s.d.)	
Age (years)	6.2 (5.4)	6.9 (5.5)	5.4 (5.5)
Weight (kg)	21.3 (13.9)	21.5 (14.2)	21.0 (14.3)
Sex (n and % male)	13 (65%)	7 (58%)	6 (75%)
Conditioning regimen		Patients (n)	
Treo, Flu, Thio, ATG	12	6	6
Treo, Flu, Thio, Alem	2	1	1
Treo, Flu, ATG	4	3	1
Treo, Flu, Alem	1	1	-
Treo, TAI, Alem	1	1	-
Diagnosis for HSCT			
Hemoglobinopathies	12	5	7
Hematologic malignancy	4	4	-
Immune deficiencies	4	3	1
Donor			
HLA id sibling	7	4	3
UD 10/10	6	4	2
UD 9/10	6	4	2
HLA id Cord Blood	1	-	1

ATG: antithymocyte globuline, Alem: alemtuzumab, Flu: fludarabine, HLA id: human leukocyte antigen identical, TAI: thoracoabdominal irradiation, Thio: thiotepa, Treo: treosulfan, UD: unrelated donor.

1 patient X-linked lymphoproliferative disease. Two patients received this conditioning regimen without thiotepa. In 1 case, treosulfan was combined with thoracoabdominal irradiation.

Validation of the treosulfan assay

A calibration range of 10–500 mg/L treosulfan in serum was selected. Average lower limit of quantification over 8 calibration lines was 10 mg/L, which is lower than the lowest calibrator. Chromatograms of blank, QC low, and a patient serum sample are shown in Supplemental Figure 6.1. No interference at the retention times of treosulfan and busulfan was observed. Accuracy of the samples with a concentration of 100% and 150% of the highest calibrator was 100.3% and 95.5%. Accuracies for each of the 3 QC samples were within the 90%–110% limit. The coefficient of variation of the within-day imprecision and between-day imprecision was less than 5% (Table 6.2). The selectivity of the assay was appropriate, resulting in a CV of less than 5% when measuring treosulfan in 6 different blank samples at the lower limit of quantification level (10 mg/L). Furthermore, in the chromatogram of a patient, no interference due to comedication was observed (see Supplemental Figure 6.1). This is due to the selectivity of the derivatization of treosulfan and busulfan. No carryover was observed after the analytical run of a sample with a concentration of 400 mg/L. The recoveries of treosulfan were appropriate for all 3 QC levels showing recovery rates of 95%, 96%, and 84% at 15, 80, and 400 mg/L, respectively. An overview of stability data is shown Supplemental Table 6.1. Stock solutions of treosulfan in water and busulfan in n,n-dimethylacetamide stored at -20 or -80°C are stable for at least 1 year (data not shown). Treosulfan serum samples are stable for 5 hours at room temperature and 4°C. Serum samples stored at -20°C are stable during 2 months, storing at -80°C resulted in stable samples for at least 1 year. Treosulfan in whole blood samples is stable during 5 hours at room temperature (data not shown). With this information, we conclude that blood samples should be centrifuged within 5 hours after withdrawal and obtained serum should be stored directly

Table 6.2 Accuracy, within-day imprecision, and between-day imprecision at 2 concentrations of treosulfan

Concentration treosulfan	N	Accuracy (%)	Within-day imprecision CV (%)	Between-day imprecision CV (%)
15 mg/L	5	102.8	2.02	2.84
80 mg/L	5	106.2	3.33	2.31
400 mg/L	5	105.8	1.96	2.13

Accuracy, within-day imprecision, and between-day imprecision obtained with 5 samples at treosulfan concentrations of 15, 80, and 400 mg/L. CV: coefficient of variance.

at -20°C for treosulfan determination. Additionally, treosulfan serum samples are stable after 3 freeze and thaw cycles at -80°C and during 5 hours postprocessing.

Pharmacokinetic model

The time course of the plasma concentration of treosulfan was adequately described using a 1-compartment model. Using a 2-compartmental model, no significant improvement in model fit could be obtained, as judged from the difference in MVOF (difference in MVOF > -10.8). Parameters were allometrically scaled using body weight according to the following equation (Equation 3):

$$\theta_i = \theta_j \cdot \left(\frac{BW}{20kg} \right)^{\alpha} \qquad (3)$$

Where θ_i is the individual CL or V and θ_j is the population CL or V, based on a weight of 20 kg. When scaling CL α is fixed to 0.75 and for V α is fixed to 1.0. The population PK model adequately describes the concentration–time profile of treosulfan in the first cohort of 12 patients (Figure 6.1). As observed in the VPC (Figure 6.2), the predicted and observed intervals (median, 10th and 90th percentiles) show good correspondence, demonstrating a good predictive performance of the final model. The population estimates of CL and V for a patient of 20 kg were 6.3 L/h and 12.3 L, respectively. An overview of pharmacokinetic parameters is given in Table 6.3a.

Limited sampling strategy

Predictive performance (MPE and MAPE) of the different limited sampling strategies is listed in Table 6.4. Two samples were sufficient to estimate the CL and V at the population level. Two time points at 4 and 7 hours are preferred from a practical perspective (no sampling during infusion). Samples taken between 4–5 hours and 6–7 hours after the start of a 3-hour infusion will give a good estimation of treosulfan exposure.

External validation

The model, developed using the data from the first cohort, was fitted to the data of a second cohort of 8 patients. The diagnostic plots (Figure 6.3) indicate a small bias in observed concentrations versus population predicted concentrations. However, individual concentrations could be well predicted. In addition, the limited sampling design with 2 samples around 4 and 7 hours after the start of infusion allows estimation of the individual CLs and AUCs with little bias (<15%). In 7 of the 8 patients, the difference in AUC was less than 10%. Only in 1 patient,

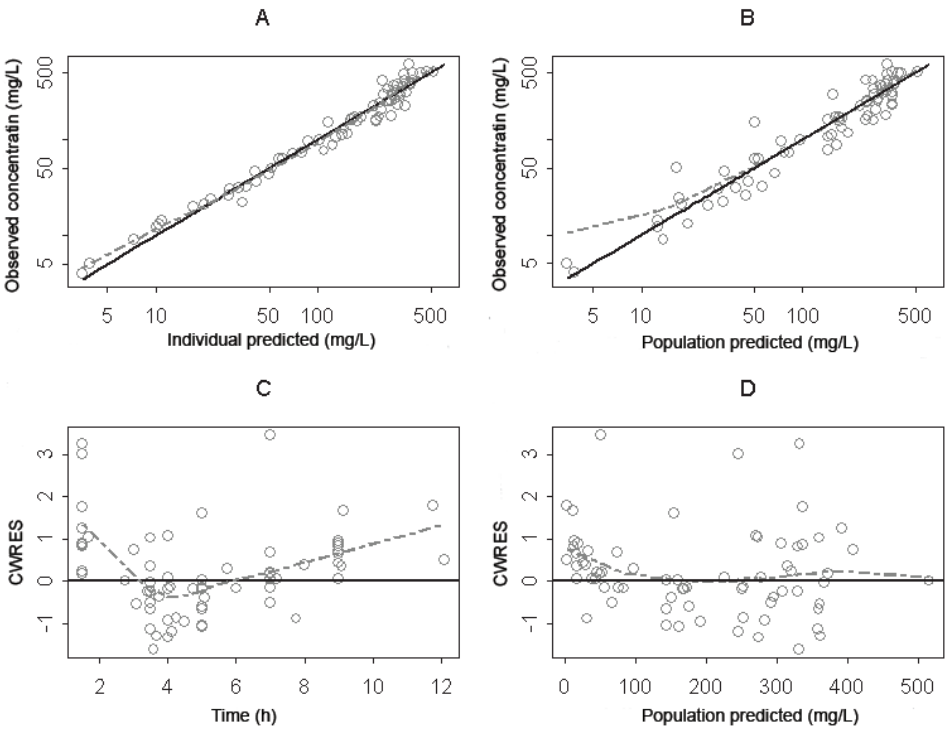

Figure 6.1 Diagnostic plots of treosulfan pharmacokinetic model. A) Observed concentrations versus individual predictions; data should be randomly distributed around the identity line. B) Observations concentrations versus population predictions (PRED); data should be randomly distributed around the identity line. C) Conditional weighted residuals (CWRES) versus time; data should be randomly distributed around zero line for the complete time course. D) CWRES versus PRED; data should be randomly distributed around zero line for all population predictions. Gray open circles: observations; black line: identity or zero line; gray intermittent line: smoother trough the data.

the difference in AUC was above 10% (e.g. 14%). This patient was a relatively older child of 16 years weighing 52 kg. The difference in AUC is probably attributable to the small data sets for model development and validation; especially the number of older patients was limited. The parameter estimates were updated by fitting the final model to the data from 2 cohorts (n_{total} = 20) (Table 6.3b). In addition, the LSS was assessed using the updated model parameters based on the combined data set of 20 patients. As a result, with only 2 samples of each individual, a good estimate of individual CL and AUC was obtained. A mean deviation of 3% in individual CL and AUC was observed when comparing the reduced with the full data set (66 samples), with

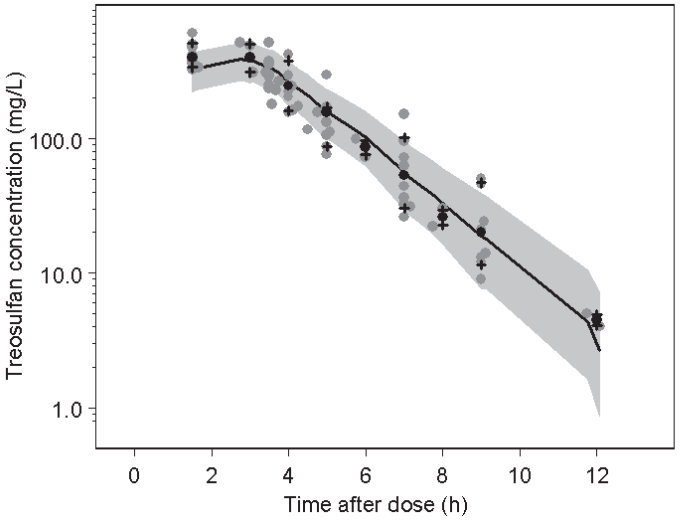

Figure 6.2 Visual predictive check. VPC with 80% prediction interval (gray area). Black line represents the median of the model prediction. Gray dots: observed concentrations. Black dots: median of the observed concentrations per time point and black plus sign: lower and upper boundary observation interval (80%).

Table 6.3a Population pharmacokinetic parameters for the treosulfan model based on the first cohort

Parameter	Value	SE	CV	95% CI	% variation
CL (20 kg)	6.3	0.462	7.33	5.39–7.21	NA
V (20 kg)	12.3	0.71	5.77	10.9–13.7	NA
Eta CL	0.0177	0.0107	60.5	-0.0033–0.0387	13.36
Residual error	0.0513	0.0092	17.8	0.0334–0.0692	22.94

CI: confidence interval, CV: coefficient of variance, Eta CL: random effect describing the deviation of the individual parameter estimate, SE: standard error, NA: not available.

Table 6.3b Updated population model based on data set of 20 patients

Parameter	Value	SE	CV	95% CI	% variation
CL (20 kg)	6.85	0.388	5.7	6.09–7.61	NA
V (20 kg)	13.2	0.626	4.7	12.0–14.4	NA
Eta CL	0.0174	0.00864	49.7	0.0005–0.0343	13.25
Residual error	0.0477	0.00673	14.1	0.0345–0.0609	

CI: confidence interval, CV: coefficient of variance, Eta CL: random effect describing the deviation of the individual parameter estimate, SE: standard error, NA: not available.

a maximum of 13.1% for CL and 11.6% for AUC. Furthermore, no clear dependency between age/weight and deviation in AUC could be observed anymore. The population estimates for treosulfan CL in the total study cohort for CL and V were 6.85 L/h and 13.2 L for atypical patient

Table 6.4 Prediction performance of different limited sampling strategies

LSS	MPE (%) CL	MAPE (%) CL	MPE (%) V	MAPE (%) V	Cov. suc
1.5, 4, 5, 7h	0.92	3.92	-0.12	4.66	100
1.5, 5h	1.64	4.77	-0.66	6.72	87
4, 7h	1.10	6.36	0.1	10.2	97
5h	-8.55	23.1	42.8	79.8	26

Four different limited sampling strategies were evaluated. Cov. suc: successful covariance steps, MPE: mean predictive error.

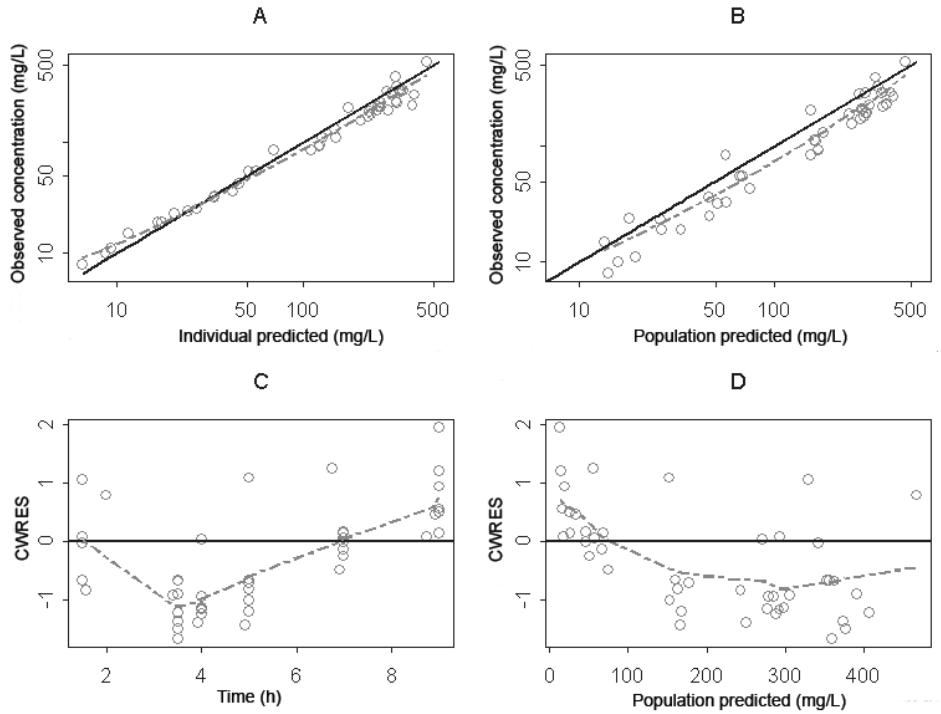

Figure 6.3 Diagnostic plots of external validation. A) Observed concentrations versus individual predictions; data should be randomly distributed around the identity line. B) Observations concentrations versus population predictions (PRED); data should be randomly distributed around the identity line. C) Conditional weighted residuals (CWRES) versus time; data should be randomly distributed around zero line for the complete time course. D) CWRES versus PRED; data should be randomly distributed around zero line for all population predictions. Gray open circles: observations; black line: identity or zero line; gray intermittent line: smoother trough the data.

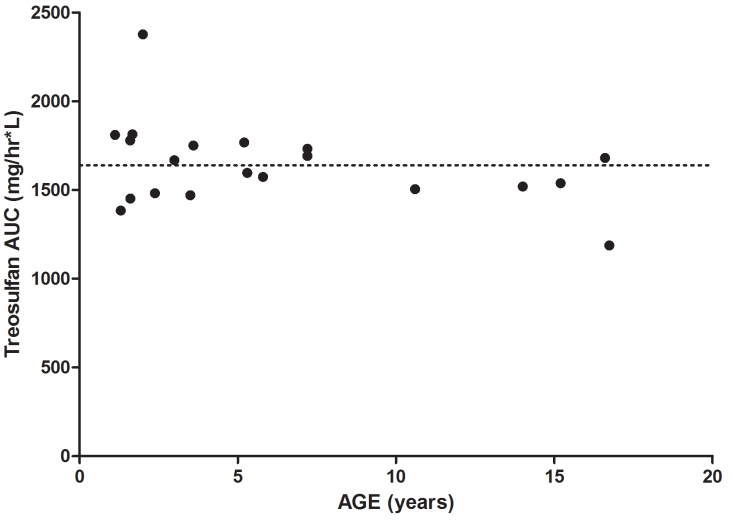

Figure 6.4 AUC values of 20 individual pediatric patients. Distribution of AUC per age. AUC calculated after dose 1, 14 mg/m^2. Horizontal line: average AUC of 1640 mg*h/L.

of 20 kg, respectively. The mean AUC in the studied population of 20 patients was 1639 ± 237 mg*h/L (calculated by individual dose/individual CL). The interpatient variability in AUC was calculated as 14.5%. In Figure 6.4, graphical presentation of all individual AUCs is provided.

DISCUSSION

We successfully developed and validated a bioanalytical method to quantify treosulfan concentrations in serum and a pharmacokinetic model to describe the concentration–time profile for treosulfan in pediatric patients. Furthermore, an LSS was developed to estimate treosulfan exposure, expressed as AUC, in pediatric patients based on only 2 serum samples. This bioanalytical method combined with a PK model is crucial to study whether outcome after conditioning with treosulfan is related to exposure and if treosulfan conditioning regimens can be optimized by targeting exposure. Furthermore, the limited sampling model is essential in studying treosulfan PK in pediatric patients, especially the youngest.

Two analytical methods for analyzing treosulfan have been described previously. Glowka *et al*.[11] presented methods for analyzing treosulfan in biologic fluids based on RP-HPLC with refractometric detection. Another article of Glowka *et al*.[16] describes a HPLC method using UV detection at 264 nm. Treosulfan and its metabolites were made detectable by derivatization

during 4 hours at 50°C with 3-nitrobenzenesulfonicacid (3-NBS) with an analytical runtime of 30 minutes. UV detection is also used in the method described in this article because this is a more selective and sensitive detection method compared with refractometric detection. In addition, UV detection is more commonly available and therefore the method presented in this article is easier to implement in most laboratories. However, treosulfan and busulfan (IS) detection with UV is possible only after derivatization of the compounds, resulting in more extensive sample preparation. Our analytical method is less time consuming due to an optimized derivatization process using DDTC, resulting in derivatization of treosulfan at 50°C during only 15 minutes, and an analytical runtime of 15 minutes.

Treosulfan itself is a prodrug, non-enzymatically, and pH dependent converted into its active metabolites. In one of the analytical methods of Glowka *et al*.[16] also, the active metabolites were analyzed. However, in this study, diepoxide was not detected in patient samples because of fast elimination. Furthermore, it is assumed that the concentration of treosulfan itself is a good representation of the alkylatingactivity.[13] The precise role of treosulfan or its metabolites in effectiveness of the conditioning is yet unclear.

In both methods presented by Glowka *et al*., patient blood samples were pretreated immediately after collection with citric acid to stop degradation of treosulfan, which in the clinic can be a problematic logistic step. Therefore, we thoroughly studied the stability of treosulfan in different biologic fluids under various conditions. These experiments indicated stability of treosulfan in whole blood and serum after collecting the samples during 5 hours, resulting in a more feasible methodology for patient sample collection.

The development of the pharmacokinetic model is based on the data from 12 patients. Despite the relative low number of subjects, multiple sampling in these subjects (approximately 6 samples per patient) facilitated the development of a PK model that adequately describes the concentration–time profiles of treosulfan and the observed variability. It was observed that combining the first and second cohorts resulted in different population parameter estimates. By increasing the number of patients in the analysis, the level of confidence in the developed PK model and population parameters increases and it is therefore recommended to update model parameters in future after including the data of new (pediatric) patients. In the development of a limited sampling model, different strategies were tested on a population level. The strategy with sampling at 4 and 7 hours after the start of the infusion was chosen, although MPE and MAPE values of the t = 1.5 and 5 hours strategy were slightly better. The first strategy was chosen because of the practical reason of no sampling during treosulfan infusion. Furthermore, the predictive performance was assessed on a population level. When considering the individualized

values of CL and AUC in the total cohort, it is demonstrated that our LSS adequately predicts treosulfan CL and AUC based on the 2 samples at 4 and 7 hours. This resulted in a total cohort of 20 patients a mean deviation of 3% in individual CL and AUC when comparing the limited sampling set with the full data set.

In our study, 6 blood samples of 1–2 mL on 1 day were collected. Therefore, one of the exclusion criteria was bodyweight under 10 kg. Because of this criterion, the treosulfan exposure and PK of patients under 10 kg body weight could not be studied. The LSS reduces the burden of sampling significantly and may allow investigating the PK of children with lower body weights in the future.

To date, pharmacokinetic data of treosulfan are scarce. In adult patients, treosulfan shows predictable linear pharmacokinetics.[6] For pediatric patients, only 1 study is published presenting PK data in a reasonable number of pediatric patients.[10] In this study of Glowka *et al*., 7 patients were analyzed, receiving different doses of treosulfan (10, 12, or 14 g/m^2), the exposure levels found were in accordance with the levels of the 20 patients in our study. The variability in exposure of 5 patients receiving the same dose was very large (CV = 70%). The cause of this large variability is unknown; however, it is based on a very small number of patients. In our study, the interpatient variability in exposure was found much smaller, 14%. This difference in variability could be due to the number of patients, Glowka *et al*. studied only 5 patients with the same dose. Alternatively, the trapezoidal rule was applied for AUC calculation of the individual patients, which could lead to less precise determination of the AUC.

Treosulfan is an attractive candidate for use in conditioning regimens before HSCT and is more often used in pediatric patients due to its rapid and stable immunosuppressive activity and favorable toxicity profile. In the study, a bioanalytical method, population PK model, and LSS for treosulfan were developed and validated. In the future, more data need to be collected to study the relationship between outcome and treosulfan exposure, especially in the youngest children with an age under 2 years.

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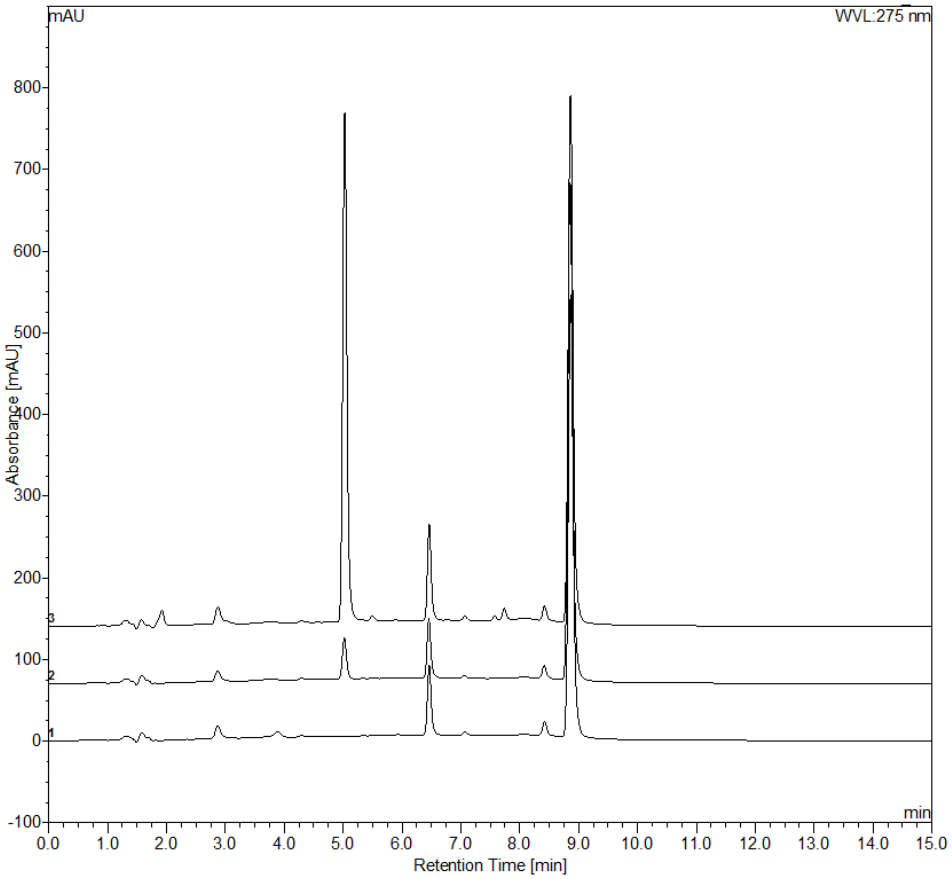
SUPPLEMENTAL MATERIAL



Supplemental Figure 6.1 Chromatograms of treosulfan assay. 1) Blank with internal standard; 2) Quality Control (low) 15 mg/L; 3) Patient sample. Retention time treosulfan: 5.0 min, retention time busulfan: 8.9 min.

Supplemental Table 6.1 Short term, long term, freeze-thaw and post processing stability data of treosulfan samples

		Percentage of initial concentration		
Short term	***Sample concentration***	***2 hours***	***5 hours***	***8 hours***
Room temperature	20 mg/L	110 (99.9–121.7)	97 (88.7–110)	81 (75.5–86.6)
	400 mg/L	92 (92.6–93.8)	89 (86.8–93.2)	88 (85.6–93.3)

		Percentage of initial concentration				
Long term	***Sample concentration***	***1 week***	***2 weeks***	***2 months***	***6 months***	***1 year***
-20°C	20 mg/L	94 (89.0–95.4)	97 (96.5–98.5)	96 (91.0–100.5)	79 (78.0–79.5)	55 (53.0–56.5)
	400 mg/L	94 (86.2–103.0)	98 (88.8–105.0)	89 (88.7–99.0)	72 (71.7–72.5)	76 (71.9–80.3)
-80°C	***Sample concentration***	***1 week***	***2 weeks***	***2 months***	***6 months***	***1 year***
	20 mg/L	88 (83.2–93.6)	104 (100.5–108.9)	102 (100.0–104.5)	99 (97.0–102.5)	102 (96.5–110.5)
	400 mg/L	103 (94.3–109.6)	100 (96.4–102.0)	96 (94.5–97.2)	95 (94.2–96.0)	99.8 (96.5–108.8)

	Sample concentration	***3 freeze-thaw cycles***	***Post-processing (5 hours)***	
-20°C	20 mg/L	83.7 (79.5–86.0)	20 mg/L treosulfan	101 (99.8–102.3)
	400 mg/L	91 (87.7–93.0)	400 mg/L treosulfan	100 (98.4–104.5)
-80°C	20 mg/L	108 (89.5–117)	Internal standard (busulfan)	102 (100.6–103.8)
	400 mg/L	103 (102.1–103.2)		

Chapter 7

Treosulfan-based conditioning in pediatric hematopoietic stem cell transplantation: a prospective study on pharmacokinetics and early clinical outcomes

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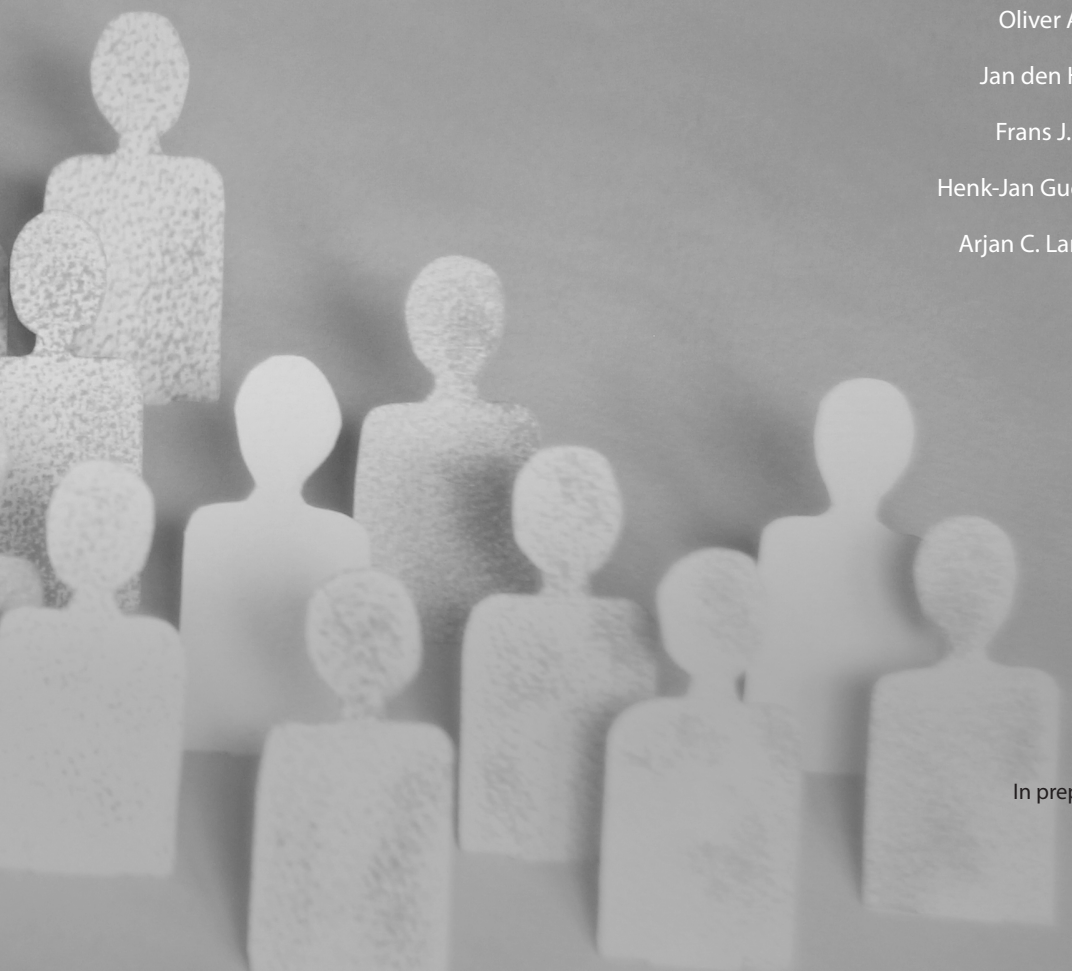

In preparation

ABSTRACT

Treosulfan is an alkylating agent applied in regimens prior to allogeneic hematopoietic stem cell transplantation (allo-HSCT) in children. It has strong myeloablative and immunosuppressive activity and a relatively mild toxicity profile. In this article we describe the first results of an ongoing prospective study on the relation between the pharmacokinetic profile of treosulfan in pediatric patients undergoing HSCT and clinical outcome.

A total of 21 patients were included in the study with a median age of 5.2 (0.13–16.8) years and a median follow-up of 1 year (168 days – 1.9 years). Patients received intravenous treosulfan prior to their HSCT for various malignant and non-malignant indications combined with fludarabine and thiotepa. Nine of 21 patients received treosulfan as a reduced toxicity conditioning, due to preexisting comorbidities. A one-compartment model was used and clearance and volume of distribution were allometrically scaled using body weight. The mean AUC was 1534 mg*h/L and the interpatient variability was 14%.

The overall toxicity profile was relatively mild and consisted mainly of liver toxicity (48%), skin toxicity (28%) and mucositis (38%). Of 19 evaluable patients, 95% had a primary engraftment. In this study we did not find a relation between treosulfan exposure and HSCT outcome parameters engraftment, chimerism, toxicity, and survival. This is the first study report in which the treosulfan pharmacokinetic profile in pediatric patients is described and exposure is related to clinical outcome. Analysis of larger cohorts of pediatric HSCT recipients is warranted to determine the relation between treosulfan exposure and HSCT outcome in age- and disease-specific subgroups.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative option for a variety of malignant and non-malignant hematologic diseases in pediatric patients. The conditioning regimen given prior to an allo-HSCT has two goals: suppression of the immune system of the host to prevent graft rejection and allow donor engraftment, and ablation or suppression of host hematopoiesis, including malignant cells. Based on differences in underlying diseases, various conditioning regimens have been developed which differ in immuno- and myeloablative potential. One of the strategies to improve HSCT outcome is reducing the toxicity caused by the conditioning regimen given prior to the HSCT. Treosulfan is an alkylating agent, which is increasingly applied in HSCT due to its beneficial toxicity profile in comparison to busulfan and total body irradiation.

Treosulfan (L-threitol 1,4-bismethanesulphonate, Ovastat®) is a prodrug and a water-soluble alkylating agent. It is non-enzymatically, pH-dependent converted into a monoepoxide and a diepoxide, which are responsible for DNA alkylation and DNA crosslinking.[1,2]

In vivo, treosulfan gives a rapid and sustained myeloablation, which is comparable to busulfan. Furthermore, the immunosuppressive profile of treosulfan was demonstrated to be stronger in comparison to busulfan and more durable than cyclophosphamide.[3] *In vitro* data suggest that treosulfan has a stronger cytotoxic effect against leukemic cells in pediatric patients than busulfan.[4]

In several studies treosulfan was applied in combination with other cytostatic and immunosuppressive drugs prior to allo-HSCT and demonstrated a safe and effective regimen.[5–7] Clinical outcome of allo-HSCT using busulfan-based condtioning is associated with the exposure of busulfan, and therapeutic drug monitoring is often applied to target exposure. Similarly, we assume that clinical outcome after allo-HSCT with a treosulfan based regimen might also be dependent on treosulfan exposure. To date, only two studies investigated the pharmacokinetic profile in pediatric patients. However, these studies focused on PK and did not report the correlation of exposure with clinical outcome.[8,9] In the first study, interpatient variability in exposure was large (CV = 70%). The second study was performed by our group and included 20 patients.[9] It comprised the development of a bioanalytical method to determine treosulfan in serum, a pharmacokinetic model and limited sampling strategy in order to determine treosulfan exposure in pediatric patients.

In the present prospective study, we describe the pharmacokinetics treosulfan in pediatric HSCT recipients and we studied whether the exposure could be related to clinical outcome.

MATERIALS AND METHODS

Patients and donor characteristicsand conditioning regimen

Pediatric patients (≤ 18 years) receiving treosulfan based conditioning prior to their first allo-HSCT in the Leiden University Medical Center in The Netherlands were included. The institutional Ethics Committee approved the treosulfan PK study protocol. Written informed consent was obtained from all parents of the study patients and patients themselves when they were older than 12 years according to the Helsinski Declaration.

Patients received an allo-HSCT for various malignant and non-malignant indications. Donor matching was based on HLA typing. All patients received treosulfan in a dose of 42 g/m^2 or 30 g/m2 if BSA < 0.5 m^2, divided over 3 days. Treosulfan was combined with fludarabine and in some patient also thiotepa was added. Thiotepa (8 mg/kg) was administered at day -8, treosulfan was administered at day -7 to day -5 and fludarabine 30 mg/m^2/day on day -7 to day -3. Serotherapy consisted of anti-thymocyteglobuline (ATG) 2.5 mg/kg/day from day -5 till day -2 or alemtuzumab 0.2 mg/kg/day from day -6 till day -2. All drugs were intravenously administered.

Graft-versus-Host disease prophylaxes consisted of cyclosporine A (CsA:1.5 mg/kg i.v. twice daily, and following engraftment equivalent oral dose),with dose adjustments based on through levels, which were 100–150 µg/L in malignant disease and 150–200 µg/L in non-malignant disease. CsA was combined with methotrexate in T cell replete transplants (10 mg/m^2 on day +1, +3 and +6) or prednisolone in cord blood transplants (1 mg/kg/day). In case of suspected toxicity or adverse events CsA was switched to mycophenolate mofetil or tacrolimus. In 9/21 patients G-CSF (5 µg/kg/day) was given to support engraftment on individual indication, mostly because of co-morbidity.

All patients were cared for in high-efficiency, particle-free air (HEPA)-filtered positive-pressure isolation rooms with total gut decontamination using non-absorbable antimicrobials and antifungal prophylaxis using azoles according to institutional guidelines.

Treosulfan assay

Patients received 10 or 14 g/m^2 treosulfan in a 3 hour infusion on three consecutive days. On day one, patient blood samples were collected at 1.5, 3.5, 4, 5, 7 and 9 or at 4-5 and 6-7 hours after the start of a three hour treosulfan infusion. For determination of the inter-occasion variability (IOV) in a selection of patients (weight >10 kg) samples were also taken on day 3. Blood samples

were collected in serum tubes without gel through the lumen of the catheter that was not used for intravenous treosulfan administration. Samples were centrifuged as quickly as possible after collection, but at least within 5 hours. A reversed phase high pressure liquid chromatography (RP-HPLC) method using Ultraviolet (UV) detection was applied to determine treosulfan in serum, as previously described by our group.[9] Briefly, treosulfan and the internal standard busulfan were made detectable with derivatization with sodium diethyldithiocarbamate (DDTC). Treosulfan can be determined over a range of 10 to 500 mg/L with a limit of quantification of 6.8 mg/L. Accuracies for each of the three QC samples were within the 90–110% limit. The coefficient of variation of the within-day imprecision and between-day imprecision was less than 5% for all three concentration levels.

Pharmacokinetics of treosulfan

The individual pharmacokinetic profile of each patient was determined by a population pharmacokinetic model, using non-linear mixed-effects modelling as implemented in the NONMEM software package (version 7 level 2; Icon Development Solutions, Ellicott City, Maryland, USA). A one-compartment model was used and clearance and volume of distribution were allometrically scaled using body weight, as previously published.[9] The scaling exponent for clearance was fixed at 0.75 and for volume of distribution at 1.0. For a number of patients, 2 serum samples were collected and a limited sampling strategy (LSS) was applied to estimate individual pharmacokinetic parameters. Interpatient variability (IPV) was calculated by the CV% of the treosulfan exposure between individuals and IOV by the calculating the mean difference between the AUC on day 1 and day 3 of each individual. IOV calculations were based on measurements in 7 patients.

Evaluation of clinical data

Clinical endpoints of this study were stem cell engraftment, chimerism, survival, relapse, treatment related mortality and toxicity (i.e. mucositis, skin toxicity, hepatic toxicity, neurologic toxicity and metabolic acidosis) and GVHD.

Stem cell engraftment was defined as platelet count > 50 x 10^9/L, without platelet support for 3 consecutive days together with neutrophils > 0.5 x 10^9/L on 2 consecutive measurements separated by at least 3 days (72 hours) without granulocyte support. Donor–recipient white blood cell chimerism was determined by VNTR polymorphism. Occurrence of aGvHD (within 100 days) and chronic GvHD is diagnosed and graded according to the scale defined by Przepiorka *et al*.[10] Toxicities were scored according to Bearman *et al*., in which toxicities are scored which

were likely due to the preparative regimen.[11] For GvHD and toxicities, events were graded from 0 (no adverse event) to 4 (severe).

Statistical analysis

Normally distributed parameters are shown as mean ± standard deviation and all log-normally distributed parameters as median (minimum and maximum). IPV was determined by calculating the mean coefficient of variation of AUCs for the total cohort.

Study endpoints included stem cell engraftment, rejection, toxicity, and survival. The relationships between AUC and transplantation outcomes were characterized using linear regression analysis. When appropriate, an independent Student's T-test was used. All p-values were 2-tailed and considered significant when $p < 0.05$. All statistical analyses were performed with ISB SPSS statistics, version 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Patients characteristics, conditioning regimen and GvHD prophylaxis

A total of 21 pediatric patients receiving their first HSCT were included in the study. The median age was 5.2 (0.13–16.8) years and 12 patients were male. HSCT indications included hemoglobinopathies (n = 12), acute myeloid leukemia (n = 3), primary immune deficiency (n = 4), and bone marrow failure (n = 2), see also Table 7.1. Patient received treosulfan based conditioning as first line choice according to institutional guidelines (n = 12) or as reduced toxicity conditioning because of individual patient comorbidity (n = 9).

Eight patients received a transplant from an HLA identical sibling, 13 patients received a transplant from a matched unrelated donor, i.e. 9/10 allelic matching (n = 6) and 10/10 allelic matching (n = 7). Seventeen of the 21 patients received a T cell replete bone marrow graft, one patient peripheral blood stem cells and 3 patients a cord blood transplant.

All patients except one received a treosulfan dose of 14 g/m^2 per day during 3 consecutive days, the youngest patient, a 1.5-month-old boy with a body surface area of 0.3 m^2, received a dose of 10 g/m^2 per day. In 16 patients treosulfan was combined with fludarabine and thiotepa and 5 patients received only treosulfan and fludarabine. Twenty patients received serotherapy; 19 received ATG and 1 alemtuzumab.

Table 7.1 Patient characteristics

	Total (n = 21)
Characteristic	
Age (years, median (range)	5.2 (0.13–16.8)
Weight (kg, mean (sd))	26.9 (18.3)
Sex (n: M/F)	12/11
Conditioning	
Treo-Flu-Thiotepa	16
Treo-Flu	5
Treosulfan dose	
14 g/m^2	20
10 g/m^2	1
Serotherapy	
Yes	20
ATG	19
Alemtuzumab	1
No	1
Diagnosis for HSCT	
Hemoglobinopathies	12
Hematologic malignancy	3
Primary Immune deficiency	4
Bone marrow failure	2
Treosulfan indication	
Reduced toxicity	9
According to protocol	12
Stem cell source	
BM	17
PBSC	1
CB	3
Donor matching	
HLA id sib	8
MUD (≥ 9/10)	13
GvHD prophylaxis	
CsA	4
CsA / MTX	16
CsA / Pred	1

Treo: treosulfan, Flu: fludarabine, Thio: thiotepa, ATG: Anti-thymocyteglobuline, BM: bone marrow, PBSC: peripheral blood stem cells, CB: cord blood, HLA id sib: Human leukocyte antigen identical sibling, MMFD: mismatched family donor, MUD: matched unrelated donor, GvHD: Graft-versus-Host Disease, CsA: Ciclosporin A, MTX: methotrexate, Pred: prednisolone.

Treosulfan pharmacokinetics

In total 28 AUCs were determined in 21 patients; in 7 patients also on day 3 treosulfan samples were taken for determination of the IOV. After development and validation of a limited sampling strategy, it was possible to calculate AUCs with LSS. In 17 patients, AUCs were calculated based on rich data curves. A total of 7 AUCs, in 4 patients, were analyzed based on the LSS. The median clearance and volume of distribution were 5.7 L/h (1.2–20.5 L/h) and 10.8 L (3.0–42.9 L), respectively. The mean AUC of treosulfan was 1534 mg*h/L and the interpatient variability (IPV) was 14%. The IOV was approximately 5%, based on the day 1 and day 3 results of 7 patients. One patient received a lower dose because of his young age and small body surface area i.e. 10 g/m^2. The AUC of this patient was 1578 mg*h/L, which is similar to the mean AUC seen in the population receiving 14 g/m^2. There was a linear relationship between age and treosulfan exposure (p = 0.026, R2 = 23%), see Figure 7.1. Exposure was not different between the various disease groups, i.e. hemoglobinopathies, malignancies, immune deficiencies, and bone marrow failure (data not shown).

Clinical outcome and treosulfan PK

Two patients of the total cohort died on day +11 and +18 due to toxicity and infections prior to engraftment, respectively. Resulting in an overall survival of 90%, primary engraftment

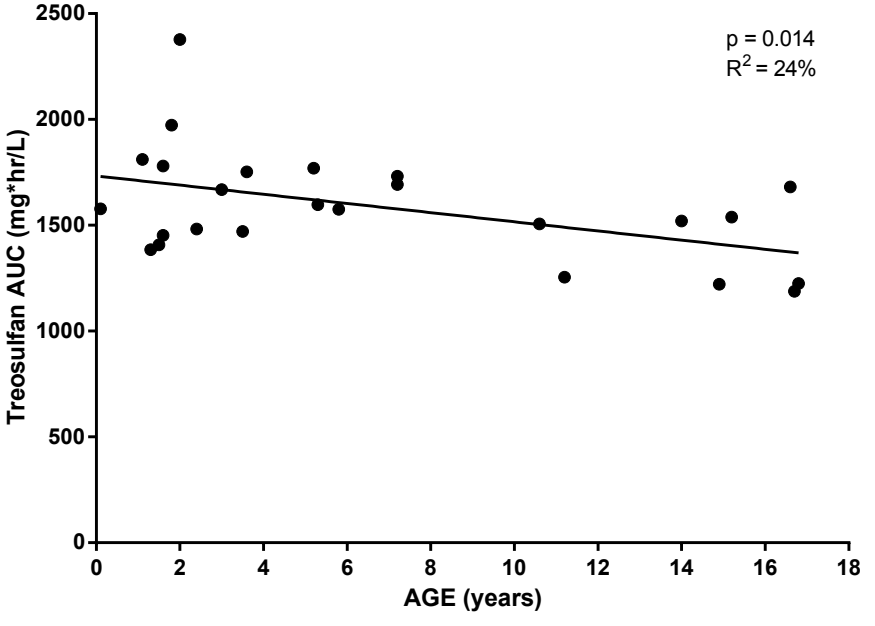

Figure 7.1 Treosulfan exposure versus age of the patient.

Table 7.2 Main toxicities and adverse events in pediatric patients receiving treosulfan based conditioning

Adverse event		N	Mean AUC ± sd		N	Mean AUC ± sd	p-value
Mucositis	Grade 0–1	13	1517 ± 187	Grade 2–3	8	1560 ± 262	0.70
SOS	No	20	1528 ± 217	Yes	1	1668	NA
Hepatic toxicity	No–mild	11	1465 ± 197	Moderate/severe	10	1610 ± 214	0.21
Skin toxicity	No	15	1535 ± 234	Grade 1–2	6	1533 ± 169	0.98
GI-bleeding	No	18	1558 ± 213	Grade 3–4	3	1392 ± 182	0.25
Neurologic toxicity	No	20	1534 ± 219	Yes	1[a]	1538	NA
Viral reactivation	No	15	1539 ± 210	Yes	6	1523 ± 243	0.89
aGvHD	Grade 0–1	18	1517 ± 190	Grade 2–3	3	1636 ± 361	0.63
cGvHD	No	20	1527 ± 216	Limited	1	1681	NA

Occurrence or grading of event, number of patients (N) and mean AUC ± standard deviation. [a] Hallucinations and disorientation in end stage disease. SOS: sinusoidal obstruction syndrome, aGvHD and cGvHD: acute and chronic graft-versus-host disease, NA: not applicable.

was seen in 95% of the 19 evaluable patients. Of the 18 patients that engrafted the median neutrophil and platelet engraftment occurred on day 23 and 35, respectively. After 100 days 11 patients had full donor chimerism and 7 patients had mixed chimerism which persisted until latest follow-up. The median follow-up time was 1 year (168 days – 1.9 years).

Of the 19 survivors, 74% are disease free (n = 14),four patients had a recurrence of their initial disease, and one patient had a primary rejection followed by rapid autologous reconstitution.

The toxicities observed were mucositis, skin- and hepatic toxicity, as shown in Table 7.2. In 38% of the patients mucositis grade 2 or 3 was observed. Twenty eight percent of the patients had any grade skin toxicity and 38% had a moderate transient hepatic toxicity within 100 days after treosulfan administration. Two patients had severe hepatic toxicity of which one patient developed sinusoidal obstruction syndrome (SOS). Both patients received treosulfan as a reduced toxicity conditioning (RTC), one patient because of pre-existent liver damage due to *Cryptosporidium parvum* infection and the second patient due to heavily pre-treatment for secondary AML after initial osteosarcoma treatment.

Gastro-intestinal bleeding occurred in 3 patients (14%) and in one patient neurological toxicity was observed. Only three patients (14%) were diagnosed with aGVHD (grade 2 (n = 1) and grade 3 (n = 2)) and one patient was diagnosed with a limited cGHVD. There is a trend towards higher toxicities in patients receiving treosulfan as a RTC in comparison to patients receiving treosulfan according to protocol, see Table 7.3. Furthermore, there was no significant difference

Table 7.3 Incidence of toxicities based on treosulfan indication

Adverse event	According to protocol (n = 12)	Reduced toxicity conditioning (n = 9)	p-value
Mucositis (grade 2–3)	25%	56%	0.33
SOS	0%	22%	0.88
Hepatic toxicity (moderate/severe)	33%	67%	0.28
Skin toxicity (grade 1–2)	17%	44%	0.36
GI-bleeding (grade 3–4)	8%	22%	0.79
Neurologic toxicity	0%	22%	0.88
Viral reactivation	25%	33%	1.0
aGvHD (grade 2–3)	8%	22%	0.79
cGvHD (limited)	8%	0%	1.0

SOS: sinusoidal obstruction syndrome, aGvHD and cGvHD: acute and chronic graft-versus-host disease.

in treosulfan exposure in these two groups. In this limited cohort, no relation was found between treosulfan exposure and HSCT outcome parameters, e.g. engraftment, chimerism, toxicity and survival.

DISCUSSION

This is the first report in which treosulfan exposure was analyzed in a comprehensive cohort of pediatric HSCT recipients and related to toxicity and clinical outcome parameters.

We found a relative small interpatient variability in PK of 14% only, which was much smaller than observed in an earlier study.[8] In this study of Glowka *et al*. three different doses were applied (10, 12, and 14 g/m^2/day) and they found a linear relationship between dose and exposure.[11] Interpatient variability based on treosulfan exposure measurements in 5 patients receiving 12 g/m^2 was 70%. The exposure in the patient receiving the highest dose of 14 g/m^2/day was relative high (e.g. 1960 mg/L*h).

In our patient population all, except one patient, received 14 g/m^2/day and the mean AUC is lower (i.e. 1534 mg/L*h) in comparison to Glowka *et al*. The inter-occasion variability was approximately 5%, which is much smaller than inter-individual variability. However, IOV is based on measurements on day 1 and 3 in only 7 patients. Notably, we found that treosulfan exposure decreases with age. This was not reported previously, but shows similarities with

the reported data on busulfan exposure and its relation with age.[12] In general, age and body weight are strongly correlated, and in the PK-model treosulfan clearance is allometrically scaled using body weight with a fixed scaling exponent of 0.75 to correct for the influence of growth.[9] The age effect observed in this study could be due to dosing based on body surface area instead of allometrically scaled body weight, or that the appropriate scaling component in the patient population diverge from 0.75. PK-data in a larger cohort is required to confirm this age effect on treosulfan exposure. One very young child received a lower dose of 10 g/m^2/day, but demonstrated an exposure similar to the older children having received 14 g/m^2/day. From these results, it may be concluded that younger patients (< 1 year) require a lower treosulfan dose to obtain an adequate exposure. Furthermore, in older patients dose-escalation could be possible to further increase efficacy of treosulfan-based conditioning. Studies in a larger cohort of children including a broad spectrum of ages, and particularly infants, will provide more insight in the pharmacokinetics of treosulfan.

Particularly in pediatric patients, the relation between treosulfan exposure and clinical outcome parameters has so far been unresolved. In this first report of an ongoing prospective pediatric study, the OS rate was 90% and primary engraftment was 95% in the 19 evaluable patients. OS and primary engraftment are similar or even better in comparison to other studies applying treosulfan-based conditioning in pediatric populations with malignant and non-malignant diseases.[13–15]

The toxicity profile in this study group was relatively mild, with mild to moderate mucositis and hepatic toxicity being most often reported. In these children, the toxicity parameters could not be related to treosulfan exposure. The high incidence of hepatic toxicity could be due to the preexistent hepatic risk profile in affected patients, which precluded the use of busulfan-based conditioning. This reflects the fact that treosulfan-based conditioning was used as reduced toxicity conditioning (RTC) in almost half of the patients because of significant preexisting co-morbidities. The incidence of acute and chronic aGvHD is low in comparison to other studies applying treosulfan based conditioning.[13,14,16] Furthermore, no relationship with any clinical outcome and treosulfan exposure was observed. However, the cohort was limited in size as to observe subtle effects of exposure on clinical outcome.

In conclusion, the optimal treosulfan exposure in pediatric allo-HSCT recipients, in relation to age and primary diseases remains to be defined. In this study we demonstrated that treosulfan conditioning is effective and well tolerated. However, primary engraftment and (long term) chimerismcould be improved, and probably a higher exposure of treosulfan may be beneficial to achieve this goal. This is not only of relevance to prevent disease recurrence in patients

with hematologic malignancies, but also to achieve adequate and long term graft function in various non-malignant diseases. Therefore, prospective treosulfan PK studies in larger cohorts of pediatric patients are warranted with the aim to define the most effective treosulfan exposure and dose while keeping its beneficial toxicity profile.

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Chapter 8

Pharmacogenetics of glucocorticoid responsiveness in treatment of acute Graft-versus-Host Disease in pediatric patients

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ABSTRACT

Acute Graft-versus-Host Disease (aGvHD), caused by the tissue destructive effect of alloreactive donor T lymphocytes is the major cause of non-relapse morbidity and mortality in pediatric allogeneic hematopoietic stem cell transplantation (allo-HSCT). Despite routine administration of pharmacological prophylaxis, 15–25% of pediatric HSCT recipients develop aGvHD ≥ grade II. Systemic treatment with high-dose glucocorticoids (GCs) is currently the gold standard as first line treatment. However, approximately half of the patients turn out to be non-responsive to high-dose GCs. To explore whether individual GC responsiveness can be predicted based on the GC pharmacogenetic signature, we studied the effect of genetic polymorphisms on gene expression in peripheral blood mononuclear cells, from healthy volunteers, cultured with or without GCs. Those polymorphisms that affect the transcriptional response to GC in this system were further studied in 56 pediatric patients with aGvHD treated with GC. We found that seven *cis*-regulatory interaction eQTLs represented regulatory polymorphisms only active in the presence of GCs, in healthy volunteers. In the pediatric patient, the HSCT donor genotypes for polymorphisms affecting the transcriptional response of the *TMEM71* and *DPYSL3* genes were found to be associated with the GC response status of the corresponding patients. The predictive value of two donor genotypes was 63%. These polymorphisms are potential predictors of clinical response to GCs in aGvHD patients and could be used to identify patients that are likely to be GC-insensitive, and therefore qualify for alternative therapies.

INTRODUCTION

Despite improvements in HLA typing, donor selection, pharmacological graft-versus-host disease (GvHD) prophylaxis and supportive care, acute GvHD (aGvHD) remains the major complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT), with significant morbidity and mortality.[1,2] aGvHD is an immunological disorder in which recipient and donor antigen-presenting cells, cytokines, and alloreactive donor T lymphocytes play a pivotal role. Triggering and activation of mature donor T cells will lead to cytokine release and tissue damage.[2,3] aGvHD affects many organ systems, including the gastrointestinal tract, skin, liver, and lungs. Despite immunosuppressive prophylaxis, severe aGvHD (i.e. grade 2–4) occurs in up to 25% of pediatric HSCT recipients,[4] depending on conditioning regimen, donor type, HLA matching, age, and other risk factors. Rapid and persistent control of aGvHD is crucial to limit morbidity and mortality associated with aGvHD and its treatment. Systemic use of glucocorticoids (GCs) (e.g. prednisolone and methylprednisolone), with their anti-lymphocyte and anti-inflammatory activity, is the gold standard for first-line treatment of moderate to severe aGvHD.[5–7] However, only half of the patients show a satisfactory response to GC therapy and an even smaller number shows a complete long-term response.[8,9] When a patient does not respond to high dose GC, a second line therapy will be added, including conventional immunosuppressive drugs or mesenchymal stromal cells (MSC).[10] Early non-responsiveness to GCs is associated with high morbidity and mortality as a consequence of both ongoing aGvHD and infectious complications secondary to prolonged treatment with GCs and other immunosuppressive agents.[11] Therefore, early detection or prediction of GC non-responsiveness would allow for timely initiation of alternative therapeutic strategies. However, reliable clinical parameters and biomarkers to early identify individuals at risk are currently lacking.

GCs enter the cell by passive diffusion and bind intracellular to the nuclear glucocorticoid receptor (GR), which is encoded by the *NR3C1* gene. Binding of GCs to the GR leads to formation and transport of the GC-GR complex to the nucleus, where it functions as a transcription factor.[12] Several studies investigated the role of germline genetic variations in GC responsiveness. Polymorphisms in the GR gene itself and genes involved in the mechanisms upstream or downstream of the binding of GC to its receptor were identified as potential variants for GC resistance.[13,14] However, most of these studies identified rare variants which do not explain common non-responsiveness in the general population.

Recently, Maranville *et al*. studied the molecular basis for variation in *in vitro* GC responsiveness among healthy individuals.[15,16] This study identified a quantitative trait locus (QTL) for

lymphocyte GC sensitivity. This expression QTL (rs11129354) lies 68kb upstream of the *RBMS3* gene and affects transcriptional response through GC-dependent *cis*-regulatory effects on *RBMS3*. *RBMS3* in turn mediates effects in *trans* on transcriptional response at other genes, and ultimately influences lymphocyte proliferation as a negative regulator.

In this study we used expression and genotype data from this previous study (Maranville *et al*.[16]) to identify additional regulatory polymorphisms and investigated the predictive value of these regulatory polymorphisms for GC non-responsiveness in a retrospective cohort of pediatric patients diagnosed with aGvHD who received systemic GC therapy.

PATIENTS AND METHODS

GC interaction eQTLs

To identify interaction expression QTLs (eQTLs), we tested for associations between genetic polymorphisms and expression of each gene in peripheral blood mononuclear cells (PBMCs) from healthy donors that were cultured *in vitro* in the presence and absence of GCs, as previously described.[16] Briefly, peripheral blood was collected from 88 healthy volunteers that self-identified as African-Americans (AA) and lymphocyte GC sensitivity (LGS) was measured in cells treated with phytohemoagglutinin (PHA) and either vehicle (EtOH) or dexamethasone (a synthetic GC) in different concentrations, as described previously. Cellular proliferation was measured after 48 hours of treatment using H^3-thymidine incorporation. DNA from each individual was genotyped with Illumina Omni1-Quad BeadChip arrays (n = 58) or Illumina Omini2.5-QuadBead Chip arrays (n = 30) and SNPs genotypes were imputed from the 1000 genome project using the program IMPUTE2.[17]

For gene expression measurements, RNA was extracted from dexamethasone- and vehicle-treated PBMCs. Expression was profiled using Illumina HumanHT-12 v4 Expression BeadChips. Low-level microarray analysis was performed as previously described.

To identify genes that were differentially expressed between treatment conditions, expression levels for each gene were corrected for measured covariates and principal components to account for unmeasured covariates. We then used the Bayesian statistical framework BRIdGE[15] to identify *cis*-regulatory polymorphisms that interact with treatment, testing all SNPs within 100kb of the transcriptional start and end sites of each autosomal gene. This method compares distinct patterns of interaction between treatment and genotype to a null model of no effect as well as to each other.

aGvHD patient characteristics

Pediatric patients receiving allo-HSCT between 2001 and 2013 in the Leiden University Medical Center and diagnosed with aGvHD grade 2 or higher were included in this retrospective study. Patients received an allo-HSCT for various malignant and non-malignant indications. Graft-versus-Host disease prophylaxes consisted of cyclosporine A (CsA: 1.5 mg/kg i.v. twice daily, and following engraftment equivalent oral dose), with dose adjustments based on through levels, which were 100–150 µg/L in malignant disease and 150–200 µg/L in non-malignant disease. CsA was combined with methotrexate in T-cell-replete transplants (10 mg/m^2 on day +1, +3 and +6) or prednisolone in cord blood transplants (1 mg/kg/day). When CsA caused toxicity or adverse events, patients were switched to mycophenolate mofetil or tacrolimus. The grade of aGvHD was defined according to the scale described by Przepiorka *et al*.[18] To treat aGvHD grade 2 or higher all patients received prednisolone 2 mg/kg/day divided into two doses, orally or intravenously. Response was defined as a decrease of at least one grade within the first 5–7 days. When patients did not respond to prednisolone within 5–7 days they were classified as non-responders, and a second line therapy for aGvHD was started. In most of the patients (89%) this consisted of administration of mesenchymal stromal cells.[10] The applied conditioning regimen was based on the guidelines of EBMT – Working Party Inborn Errors and Pediatric Diseases.

Genotyping of retrospective cohort

DNA extracted from donor and recipient PBMC was used for genotyping. Blood samples of the patients were taken before their allo-HSCT and in the case of malignant disease, when the patient was in remission.

Patients and donors were genotyped for 8 eQTLs: rs11129354 in *RBMS3* as reported by Maranville *et al*.[16] and 7 *cis*-eQTLs first reported in this paper: rs2739024 for *TMEM71*, rs2288807 for *DPYSL3*, rs13354714 for *GZMA*, rs12022333 for *TTF2*, rs9963737 for *LPIN2*, rs3766236 for *MOB3C*, and rs116735324 for *FAM117B*, see also Table 8.1.

All samples were anonymized according to the instructions stated in the Codes for Proper Use and Proper Conduct (www.federa.org). eQTLs for *RBMS3*, *TMEM71*, *DPYSL3*, *GZMA*, *TTF2*, *LPIN2*, and *MOB3C* were determined using commercially available realtime PCR genotyping assays (Lifetechnologies, Bleiswijk, the Netherlands) and analyzed on Lightcycler 480 (Roche, Almere the Netherlands). Because the available amount of DNA was limited, a pre-amplification step was performed prior to genotyping analysis. Briefly, genotyping assays were pooled and 200 times diluted. Amplification was performed using 3 ml genotyping mastermix (Lifetechnologies,

Bleiswijk, the Netherlands), 2 ml of DNA, and 1 ml of assay pool. PCR conditions were as follows, 10 minutes at 95°C followed by 18 cycles of 15 seconds at 95°C, and 4 minutes at 60°C. After this, 114 ml water was added and 4 ml was used for further analyses using standard conditions.

Since no real-time PCR genotyping assay could be designed for *FAM117B*, this SNP was genotyped by pyrosequencing using standard conditions (Qiagen, Venlo, The Netherlands). Used PCR primers were GTGTGAGCAGAACCTAGTG (forward, biotinylated)and GTAGCTGGGATTACGGGC (reverse). Pyrosequence primer was ACCTCGGCCTCCCAGAAT with sequence to analyse T/GCTAGGATTAC.

Table 8.1 Patient characteristics

Characteristic	Total	Responders	Non-responders	p-value
Number of patients	56	28 (50%)	28 (50%)	
Age (years)	8.8 (5.4)	8.3 (5.1)	8.8 (5.8)	0.70
Sex (% male)	66%	82%	54%	0.045
Diagnosis for HSCT (n)				
Hematological malignancy	41	21	20	0.77
Bone marrow failure	4	1	3	
Hemoglobinopathy	2	1	1	
PID/inborn errors	9	5	4	
Graft type				
Bone marrow	34	22	12	0.002
Peripheral blood stem cells	9	4	5	
Cord blood	6	1	5	
DLI/Boost	7	1	6	
Donor matching				
Identical related donor (10/10)	17	9	8	
Matched unrelated donor				0.12
10/10 or 6/6	22	14	8	
9/10 or 5/6	15	5	10	
8/10	2	0	2	
GvHD prophylaxes				
CsA	16	8	8	0.20
CsA+MTX	30	18	12	
CsA+pred	6	1	5	
No*	4	1	3	
Maximum aGvHD grade				
2	21	20	1	<0.001
3	24	8	16	
4	11	0	11	

Table 8.1 continues on next page

Table 8.1 *Continued*

Characteristic	Total	Responders	Non-responders	p-value
aGvHD organ involvement				
Skin	45	26	19	0.04
Liver	15	4	11	0.07
Gut	43	15	28	<0.001
Conditioning regimen				
TBI-based	18	12	6	0.06
Busulfan-based	27	13	14	
Treosulfan-based	1	0	1	
Other	2	1	1	
No conditioning**	8	2	6	
Serotherapy	46%	61%	57%	1.0
Overall survival	52%	68%	36%	0.032

Malignant diseases: acute lymphoblastic leukemia (n = 18), acute myeloid leukemia (n = 9), chronic myeloid leukemia (n = 3), juvenile myelomonocytic leukemia (n = 3) and myelodysplastic syndrome (n = 10).
Non-malignant diseases: 2 patients with congenital anemia, 1 patient with Blackfan Diamond anemia and 1 with Fanconi anemia, 1 patient with congenital amegakaryocytic thrombocytopenia, 2 patients with β-thalassemia, 9 patients with a primary immune deficiency.
CsA: Ciclosporine A, MTX: methotrexate, pred: prednisolone, TBI: total-body-irradiation.
* Three patients receiving donor lymphocyte infusion or a boost and one patient with severe combined immune deficiency and an identical related donor did not receive GvHD prophylaxis.
** Patient that received donor lymphocyte infusion or a boost did not receive conditioning and one patient with severe combined immune deficiency and an identical related donor did not receive conditioning, according to protocol.

Initial call rate of *RBMS3* by real-time PCR genotyping assay was insufficient and therefore *RBMS3* was genotyped by pyrosequencing. Pyrosequence primer was TCAGCAAAGTCAGAAAAT with sequence to analyze TAGT/CAGTGTTACCA. A pre-amplification step was performed prior to pyrosequencing using 1 pmol of primers and 18 cycles. Pre-amplified DNA was 20-fold diluted and 4 µL were used for second PCR. Of note, PCR was performed at 63°C because of aspecific PCR products at lower temperatures.

As quality control at least 5% of the samples were genotyped in duplicate and negative controls (water) were used.

GC responsiveness and eQTLs

The genotypes were tested for association with response status by logistic regression. Donor genotypes and patient genotypes were tested separately. In a full model the most strongly associated eQTLs were selected ($p < 0.05$). Akaike information criterion (AIC) and Bayesian

information criterion (BIC) were applied to select the model that best fit the data. Model performance was assessed with leave-one-out cross validation and by calculating the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. Results were analyzed using statistical software package R (v2.10.1).

RESULTS

GC interaction eQTLs and genotyping of patient material

To identify candidate genetic variants for predicting GC non-responsiveness in patients with aGvHD, we used a Bayesian regression method (implemented in BRIdGE) to identify GC interaction eQTLs in PBMCs from healthy donors. We found that all *cis*-regulatory interaction eQTLs represented regulatory polymorphisms only active in the presence of GCs; 7 eQTLs were only active in the presence of GCs (false discovery rate (FDR) = 0.14) and no eQTLs were found that were only active in presence of the control (posterior probability > 0.7). None of these interaction eQTLs were significantly associated with in vitro lymphocyte GC sensitivity (e.g. I_{max}, corrected p > 0.1). The *cis*-eQTLs affect the expression of the following genes: *LPIN2*, *DPYSL3*, *TMEM71*, *GZMA*, *MOB3C*, *TTF2*, and *FAM117B*, see Figure 8.1.

When genotyping the pediatric patients, one SNP (*FAM117B*) had only one heterozygous individual, all other individuals were homozygous wild type; this SNP was excluded from further analysis.

All other SNPs had a call rate between 80–100%, and genotype frequencies were in Hardy-Weinberg equilibrium (p > 0.05). A summary of minor allele frequencies and call rates of the seven remaining SNPs is given in Table 8.2.

aGvHD patient characteristics and GC responsiveness

Fifty-six pediatric patients diagnosed with aGvHD grade 2 or higher were included in the study. The mean age of the study cohort was 8.8 (± 5.4) years and 68% of the patients were male. Seventy-three percent of the patients had a malignant disease for which they received the allo-HSCT. The remaining 16 patients were diagnosed with non-malignant diseases. A summary of the transplantation characteristics is given in Table 8.1. Systemic prednisolone treatment was started in patients diagnosed with aGvHD grade 2. Furthermore, the maximum aGvHD grade of each patient was registered; 38% of the patients had grade 2 aGvHD, 43% had grade 3, and 20% of patients had grade 4 aGvHD. Twenty-eight patients (50%) patients did not respond

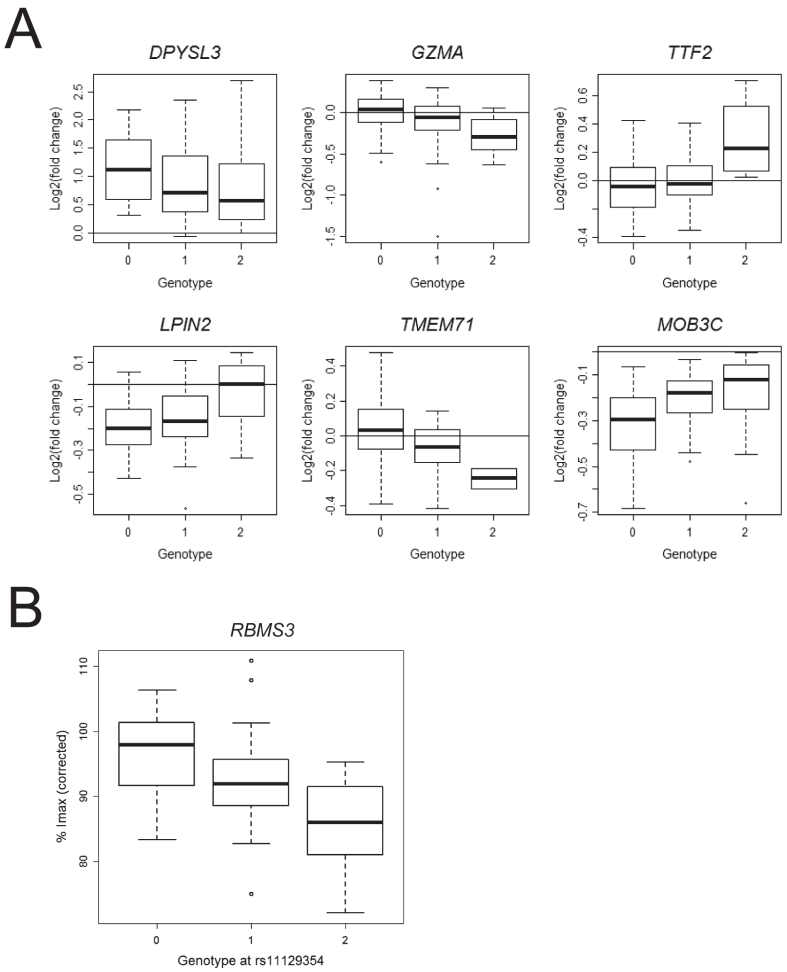

Figure 8.1 Effect of eQTLs on expression in PBMCs of healthy volunteers. a) Boxplot of effect of eQTL genotype on log 2 fold change in expression of the corresponding gene in absence and presence of dexamethasone, for *DPYSL3*, *GZMA*, *TTF2*, *LPIN2*, *TMEM71*, and *MOB3C*. b) Boxplot of effect of rs11129354 genotype on $\%I_{max}$, as a measure of lymphocyte GC sensitivity, (previously published[16]).

adequately to prednisolone within 7 days after initiation of the therapy and were considered GC non-responders. In accordance with response-status, maximum grade of aGvHD was higher in the non-responders ($p < 0.001$). In line with this observation the gastrointestinal tract was always affected in the non-responders (versus 54% in the responders, $p < 0.001$), whereas in the responders aGvHD grade 2 often remained limited to the skin. The overall survival of the study cohort was 52% and was significantly lower in the non-responders (36% versus 68%, $p = 0.036$).

Table 8.2 Minor allele frequencies and call rates of 7 eQTLs

Gene	eQTLS	Minor allele frequencies		Call rate aGvHD patients	Minor allele
		Healthy volunteers	aGvHD patients and donors		
RBMS3	rs11129354	48%	37%	80%	G
LPIN2	rs9963737	33%	43%	91%	C
TMEM71	rs2739024	18%	38%	93%	A
DPYSL3	rs2288807	29%	43%	96%	C
GZMA	rs13354714	23%	15%	100%	C
MOB3C	rs3766236	39%	31%	99%	G
TTF2	rs12022333	20%	9%	93%	C

Gender and stem cell source were the only variables that were significantly different between responders and non-responders. Patients developing aGvHD after a donor lymphocyte infusion or a stem cell boost were more likely to be non-responder.

GC responsiveness and patient and donor genotypes

The eQTL genotype of each patient and its donor was determined. Donor and patient immune cells are both involved in the process of aGvHD. However, donor T cells are considered the major target of GC therapy. At first, we compared the predictive value of all 7 eQTLs in patients and in donors separately. When excluding individuals with missing data in any of the donor genotypes, 34 individuals remained for further analysis. Forty-two patients were analyzed for association with patient genotype, due to missing data in 14 patients of any of the patients' genotype.

Based on likelihood ratio tests, the donors' genotypes were significantly associated with responsiveness ($p = 0.011$), this was not the case for patients' genotypes ($p = 0.56$).

Predictive model

We conducted further analyses to determine which eQTLs genotype in the donors contributed most to the patient responsiveness to GC therapy (see Table 8.3). Donor eQTLs for *TMEM71* ($p = 0.008$) and *DPYSL3* ($p = 0.022$) were significantly associated with responsiveness. *TMEM71* genotype was borderline significantly associated when correcting for multiple testing for 7 eQTLs ($p = 0.055$). Furthermore, based on both AIC and BIC, *TMEM71* and *DPYSL3* donor genotype were found to be most strongly associated.

Table 8.3 Logistic regression of donor genotype and patient responsiveness

Gene	eQTL	Direction of expression data	OR (CI)	p-value (one-tailed)	
RBMS3	rs11129354	-	0.61 (0.11–3.30)	0.283	
LPIN2	rs9963737	-	1.60 (0.30–8.46)	1.158	
TMEM71	rs2739024	+	27.92 (1.88–416)	0.008	**
DPYSL3	rs2288807	-	0.12 (0.01–0.95)	0.022	*
GZMA	rs13354714	+	2.66 (0.29–24.39)	0.193	
MOB3C	rs3766236	-	1.65 (0.31–8.80)	0.280	
TTF2	rs12022333	+	1.59 (0.08–29.9)	0.379	

Based on n = 34, due to missing data in individuals. The direction of *DPYSL3* expression data is based on the effect of the C-allele. OR (CI): odds ratio (confidence interval).

When *TMEM71* and *DPYSL3* donor genotype were tested separately, *TMEM71* genotype remained significantly associated with response status (p = 0.023, n = 52) but *DPYSL3* genotype was not (p = 0.08, n = 56).

In order to give an estimate of the predictive value of both eQTLs in donors, *leave-one-out* cross validation was performed. The predictive value of *TMEM71* genotype alone was 65%; when the two donor genotypes were combined the predictive value was 63%. The AUC of the ROC curve for the combination of both donor genotypes was higher (70% (52% – 85%)) than for *TMEM71* genotype alone (66% (53 – 79%)), however confidence intervals were overlapping.

TMEM71 and *DPYSL3* expression

Patients in whom their donor is a carrier of the minor allele of the *TMEM71* eQTL are more likely to be responders of GC therapy, see also Figure 8.2. Furthermore, when the patients themselves were carriers of the minor allele of *TMEM71* eQTL, it was more likely that the patients are responders, however, this relationship was not significant. In the healthy volunteers, the minor allele was associated with down regulation of *TMEM71* in GC treated lymphocytes, see Figure 8.1. These findings suggest that down regulation of *TMEM71*, results in increased responsiveness towards GCs.

Donors that were *DPYSL3* major allele homozygous were more likely to be GC responders. In the healthy volunteers the major allele was associated with a higher expression of the *DPYSL3* gene in comparison to individuals carrying the minor allele. Therefore, increased expression of *DPYSL3* could lead to improved responsiveness towards GCs.

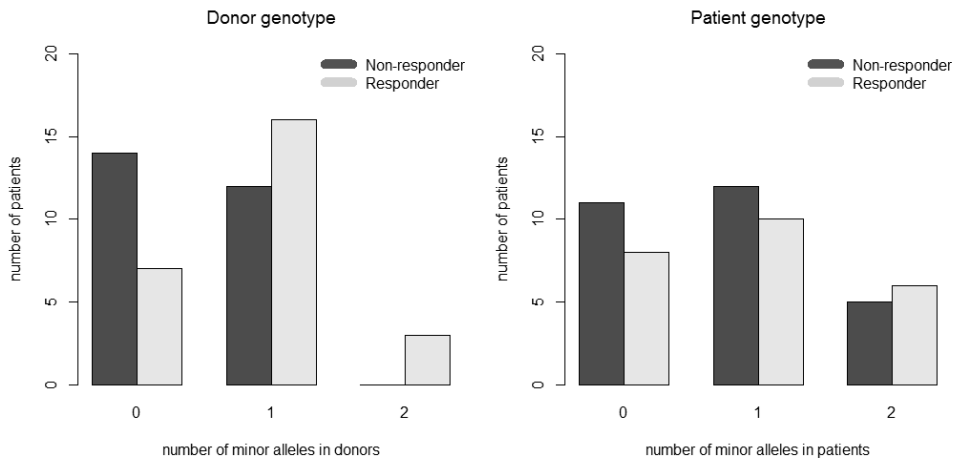

Figure 8.2 Allele frequencies in donor and patient genotypes for *TMEM71* in responders and non-responders.

As a further validation of the effect of eQTLs, we compared the expected direction of effect of the eQTLs based on transcriptional data (e.g. is the minor allele associated with greater or lesser response). In donors, we found that the odds ratios of 5 eQTLs were in line with the expression data in healthy volunteers. In contrast, the effects of only 3 of 7 eQTLs in patients were in line with the expression data, see Table 8.3. This is a pattern expected based on the physiological mechanism of aGvHD.

DISCUSSION

This is the first study demonstrating an association between genetic polymorphisms and glucocorticoid responsiveness in aGvHD patients. Specifically, the genotype at two regulatory polymorphisms in allo-HSCT donors is associated with GC responsiveness. Based on TMEM71 donor genotypes it was possible to predict response status with 65% accuracy.

A recent study identified the biomarker suppression of tumorigenicity 2 (ST2) for treatment resistant GvHD and outcome after HSCT. ST2 level measurement can improve risk stratification of patients with treatment-resistant GvHD.[19] In this study, we searched for genetic markers related to the GC-treatment effect. Several studies tried to identify genetic markers to explain GC non-responsiveness in a variety of diseases, mainly applying a candidate gene approach.[13,14,20] However, the pharmacological mechanism of GCs is complex, involving a multi-level signaling

pathway. Earlier attempts to move beyond the candidate gene approach identified *cis*-regulatory polymorphisms associated with GC-response in asthma patients.[21,22]

The regulatory polymorphisms we tested in the pediatric patients were identified in healthy volunteers in which information from genome wide polymorphisms and *in vitro* transcriptional response toward GC treatment in lymphocytes was combined. This method of applying multiple levels of genotypic and phenotypic information allowed us to identify polymorphisms that interact with GC treatment to influence response and are therefore excellent candidates.

Our study in healthy donors was performed in a relevant cell type for this clinical phenotype since lymphocyte GC sensitivity has been shown to be correlated with patient response across a wide range of diseases, including asthma,[23,24] rheumatoid arthritis,[25] and renal transplant rejection.[26] In aGvHD alloreactive donor T lymphocytes interact with recipient immune cells and cause cytokine release and tissue damage. The healthy volunteers were from AA ancestry; the majority of the aGvHD patients and their donors are Caucasians. Response to GC treatment differs among ethnic groups, with African-Americans having a weaker response to GC treatment in comparison to Europeans.[27] It is most likely that differences in response are due to allele frequency differences rather than a differential effect of polymorphisms in different ethnicities. This is also observed in our results; *FAM117B* was excluded from further analysis since the MAF was 0.1%; in the AA volunteers the MAF was 21%, which is in line with findings from the 1000 genome project. When there is a big difference in allele frequencies in the two populations, potential interesting genetic markers in Caucasians, could have been not identified in the healthy volunteers with AA ethnicity, due to a low allele frequency in the AA population.

The *TMEM71* and *DPYSL3* eQTLs were not associated with PHA induced T cell proliferation in the healthy volunteers. Furthermore, *RBMS3* was strongly associated with PHA induced T cell proliferation and appeared to be a promising candidate eQTL. However, we did not find an association of *RBMS3* with GC responsiveness in aGvHD patients. This is could be due to different immune response mechanisms in aGvHD and in PHA mediated T cell proliferation.

In contrast to GvHD grade at start of the treatment, maximum aGvHD grade was higher in the non-responders. In all non-responders GvHD progressed to higher grades with liver or gastro-intestinal involvement of their disease, due to inadequate treatment with GCs. Otherwise, the patient and donor characteristics of responders and non-responders were very comparable, except for gender and the source of donor cells. Patients receiving donor lymphocyte infusion or a stem cell boost were more likely to be non-responder. The clinical significance of prevention or early detection of GC non-responsiveness is demonstrated in this study by the significantly lower overall survival of GC non-responsive patients.

To more accurately estimate the predictive value of *TMEM71* it is essential to replicate current findings. We performed simulations to calculate sample size for a replication cohort, using the same minor allele frequencies of *TMEM71* and the same case-control ratio. A replication cohort should contain at least 110 patients in order to detect an odds ratio of 2.5, based on a statistical power of 0.8 at a significance threshold of $p < 0.05$. This replication of current findings is scheduled in the context of a multi-center study.

We hypothesized that by analyzing genetic susceptibility factors, clinical responsiveness to GC can be predicted in individual patients before treatment is started. *TMEM71* and *DPYSL3* donor genotype are potential genetic markers in the prediction of GC responsiveness in patient with aGvHD. Furthermore, this knowledge should be implemented in prospective studies in which patients with a GC non-responsive genetic signature could be candidates for intensified GvHD prophylaxis and early switch to or direct initiation of alternative GvHD treatment modalities that could replace or complement GCs and, thereby, circumvent non-efficacious GC treatment.

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Chapter 9

Pharmacogenetics in transplant patients; Mind the mix

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INTRODUCTION

Pharmacogenetics is aimed at individualizing drug treatment and increasing drug efficacy while reducing toxicity. Currently, pharmacogenetic information is accumulating rapidly and is beginning to show consistent reproducible results for a growing number of genetic markers for drug response. Several consortia have published guidelines to aid clinicians with the interpretation of pharmacogenetic test results[1,2] and an increasing number of medical centers have acquired clinical genotyping facilities and centers are implementing prospective genotyping.[3] Among these, there are many highly specialized care centers with complex patient populations. These patients may present unexpected challenges, as demonstrated by this case.

CASE DESCRIPTION

A 20-year-old woman was admitted for a living–related kidney transplant. A standard quadruple immunosuppressive regimen was prescribed (basiliximab induction, tacrolimus, mycophenolate, and prednisolone). Tacrolimus is metabolized by cytochrome P450 (CYP) 3A4 and CYP3A5. To attain therapeutic concentrations, carriers of at least one copy of the *CYP3A5*1* allele require a significantly increased tacrolimus dose.[4] Therefore, all kidney transplant patients at our institution are preemptively genotyped for the *CYP3A5*3* (rs776746) and *CYP3A5*6* (rs10264272) polymorphisms. Clinical genotyping is performed by two independent techniques: a commercially available TaqMan assay and a home-brew pyrosequencing method. For the case patient, results were conflicting. Pyrosequencing identified the patient as *CYP3A5*1/*3*, whereas TaqMan software called the patient as *CYP3A5*3/*3* (Figure 9.1a and e). Repeated genotyping on a second blood sample showed identical results for pyrosequencing (data not shown), and TaqMan software identified the patient as *CYP3A5*1/*3*. Moreover, pyrosequencing results showed inconsistencies in peak proportions between the A and the G peak as compared with results obtained with plasmid controls,[5] indicating that the genotyping result may be questionable (Figure 9.1a and b). Consultation with the attending nephrologist revealed a patient history including allogeneic hematopoietic stem cell transplantations (allo-HSCT). A first allo-HSCT in 1992 to treat β-thalassemia major was rejected. A second allo-HSCT in 2009, from a second donor, resulted in a mixed hematopoietic chimerism (28% autologous, 72% donor). Saliva samples from both the patient and the second stem cell donor were obtained and regenotyped. The donor was autocalled as *CYP3A5*3/*3* and the patient was autocalled as *CYP3A5*1/*3*. Pyrosequencing and TaqMan results were in concordance (Figure 9.1c, d, and g), and the patient's genotype was finally reported as *CYP3A5*1/*3*. This genotype is in line with the relatively low trough

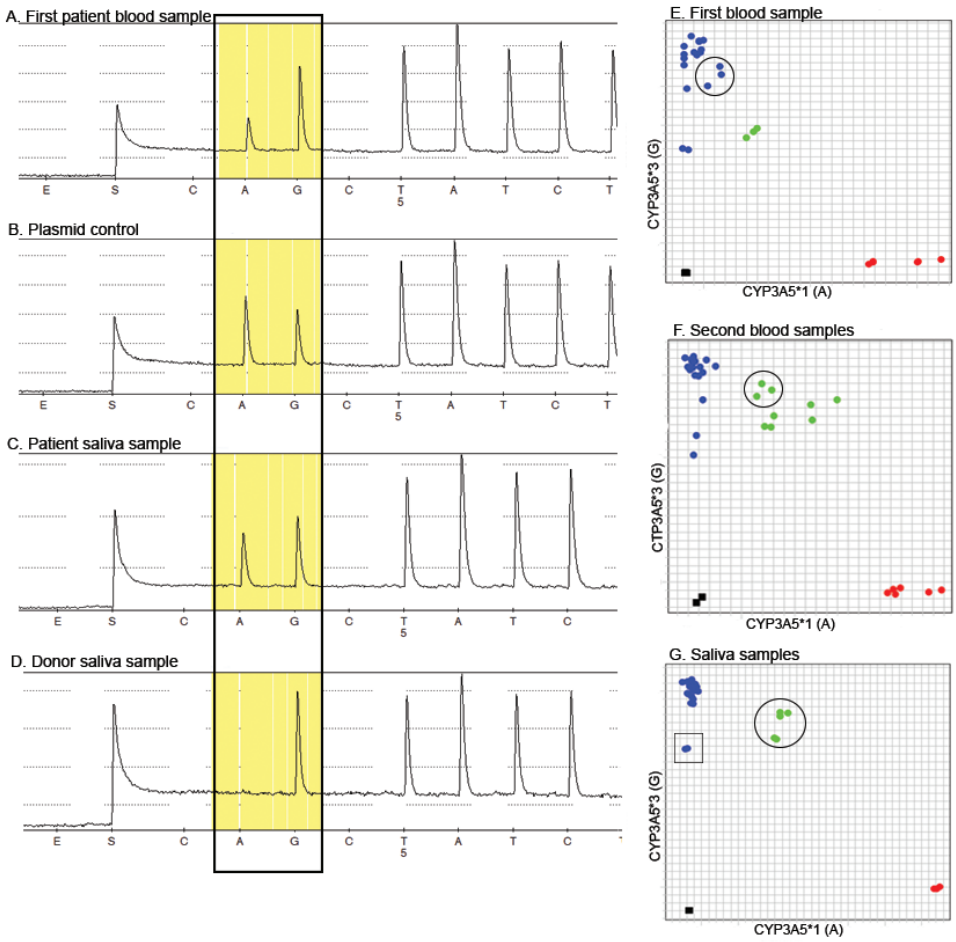

Figure 9.1 Genotyping results of different samples based on pyrosequencing and the TaqMan assay. (a–d) Pyrosequencing results. Results for a) patient blood sample b) plasmid *CYP3A5*1/*3* control c) saliva sample of the patient and d) saliva sample of the donor. A peak indicates presence of the *CYP3A5*1* allele; G peak indicates presence of the *CYP3A5*3* allele. Pyrosequencing results from the patient's blood sample (a) showed inconsistencies in peak proportion between the A and the G peaks as compared with the results obtained with (b) the plasmid control. As a result, the pyrosequencing software was not able to autocall the genotype of the patient. The saliva sample from the patient is autocalled as *CYP3A5*1/*3*, and the sample from the donor is autocalled as *CYP3A5*3/*3*. (e–g) TaqMan results. Blue dots indicate samples called as *CYP3A5*3/*3*; green dots indicate samples called as *CYP3A5*1/*3*; and red dots indicate samples called as *CYP3A5*1/*1*. Encircled dots are results obtained with DNA from the patient (triplos). Squared dots are results obtained with DNA from the donor. Conflicting results obtained with the e) first and f) second blood samples. g) Saliva sample from the patient is called as *CYP3A5*1/*3*, and the sample from the donor is autocalled as *CYP3A5*3/*3*.

level (5.5 µg/l) and area under the concentration-time curve (110 µg*h/l) achieved with a dose of 8 mg b.i.d. tacrolimus. It is essential to consider the source of the sample used to collect DNA for genotyping.

DISCUSSION

This case description demonstrates the challenging aspect of pharmacogenetic testing in an allo-HSCT recipient and illustrates the importance of proper quality control mechanisms when performing pharmacogenetic testing. In our opinion these challenges are not limited to the allo-HSCT setting. Also pharmacogenetic testing in solid organ transplantation recipients should be handled with great care, taking the transplant type and the metabolic pathway, mechanism of action and toxicity of the applied drugs into consideration. Therefore, we now routinely request transplantation status on all our pharmacogenetic laboratory orders.

In liver transplant recipients, the donor genotype will control enzyme expression patterns in the liver, while the recipient's genotype can affect absorption of drugs through drug transporter expression patterns in the intestine. Indeed, several studies have reported that donor and recipient *CYP3A5* genotype have a combined influence on the pharmacokinetics of tacrolimus following liver transplantation. This relation is time dependent and it appears that there is a shift in the major organ influencing tacrolimus disposition, from native intestine directly after the transplantation to donor liver due to recovery of the metabolic function of the liver.[6]

In renal transplant recipients, the hepatic metabolic capacity is not influenced by the transplantation and recipient genotyping would be sufficient in the case of drugs that are only subject to hepatic clearance. However, many drugs also undergo renal elimination and this can be influenced by drug transporters i.e. P-glycoprotein which are expressed in the kidney. Assuming that the donor kidney is the only functional kidney of the patient, both donor (locally in the kidney) and recipient (in the rest of the body) drug transporter genetic polymorphisms will affect elimination of these drugs. In addition the donor genotype potentially will also have influence on the development of toxicity in the donor graft. Hauser *et al*. demonstrated a higher incidence of cyclosporine nephrotoxicity when the donor, but not the recipient, had the *ABCB1* 3435TT genotype.[7]

These examples demonstrate that it is challenging, yet of utmost importance to select the correct material for genotyping. In allo-HSCT recipients peripheral blood samples are not adequate for genotyping due to mixed chimerism or complete donor genotype. We collected saliva samples of the patient and donor to determine both genotypes. The pyrogram obtained

with DNA derived from the patient's saliva is more similar to that obtained with a plasmid control (*CYP3A5*1/*3*) than the pyrogram obtained from DNA derived from the patient's blood sample. However, the saliva sample was still not identical to the plasmid control. This could be due to the fact that saliva samples obtained after allo-HSCT have been reported to display a chimeric or even complete donor genotype.[8] A buccal swab is considered to be more informative about the germline genotype of a recipient of an allo-HSCT.

In our case, the use of two independent validated genotyping assays and standardized plasmid controls resulted in the discovery of the mixed chimerism of the patient. These findings show the importance of proper quality control protocols when performing pharmacogenetic testing in routine patient care. With the increasing number of medical centers acquiring clinical genotyping facilities, the need for external quality control programs for pharmacogenetic testing is rising.

In conclusion, it is essential to consider the source of the sample used to collect DNA for genotyping in transplantation patients. In addition, quality control e.g. the use of validated assays and standardized control materials is of utmost importance to guarantee a safe implementation of pharmacogenetics.

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Chapter 10

General discussion & future perspectives

INTRODUCTION

Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) is basically a curative treatment in various hematologic malignancies and non-malignant diseases in both adult and pediatric patients, it may be accompanied by various complications, such as recurrence or persistence of the initial disease, severe infections, graft-versus-host disease (GvHD) and both acute and late toxicity related to the conditioning regimen. Pharmacotherapy is an essential part in the allo-HSCT procedure and therefore optimization of pharmacotherapy can help to reduce the risks of adverse events in allo-HSCT. Busulfan and treosulfan are both alkylating agents, which are widely used and play an important role in the conditioning regimen prior to allo-HSCT. Busulfan exposure has been related to allo-HSCT outcome and toxicity and therapeutic drug monitoring is often applied in clinical practice to target the exposure in the individual patient.[1–3] In addition, several population pharmacokinetic (pop-PK) models have been developed to describe busulfan PK. However, significant interpatient variability remains, caused by unknown variables. In this thesis the role of genetic markers in busulfan PK is explored.

Treosulfan was only recently introduced as myeloablative agent in conditioning prior to allo-HSCT. It is a promising drug with strong myeloablative and immunosuppressive capacity and a mild toxicity profile.[4–6] It is currently unresolved whether treosulfan therapy could be optimized when targeting exposure, similar to busulfan therapy. Treosulfan PK in adults is linear, however, there are limited data in pediatric patients showing a wide interpatient variability.[7] In this thesis further research on treosulfan PK in pediatric patients was performed, by development of a bioanalytical method and pop-PK model.

A major risk after allo-HSCT is the occurrence of aGvHD, which is accompanied with significant morbidity and mortality.[8] Despite improvements in donor matching and prophylaxis still approximately 25% of the patients will develop aGvHD requiring treatment with systemic high-dose glucocorticoids (GCs) as the first line therapy.[9,10] Unfortunately, about half of the patients do not respond to GCs and require alternative therapy.[11,12] In other diseases non-responsiveness towards GCs has been observed too.[13,14] Therefore it is suggested that GC-unresponsiveness is an intrinsic property of a subset of the population and differences in genetic make-up might contribute to this phenomenon.[13,14] Further insight in biological factors causing GC resistant aGvHD will be instrumental in designing the most effective aGvHD treatment for the individual patient.

BUSULFAN

In this thesis it was investigated whether genetic markers could be identified to explain the interpatient variability in busulfan clearance. Genetic markers were studied in both an adult and a pediatric population. In adults, the absence of maturation effects and relative small variability in body weight makes the association of busulfan clearance and genetic markers probably more straight forward. In **chapter 3** we studied the effect of SNPs in three genes encoding for glutathione-S-transferases (GSTA1, GSTM1, and GSTP1). These enzymes are involved in conjugation of busulfan with glutathione, with GSTA1 being the predominant enzyme.[15] A SNP (rs3957357) in *GSTA1* was associated with busulfan clearance and could explain 18% of the interpatient variability in this adult study population. No association between *GSTM1*, *GSTP1* and busulfan exposure was observed, nor could any relation between *GSTA1* genotype and clinical outcome be found in our study population. The latter is most probably due to the application of busulfan in a non-myeloablative regimen resulting in very low toxicity levels. The association of *GSTA1* genotype with busulfan PK parameters has been observed in a few studies in adults as well.[16,17] However, Krivoy *et al*.[18] did not observe an association with *GSTA1*, but found an association with *GSTP1* and two SNPs in *ABCB1* in combination with *GSTM1* and busulfan clearance. However, in this study the patients received busulfan orally and therefore other factors might contribute to this contradictious finding.

In **chapter 4** an exploratory pharmacogenetic approach was applied to investigate whether the remaining interpatient variability in busulfan clearance in adults could be explained. This analysis included a wide range of genes involved in drug ADME (absorption, distribution, metabolism and excretion). Two cohorts were composed; an exploratory and a validation cohort. Patients in the exploratory cohort were genotyped with the DMET array. This array contains 1936 SNPs in 225 genes involved in drug metabolism and transport. This array is a comprehensive platform to identify potential new genetic markers involved in busulfan PK. Again, the role of the *GSTA1* in association with busulfan clearance was confirmed both in the exploratory and validation cohort and no additional genetic markers were identified explaining the remaining part of the variability in busulfan PK. This could have been due the relative small cohort sizes resulting in low statistical power to detect rare variants or variants with a small effect size. Although it has been suggested that transporters are involved in busulfan metabolism, we could not identify any genetic markers in a wide variety of transporters affecting busulfan metabolism.

Clinical relevance of the *GSTA1* genotype in busulfan-based allo-HSCT in adults is minor. The effect of *GSTA1* genotype is potentially of more interest in pediatric patients, since in children

a high interpatient variability in exposure is observed, pediatric patients receive more often a myeloablative conditioning and a tighter control of exposure is aimed for in clinical practice.

However, the effect of genetic markers on busulfan PK in pediatric patients seems to be more complicated to determine, which is illustrated by several studies in pediatric patients presenting conflicting results. Either no association between *GSTA1* genotype and busulfan PK[19,20] was observed or the association was found only in a subset of the study cohort receiving busulfan orally.[21] However, there are also a number of positive studies,[22–25] including **chapter 5** of this thesis.[26] To our opinion, several factors might account for the discrepancies between the positive and negative studies towards the effect of genetic variation in *GSTA1* on busulfan clearance in children, such as sample size,[20] correction of busulfan clearance for body size[19,20] or administration route.[21]

Interpatient variability of busulfan PK in children is higher in comparison with adults and several mechanisms from birth until adulthood will affect busulfan PK and with that the effect of genetic markers on busulfan PK. Young children have a higher clearance (expressed per unit of body weight) in comparison to adults, which is partially caused by an increased liver size to body weight ratio. To capture the effect of physiological growth and liver size, busulfan clearance should be expressed as function of allometric scaled body weight or body surface area.[27,28] However, when clearance is expressed by body surface area there is still an increased clearance in younger children (< 4 years). This is most probably caused by an increased GST expression.[29] Furthermore, in the earliest years after birth, maturation of enzyme function occurs as demonstrated by a 1.7 fold increase in busulfan clearance in the first 2 years after birth.[28]

A genotype of each individual is set at conception. However, one's phenotype is affected by environmental factors and can change over time. *GSTA1* genotype affects GST activity and with that busulfan metabolism, however, the magnitude of effect of the genotype on busulfan metabolism is also determined by other contributing factors, such as administration route, co-medication and age of the patient. Furthermore, the expression of the *GSTA1* gene changes with age (as described above) and with that the relative contribution of this genotype to busulfan metabolism will change. A schematic overview of factors influencing busulfan PK is given in Figure 10.1. We hypothesize, that when there is abundant GST activity, which is the case at the age of 2–4 years, a less functional allele in *GSTA1* gene will have limited effect on busulfan PK. This was demonstrated in **chapter 5** of this thesis; a larger effect of *GSTA1* genotype was observed in children under 2 years of age in comparison to the older children; explaining 20% versus 5% of interpatient variability, respectively. Therefore, genotyping of *GSTA1* in pediatric patients seems only to be clinical relevant in a certain subpopulation based on age.

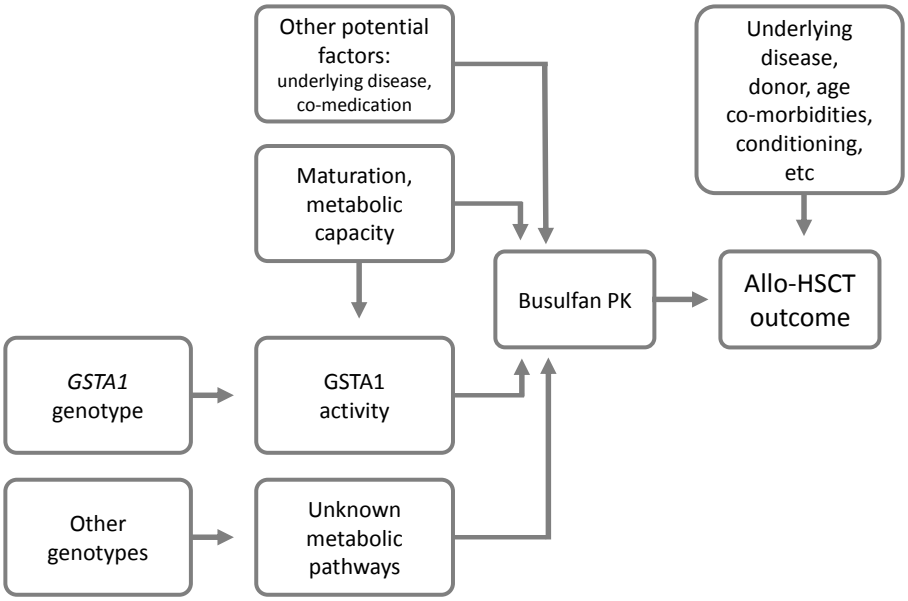

Figure 10.1 Factors affecting busulfan pharmacokinetics in pediatric patients. Allo-HSCT: allogeneic hematopoietic stem cell transplantation, PK: pharmacokinetics.

In **chapter 5** also a novel genetic marker (*CYP39A1*) was associated with busulfan clearance in pediatric patients. The role of this genetic marker in general and in busulfan metabolism in particular is unclear yet and functional analysis should be performed. Furthermore, the effect of *CYP39A1* in combination with *GSTA1* needs to be replicated in a second cohort.

In this thesis we searched for a genetic explanation of interpatient variability in busulfan PK in adults and in children. Investigating genetic markers in adults and children demonstrated that the effect of genetic variants is relative small to other patient related factors, such as age, maturation and body weight. Furthermore, not all the interpatient variability in busulfan PK cannot be explained by genetic markers involved in drug transport and metabolism.

TREOSULFAN

Treosulfan is an interesting alternative for busulfan in the conditioning prior to allo-HSCT. It has a mild toxicity profile and strong myeloablative capacity. It is most often applied in reduced toxicity or reduced intensity conditioning regimens. Data on PK of treosulfan in pediatric

patients is limited.[7] To assess the PK profile of treosulfan in pediatric patients we developed and validated a bioanalytical method for determination of treosulfan in serum, a pop-PK model and a limited sampling strategy, described in **chapter 6** of this thesis. Furthermore, in **chapter 7** we analyzed the PK profile of treosulfan in 21 pediatric allo-HSCT patients receiving treosulfan as part of their conditioning regimen.

The presented population PK model performs adequately, which is mainly due to the predictive PK profile of treosulfan. We have demonstrated in **chapter 6** and **7** that the interpatient variability in treosulfan exposure is relative small (14%) in pediatric patients above the age of 1 year. Furthermore, we could not relate early clinical outcome and toxicity to treosulfan exposure, This could be due to several reasons. First, such a relationship could be non-existent. Second, the interpatient variability may be too limited to observe such a relationship in this heterogeneous cohort of children. At first, a larger cohort and single-disease subgroups should be studied to more precisely assess the interpatient variability and the relationship of exposure with clinical outcome. Furthermore, it should be investigated whether treosulfan-based conditioning is more effective when increasing the dose of treosulfan while keeping its beneficial toxicity profile. The optimal dose of treosulfan for the youngest and smallest patients remains to be established, since we were only able to study patients above the age of 1 year and with a body weight of more than 10 kg, when developing the limited sampling strategy. One patient was much younger (2 months and weighing 5 kg) and received a lower adjusted dose of 10 g/m^2. Treosulfan exposure was assessed with the limited sampling strategy and the exposure of this young patient was similar to the mean exposure observed in the older patients. This suggests that younger patients may need a lower dose (based on body surface area). However, this is based on the finding in one patient and more data on the efficacy and toxicity profile in infants is required.

All patients above 1 year of age received 14 g/m^2 of treosulfan and a decrease of treosulfan exposure with age was observed. However, the higher exposure in the younger patients was not related to increased toxicity and this implicates that dose escalation in the older patients is possible. Treosulfan-based conditioning was relatively well tolerated and effective, as demonstrated by the mild toxicity profile in comparison to other conditioning regimens, a high overall survival (90%) and good disease free survival (74%). However, chimerism levels after 1 year and primary engraftment could be improved, especially in diseases where effective myeloablation and donor chimerism are mandatory, such as hematologic malignancies. It is therefore warranted to continue the research of treosulfan PK in relation to clinical outcome, in order to identify the optimal exposure and dosing regimen.

GLUCORTICOID RESPONSIVENESS IN AGVHD

Acute GvHD is one of the major complications after allo-HSCT which occurs when donor cells engraft and mature donor T-cells react with recipient immune cells. In malignant diseases a beneficial effect of aGvHD has been demonstrated, since it may also be accompanied with graft-versus-leukemia (GvL) effect. However, the right balance between the beneficial GvL effect and the adverse event of aGvHD is difficult to achieve. In patients diagnosed with aGvHD grade 2 or higher, first line treatment with high-dose glucocorticoids remains the gold standard. However, approximately only half of the patients responds adequately to this therapy. Non-responsiveness to GCs is associated with high morbidity and mortality. Furthermore, non-responsiveness will delay adequate treatment of the aGvHD, very often resulting in worsening of the disease. Overall, non-responsiveness towards GCs in aGvHD treatment is of major clinical concern. In **chapter 8** of this thesis 7 expression quantitative trait loci (eQTLs) were identified in healthy volunteers and these 7 eQTLs and an eQTL of *RBMS3* were investigated for association with GC non-responsiveness in pediatric patients with aGvHD. In this study it was demonstrated that genetic markers are involved in GC responsiveness and that donor genotype has a predominant effect on responsiveness. This finding is in line with the physiological process of aGvHD where alloreactive donor T-cells interact with immune cells of the recipient and GC therapy is considered to mainly target donor lymphocytes activity.

The pharmacologic effect of GCs is complicated and is affecting multiple targets and gene expression. Several studies sought to indentify genetic markers for association with GC-responsiveness and most of them were based on a candidate gene approach and mainly studied SNPs in genew encoding for the GC receptor and transcription factors. Combining genome wide genotyping data and multiple levels of phenotypic data provides the opportunity to identify polymorphisms that interact with GC treatment and influence response. These polymorphisms are therefore excellent candidates. The strength of the study in **chapter 8** is that findings from genome wide information were combined into a candidate gene approach study on a retrospective patient cohort.

PHARMACOGENETIC TESTING IN ALLO-HSCT RECIPIENTS

In this thesis pharmacogenetic testing was applied in 2 widely used drugs in allo-HSCT. When pharmacogenetic testing is more broadly applied in allo-HSCT patients it is essential to choose the right biospecimen for collecting DNA as we described in **chapter 9**. Patient material should preferably be acquired before the allo-HSCT. When genotyping patients with malignant disease

it is important to obtain germline DNA, therefore a blood sample in remission is a good option. Furthermore, we have demonstrated in **chapter 9** that after allo-HSCT, mixed chimerism in a patient could affect genotyping outcomes, emphasizing the need for proper quality control procedures.

FUTURE PERSPECTIVES

Busulfan conditioning can be optimized with therapeutic drug monitoring and evidence has been provided that tight control of exposure results in a better outcome.[3,30] It is essential to identify the optimal target exposure giving the optimal balance between efficacy and toxicity for different conditioning regimens. Research on the optimal busulfan exposure should be performed in more homogenous cohorts; patients receiving the same conditioning regimen and having the same underlying disease. This may result in disease and regimen specific recommendations.

The same accounts for treosulfan-based conditioning, however, the experience with treosulfan-based conditioning is limited and with that the knowledge on PK of treosulfan. First, the optimal dose and combination of drugs in treosulfan-based conditioning should be investigated. We hypothesize that the efficacy of treosulfan-based conditioning can be improved if a higher dose of treosulfan is administered and keeping its beneficial toxicity profile. At this point, busulfan is considered the most effective myeloablative conditioning regimen, whereas treosulfan appears to have a more favorable toxicity profile. Therefore, in pediatric patients, treosulfan is preferentially used in patients with co-morbidities or in non-malignant diseases. In malignant diseases a strong myeloablative conditioning is required. Treosulfan effectiveness might be improved at a higher dose. Therefore, it would be interesting to expand research on treosulfan PK and whether higher exposure translates into improved outcome. Ultimately, this may lead to a dose-escalation study in patients requiring a potent myeloablative conditioning regimen. Another advantage of treosulfan is the relative small interpatient variability in exposure. To further optimize the clinical use of both alkylating drugs, a direct comparison is warranted. Busulfan- and treosulfan-based conditioning should be compared prospectively in a large homogenous cohort in patients with the same underlying disease and disease status. A good example of such a prospective randomized study is the recently started ALL SCTPED 2012 FORUM study (EudraCT: 2012-003032-22) in which busulfan- and treosulfan-based conditioning regimens are directly compared to a traditional total body irradiation-based regimen in pediatric patients with ALL.

The finding that genetic markers are involved in non-responsiveness towards GCs in aGvHD is very promising. At first, the results should be replicated in a second and larger cohort. This will

lead to a more accurate measure of the predictive value of the two eQTLs, giving the possibility to study the effect of the eQTLs prospectively in pediatric patients with aGvHD. Furthermore, the physiological effect of the SNPs in *TMEM71* and *DPYSL3* should be further elucidated. At first, the SNPs should be studied in an *in vitro* model which resembles immune response in aGvHD accurately. When the SNPs remain to be associated with GC non-responsiveness in aGvHD, it could be of great interest to study them in other diseases such as asthma, inflammatory bowel disease and rheumatoid arthritis. For allo-HSCT patients ultimately, HSCT donors should be genotyped for the SNPs related to GC non-responsiveness and these biomarkers can be used to identify patients that will not benefit from GC therapy when encountering aGvHD. When aGvHD occurs in these patients, an alternative aGvHD therapy can be started directly and more stringent aGvHD prophylaxes can be applied in these patients. In addition, anti-thymocyte globuline (ATG) dose may be adapted based on the genotype of the donor, to reach the optimal balance between T-cell depletion to reduce the risk of aGvHD and rapid immune reconstitution. Furthermore, PK-models for estimation of ATG exposure are being developed and matching of ATG exposure to donor genotype could even be more beneficial.

Pharmacologic research in the field of allo-HSCT is often focused on one agent and one mechanism to optimize the studied drug. However, during allo-HSCT multiple factors, such as drugs, patient and donor characteristics, have also an effect on outcome. For example, the conditioning regimen can affect the risk of aGvHD. Therefore, future pharmacological studies should emphasize on the effects of combination of drugs, together with specific patient conditions, in order to further improve allo-HSCT outcome.

CONCLUSION

In this thesis, studies on three different pharmacological agents applied in pediatric and adult allo-HSCT have been performed. The goal of these studies was to elucidate mechanisms causing interpatient variability and thereby optimize current therapies for each individual patient. We demonstrated that *GSTA1* is involved in busulfan PK in adults and pediatric patients and in children a novel genetic marker, *CYP39A1*, was demonstrated to affect busulfan PK. Interpatient variability in treosulfan PK in pediatric patients is not as large as observed with busulfan, which could be a beneficial aspect of treosulfan. We also demonstrated that pharmacogenetic markers in HSCT donors are involved in GC responsiveness of patients with aGvHD. The procedure of allo-HSCT is very complex and pharmacotherapy plays an important role. Studies on the optimization of current drug therapies and combining results on different agents could have potentially great impact on morbidity and mortality rates in allo-HSCT patients.

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Summary

SUMMARY

Pharmacotherapy plays an essential role in an allogeneic hematopoietic stem cell transplantation (allo-HSCT) procedure. Busulfan and treosulfan are two alkylating agents often applied in the conditioning regimen administered prior to the allo-HSCT. Finding the right balance between efficacious conditioning and toxicity remains the challenge for both agents. In **chapter 2** of this thesis an overview of current strategies in optimization of busulfan and treosulfan therapy is given. Busulfan exposure has been related to clinical outcome and therapeutic drug monitoring is often applied in clinical practice to guide personalized dosing. Busulfan has a considerable unexplained interpatient variability in clearance. In **chapter 3**, **4** and **5** we sought to determine genetic markers that are related to interpatient variability in busulfan clearance. In **chapter 3**, three genes encoding for glutathione-S-transferases (GSTA1, GSTM1, and GSTP1) were analyzed in relation to busulfan PK and clinical outcome in 66 adult patients. In this study an association of *GSTA1* genotype (rs3957357) with busulfan clearance and exposure in adults patients was observed. However, only 18% of the interpatient variability could be explained by the *GSTA1* genotype. In **chapter 4**, a comprehensive pharmacogenetic analysis was performed by genotyping adult patients with the DMET array. This array contains 1936 SNPs in 225 genes involved in drug metabolism and transport; patients in the exploratory cohort were genotyped with this array. Based on the results from the first cohort, seven top genetic markers were selected and validated in a second independent cohort. In this validation cohort only one SNP in *GSTA5* (rs4715354) remained significantly associated with busulfan clearance and the *GSTA5* genotype explained 6.5% of variability of busulfan clearance. This SNP in *GSTA5* is in linkage disequilibrium with *GSTA1* genotype and was therefore a positive control for the association of *GSTA1* with busulfan PK. No additional genetic markers involved in drug metabolism and transport were associated with busulfan PK.

In **chapter 5**, the top 7 genetic markers from the exploratory adult cohort of chapter 4 were analyzed in pediatric patients. Since *GSTA5* was in linkage with *GSTA1*, we analyzed *GSTA1* genotype (rs3957357) instead of *GSTA5* in the pediatric cohort. In children, apart from *GSTA1*, also *CYP39A1* was associated with busulfan clearance. When combined, the two haplotypes explained 17% of the variability in busulfan clearance. Furthermore, in younger children (<2 years of age) a stronger effect of *GSTA1* on busulfan clearance was observed. We hypothesized that maturation effects account for this difference and when there is abundant GST activity, which is the case at the age of 2–4 years, a less functional allele in *GSTA1* gene would have limited effect on busulfan PK.

Chapter 6 and **7** focus on treosulfan, also an alkylating agent, in pediatric conditioning regimens. The experience with treosulfan-based conditioning is limited and only a few studies investigated the PK profile of treosulfan in pediatric patients. In **chapter 6** we developed and validated a bioanalytical method, population-PK model and limited sampling strategy. With the population-PK model and the limited sampling strategy treosulfan exposure can be determined based on two serum samples of an individual patient. These developments provide the opportunity to study treosulfan PK in a comprehensive manner in large pediatric cohorts, including infants. In **chapter 7**, a pilot study on treosulfan PK and clinical outcome was performed. It was demonstrated that treosulfan-based conditioning is very effective and toxicities are limited, in comparison to other conditioning regimens. Furthermore, there is potential of dose-escalation especially in older children, to increase the efficacy of treosulfan. This is of interest in diseases where a more myeloablative conditioning is required, such as malignant diseases. The interpatient variability of treosulfan clearance was 14% and intrapatient variability was 5%. Furthermore, no relationship between treosulfan exposure and clinical outcome was observed.

Acute graft-versus-host disease (aGvHD) is one of the major complications after allo-HSCT and is accompanied with significant morbidity and mortality. When patients are diagnosed with grade 2 or higher aGvHD, systemic treatment with high-dose glucocorticoids is started as first line treatment. However, only half of the patients respond adequately to this therapy and also in other diseases non-responsiveness to glucocorticoids has been observed. Therefore, it is assumed that non-responsiveness is an intrinsic factor of a subset of the population. In **chapter 8** of this thesis 7 expression quantitative trait loci (eQTLs) in healthy volunteers were identified. The *cis*-eQTLs affect the expression of the following genes: *LPIN2*, *DPYSL3*, *TMEM71*, *GZMA*, *MOB3C*, *TTF2*, *FAM117B* only in presence of glucocorticoids. Furthermore, the 7 eQTLs were not associated with glucocorticoids affected T-cell proliferation *in vitro*. We then analyzed the effect of the 7 eQTLs and an eQTL of *RBMS3* on glucocorticoids responsiveness in pediatric patients with aGvHD (grade 2 or higher). Since T-cells of the donor play an important role in aGvHD, we genotyped the 8 eQTLs in patient and donor DNA. In this chapter, it was demonstrated that donor eQTL genotype is associated with response-status and patient genotype is not; *TMEM71* and *DPYSL3* donor genotype were associated with GC-responsiveness and *TMEM71* alone had a predictive value of 65%.

In **chapter 9**, a case of pre-emptive genotyping of a patient receiving a kidney transplantation was described. To determine the adequate individual tacrolimus dose, *CYP3A5* genotype is determined in these patients. However, based on two genotyping methods conflicting results were obtained. Consultation with the attending nephrologist revealed a patient

history including two allo-HSCTs. This patient had a mixed hematopoietic chimerism and when genotyping peripheral blood samples from the patient, also a mixed *CYP3A5* genotype was obtained. This case shows the importance of proper quality control protocols when performing pharmacogenetic testing in routine patient care, especially in complex patients, such as allo-HSCT recipients.

The goal of these studies was to elucidate mechanisms causing interpatient variability and thereby optimize current therapies for each individual patient. We demonstrated that *GSTA1* is involved in busulfan PK in adults and pediatric patients and in children a novel genetic marker, *CYP39A1*, was demonstrated to affect busulfan PK. Interpatient variability in treosulfan PK in pediatric patients is not as wide as busulfan, which could be a beneficial aspect of treosulfan. We also demonstrated that pharmacogenetic markers in HSCT donors are involved in GC responsiveness of patients with aGvHD.

In the future, a direct comparison of busulfan- and treosulfan-based conditioning in a large homogenous cohort is warranted. The effect of the donor eQTLs on glucocorticoid non-responsiveness should be replicated in a second independent cohort and when current findings are confirmed it would be very interesting to study the eQTLs in other diseases as well.

Samenvatting

INLEIDING

Bij een allogene hematopoietische stamceltransplantatie (allo-HSCT) worden stamcellen van een donor toegediend aan een patiënt met als doel het immuunsysteem of bloedvormend systeem van de patiënt te herstellen. Een allo-HSCT wordt toegepast bij bloedkanker (verschillende typen leukemie), bij ziekten waarbij het afweersysteem niet of onvoldoende werkt of ziekten waarbij er een tekort is aan goed functionerende bloedcellen. Deze aandoeningen kunnen aangeboren zijn of zijn verworven na de geboorte. Aangeboren ziektes kunnen vlak na de geboorte al aan het licht komen, daarnaast zijn er verschillende vormen van bloedkanker die vooral bij kinderen voorkomen en allo-HSCT wordt daarom veelvuldig bij kinderen toegepast.

Wanneer lichaamsvreemde stoffen worden toegediend zal het lichaam er voor zorgen dat deze zo snel mogelijk worden opgeruimd. Dit is het geval bijvoorbeeld bij een infectie met een bacterie of een virus of bij toediening van cellen of organen in het geval van een transplantatie. Dus als een patiënt stamcellen van een donor ontvangt, is er het risico dat deze worden afgestoten door het lichaam. Er worden verschillende maatregelen genomen om de kans op afstoting zo klein mogelijk te houden. In de eerste plaats wordt er gezocht naar een donor die zo gelijk mogelijk is aan de patiënt. Dit gebeurt op basis van humaan leukocytenantigen (HLA) matching; antigenen van patiënt en donor moeten zo veel mogelijk overeenkomen, zodat het afweersysteem van de patiënt de cellen van de donor zo min mogelijk herkent als lichaamsvreemde cellen. HLA-antigenen van broers en zussen zijn soms identiek aan elkaar, of kunnen veel op elkaar lijken. Daarom is een broer of zus vaak een geschikte donor. Daarnaast zijn er mogelijkheden om stamcellen van niet-gerelateerde donoren toe te dienen waarbij vooraf is vastgesteld dat deze voldoende overeenkomsten vertonen met de cellen van de patiënt zelf.

ALLOGENE STAMCELTRANSPLANTATIE

De patiënt krijgt voorafgaand aan de allo-HSCT een chemokuur, ook wel conditioneringsfase genoemd, die bestaat uit een combinatie van geneesmiddelen. Deze conditionering heeft als doel het afweersysteem zo veel mogelijk te remmen (immuunsuppressie), zodat de donorcellen niet worden afgestoten. Verder wordt met de conditionering "myelosuppressie" beoogd, dit houdt in dat zo veel mogelijk stamcellen van de patiënt worden gedood, zodat er ruimte is voor de stamcellen van de donor om zich te nestelen en te gaan delen. In het geval van leukemie zal de chemokuur er ook voor zorgen dat kankercellen zo veel mogelijk worden gedood. De dosis van de verschillende geneesmiddelen is hoog in vergelijking met een "normale" chemokuur. Dit resulteert erin dat de hoeveelheid bloedcellen in de patiënt heel laag wordt, dit wordt aplasie

genoemd. Alleen wanneer een patiënt in aplasie is, is het mogelijk om de donor stamcellen toe te dienen. Echter, als een patiënt vrijwel geen witte bloedcellen meer heeft dan werkt het immuunsysteem niet meer en kan de patiënt gemakkelijk geïnfecteerd raken met een bacterie of een virus. Om deze reden krijgen alle patiënten preventief antibiotica en middelen tegen virusinfecties. Daarnaast worden ze verzorgd in een geïsoleerde ruimte met gefilterde, schone lucht om de patiënt zo veel mogelijk te beschermen tegen infecties.

Nadat de stamcellen zijn toegediend is het doel dat deze zich nestelen en gaan expanderen, wat uiteindelijk moet leiden tot herstel van het immuun- en bloedsysteem. In deze fase na de allo-HSCT bestaat het risico dat de stamcellen van de donor worden afgestoten of de immuuncellen van de donor kunnen reageren tegen de cellen van de patiënt zelf en schade veroorzaken. Dit laatste fenomeen heet graft-versus-host ziekte.

DOEL VAN DE STUDIE

In de verschillende fasen van een allo-HSCT procedure krijgt een patiënt geneesmiddelen toegediend, in de conditioneringsfase, in de preventie en behandeling van infecties en bij complicaties die kunnen optreden tijdens de allo-HSCT. Deze middelen kunnen toxisch zijn en schade geven bij de patiënt of kunnen soms juist iets hoger worden gedoseerd om daarmee effectiever te zijn. Het doel van het onderzoek beschreven in dit proefschrift is de behandeling met bestaande geneesmiddelen rondom een allo-HSCT verder te optimaliseren. Het onderzoek is gericht op drie geneesmiddelen: busulfan en treosulfan, beide cytostatica die in de conditioneringsfase worden toegepast, en glucocorticosteroïden, die worden toegepast bij de behandeling van acute graft-versus-host ziekte. Onderzoek naar optimalisatie van deze geneesmiddelen wordt gedaan door relaties te zoeken tussen de concentratie of blootstelling (farmacokinetiek) van een geneesmiddel in het bloed en het effect van het geneesmiddel. Of door te kijken of er genetische kenmerken zijn in een patiënt die maken dat het geneesmiddel een afwijkend effect heeft in verschillende individuen (farmacogenetica).

BUSULFAN EN TREOSULFAN

Busulfan en treosulfan zijn beide alkylerende cytostatica die worden toegepast in de conditioneringsfase bij allo-HSCT in volwassenen en kinderen. Met busulfan is al veel ervaring en in eerder onderzoek is aangetoond dat de concentratie (blootstelling) in het bloed gerelateerd is aan de effectiviteit en toxiciteit van busulfan. Als de concentratie in het bloed van busulfan te hoog is, dan bestaat er kans op toxiciteit; leverschade en mucositis (beschadiging van de

slijmvliezen). Wanneer de concentratie van busulfan te laag is, dan is er verhoogde kans op afstoting van het transplantaat. In de praktijk wordt daarom tijdens de vierdaagse kuur van busulfan de concentratie in het bloed gemeten en eventueel de dosis aangepast, zodat de juiste blootstelling wordt bereikt in een patiënt.

De ervaring met treosulfan in allo-HSCT is nog beperkt en vooral in kinderen is er weinig onderzoek gedaan naar de concentratie van treosulfan in het bloed van verschillende patiënten. Het middel lijkt op busulfan, maar geeft minder toxiciteit en is mogelijk iets minder effectief.

In **hoofdstuk 2** van dit proefschrift is een overzicht gegeven van de eerdere onderzoeken die zijn gedaan om de huidige conditioneringen op basis van busulfan en treosulfan te optimaliseren. Busulfan wordt gedoseerd op basis van het lichaamsoppervlak of lichaamsgewicht van de patiënt, maar desondanks kan de blootstelling tussen individuen erg verschillen. Dit zou kunnen komen door verschil in genetische opmaak van patiënten. In **hoofdstuk 3** is gekeken naar verschillen in het DNA (polymorfismen) van patiënten. Polymorfismen in 3 genen zijn onderzocht in 66 volwassen patiënten die busulfan kregen voorafgaand aan hun allo-HSCT. De drie genen coderen voor drie verschillende enzymen die betrokken zijn bij het omzetten van busulfan naar een inactieve stof (metabolisme); GSTA1, GSTM1 en GSTP1. De polymorfismen in deze genen werden gerelateerd aan de busulfanconcentratie in het bloed van de individuele patiënten. Eén polymorfisme in *GSTA1* (rs3957357) was gerelateerd aan de blootstelling van busulfan in volwassenen; patiënten met twee C-allelen hebben gemiddeld de hoogste klaring, patiënten met het CT-genotype hebben een lagere klaring in vergelijking met patiënten met het CC-genotype en patiënten met het TT-genotype hebben gemiddeld de laagste klaring en daarmee de hoogste blootstelling van busulfan in het bloed. Echter slechts 18% van de variabiliteit in klaring in de studiegroep kon worden verklaard met dit polymorfisme in *GSTA1*. In **hoofdstuk 4** hebben we daarom breder gezocht naar genetische polymorfismen die een effect zouden kunnen hebben op de farmacokinetiek van busulfan. Een eerste studiegroep van 65 volwassenen is gegenotypeerd met behulp van de DMET array (Drug Metabolizing Enzymes and Transporters array). Met deze array kunnen 1936 genetische veranderingen in 225 genen, betrokken bij metabolisme en transport van geneesmiddelen, worden geanalyseerd. De zeven meest belovende polymorfismen uit de eerste studiegroep werden vervolgens geanalyseerd in een tweede studiegroep van 78 volwassen patiënten. In deze groep was één polymorfisme in *GSTA5* (rs4715354) geassocieerd met busulfanklaring en deze kon 6.5% van de variabiliteit in klaring verklaren. Het polymorfisme in *GSTA5* is gekoppeld aan het polymorfisme in *GSTA1* (rs3957357); wanneer een individu een afwijkend allel heeft in *GSTA1* is de kans groot dat deze ook een afwijkend allel heeft in *GSTA5*. De bevinding dat *GSTA5* geassocieerd is met busulfanklaring is daarom een positieve controle van het effect van *GSTA1* op busulfanklaring. In

deze studie zijn geen andere polymorfismen, in genen betrokken bij geneesmiddelmetabolisme en -transport, gevonden die waren geassocieerd met de farmacokinetiek van busulfan.

De zeven meest veelbelovende polymorfismen uit het volwassenencohort van hoofdstuk 4 zijn ook geanalyseerd in een studiegroep van 84 kinderen, dit is beschreven in **hoofdstuk 5**. In dit hoofdstuk hebben we het polymorfisme in *GSTA1* in plaats van het polymorfisme in *GSTA5* geanalyseerd. Hiervoor is gekozen, omdat deze twee polymorfismen gekoppeld zijn en *GSTA1* functioneel is en *GSTA5* niet. In de kinderen die busulfan kregen toegediend voorafgaand aan hun allo-HSCT was wederom het polymorfisme in *GSTA1* (rs3957357) en een polymorfisme in *CYP39A1* (rs9381468 en rs953062) geassocieerd met busulfanklaring. Tezamen kunnen deze twee genetische varianten 17% van de variabiliteit in busulfanklaring in kinderen verklaren. Tevens is gebleken dat het effect van *GSTA1* groter is in kinderen onder de twee jaar. Een verklaring zou kunnen zijn dat in jonge kinderen de GSTA1-activiteit nog niet maximaal is en daarom het effect van genetische variatie groter is.

In **hoofdstuk 6** en **7** is onderzoek gedaan naar de farmacokinetiek van treosulfan. Er zijn enkele studies die de farmacokinetiek van treosulfan in kinderen die een allo-HSCT ondergaan beschrijven, deze studies zijn uitgevoerd in kleine patiëntengroepen. In **hoofdstuk 6** zijn ontwikkeling en validatie van een bioanalytische methode voor het bepalen van treosulfan in het bloed, een populatie-farmacokinetisch model en een "limited sampling model" beschreven. Met behulp van het populatie-farmacokinetisch model en het "limited sampling model" kan de farmacokinetiek van de populatie worden beschreven en kan met behulp van slechts twee bloedmonsters van een individuele patiënt de klaring en blootstelling worden berekend. Met behulp van deze methode kan de farmacokinetiek gemakkelijker worden bestudeerd, tevens bij baby's, aangezien er slechts twee bloedmonsters hoeven worden afgenomen van maximaal 500 µl.

In **hoofdstuk 7** is een eerste verkennend onderzoek in 21 kinderen uitgevoerd waarin de farmacokinetiek van treosulfan is gerelateerd aan uitkomst van de HSCT en toxiciteit. De variabiliteit in blootstelling tussen patiënten was 14% en de van-dag-tot-dag-variatie is 5%. Treosulfanconditionering bleek effectief te zijn en de toxiciteit was relatief mild in vergelijking met andere conditioneringsschema's. Hieruit volgt dat de effectiviteit van treosulfan mogelijk te verbeteren is, door de dosis te verhogen. Dit zou dan in eerste instantie vooral onderzocht kunnen worden in oudere kinderen en in kinderen waarbij een sterkere conditionering nodig is, bijvoorbeeld in het geval van verschillende typen leukemie.

Glucocorticosteroïden en acute graft-versus-host ziekte

Nadat de stamcellen van de donor zijn toegediend en de donorcellen zich nestelen in het beenmerg van de patiënt is er een risico op het optreden van acute graft-versus-host ziekte (aGvHD). Bij aGvHD vallen de immuuncellen van de donor cellen van de patiënt aan en zorgen voor schade, vooral in het maag-darmstelsel, de lever en de huid. Deze ziekte heeft een hoge morbiditeit en mortaliteit. De eerstelijns therapie bij patiënten met graad 2 aGvHD of hoger bestaat uit hoge doses systemische glucocorticosteroïden (GC's). Helaas reageert ongeveer slechts de helft van de patiënten voldoende op deze therapie. Het niet reageren op GC's (GC-resistentie) is ook bekend bij andere ziektes waarbij GC's worden toegepast. Men denkt dat GC-resistentie een intrinsieke eigenschap is van een deel van de populatie, waarbij mogelijk de genetische opmaak van een patiënt een rol speelt. In **hoofdstuk 8** hebben we nieuwe genetische markers ontdekt in gezonde vrijwilligers die een rol spelen bij GC-resistentie en deze vervolgens getest in kinderen met aGvHD na hun allo-HSCT. De genetische markers die onderzocht zijn hebben een effect op de expressie van genen in de aanwezigheid van GC's. Deze markers worden ook wel "expression quantitative trait loci" (eQTLs) genoemd. In gezonde vrijwilligers zijn zeven eQTLs geïdentificeerd. De expressie van de volgende genen is geassocieerd met het genotype van de eQTLs: *LPIN2*, *DPYSL3*, *TMEM71*, *GZMA*, *MOB3C*, *TTF2*, *FAM117B*. Echter, het genotype van de eQTLs hadden geen effect op *in vitro* T-cel proliferatie. In de kinderen zijn deze 7 eQTLs en een eQTL van *RBMS3* geanalyseerd. Aangezien in aGvHD de GC-behandeling vooral is gericht tegen de immuuncellen van de donor, hebben we de patiënt zelf en zijn of haar donor gegenotypeerd voor de 8 eQTLs. In **hoofdstuk 8** hebben we aangetoond dat het genotype van de donor van belang is om de GC-respons van de individuele patiënt met aGvHD te kunnen voorspellen. Met name de eQTLs die een effect hebben op *TMEM71* en *DPYSL3* zijn geassocieerd met GC-respons en *TMEM71* donorgenotype heeft een voorspellende waarde van 65%.

Genotyperen van allo-HSCT patiënten

In **hoofdstuk 9** is een casus beschreven van een patiënt die een niertransplantatie zou ondergaan. Hiervoor worden immunosuppressieve geneesmiddelen gegeven en één van de geneesmiddelen is tacrolimus. Dit geneesmiddel wordt gemetaboliseerd door CYP3A5 en de activiteit van dit enzym is afhankelijk van de allelen die de patiënt van het *CYP3A5*-gen heeft. Om direct te starten met een goede dosis wordt voor start van tacrolimus het genotype van *CYP3A5* bepaald. Dit wordt routinematig met behulp van twee onafhankelijke methoden gedaan, zodat er altijd een accurate uitslag wordt uitgegeven. Bij deze patiënt gaven de twee

methoden echter tegenstrijdige resultaten en toen de behandelend arts werd geconsulteerd bleek dat de patiënt in het verleden een allo-HSCT had ondergaan. Het bloedmonster dat was gebruikt voor de genotypering bevatte cellen van de donor en de patiënt zelf, wat vaak voorkomt na een allo-HSCT. Dit resulteerde in een gemengd genotype van *CYP3A5* in het perifere bloed. Het genotype is vervolgens bepaald in speeksel van de patiënt. Dit materiaal gaf een eenduidiger resultaat van het genotype van de patiënt zelf, welke het meest van belang is bij het metabolisme van tacrolimus. Deze casus geeft het belang van goede kwaliteitscontroles en de keuze van patiëntmateriaal aan voor genotyperen in de klinische praktijk.

CONCLUSIE

In dit proefschrift is onderzoek gedaan naar drie geneesmiddelen die worden toegepast bij allo-HSCT in kinderen en volwassenen, met als doel het optimaliseren van de huidige therapieën.

We hebben aangetoond dat variaties in het *GSTA1*-genotype in volwassen en *GSTA1*- en *CYP39A1*-genotype in kinderen een effect hebben op de farmacokinetiek van busulfan. De variatie in treosulfanklaring tussen patiënten is relatief klein, dit zou één van de voordelen van treosulfan kunnen zijn. In aGvHD-patiënten blijkt dat het donorgenotype meer van belang is voor het voorspellen van de GC-respons dan het genotype van de patiënt zelf. In de toekomst moeten busulfan- en treosulfanconditionering in grote homogene studiegroepen direct met elkaar vergeleken worden, zodat een goede keuze voor één van de middelen kan worden gemaakt voor de individuele patiënt. De resultaten met betrekking tot de donorgenotypes van *TMEM71* en *DPYSL3* en GC-respons moet nog worden onderzocht en bevestigd in een tweede onafhankelijk cohort. Wanneer de huidige resultaten worden bevestigd dan zouden deze genotypes ook kunnen worden onderzocht in andere ziekten.

About the author

CURRICULUM VITAE

Marloes ten Brink was born in Enschede, The Netherlands on February 2nd 1982. After having finished her secondary school at the Tijcollege (VWO) in Oldenzaal in 2000, she started with her bachelor's degree in Pharmacy at the University of Groningen. In 2004 she obtained her degree and continued with the master's degree also at the University of Groningen. As part of her studies she worked on a research project at the Institut Pasteur, Paris, France (supervisor Dr. T. Msadek) and in collaboration with the Medical Microbiology Division at the Faculty of Medical Sciences, University of Groningen (supervisor Prof. Dr. J.M van Dijl). The research was related to the *luxS* gene in *Staphylococcus aureus* and its role in quorum sensing and biofilm formation. In 2007, Marloes received her Master's degree in Pharmacy and started working at the hospital pharmacy of the Leiden University Medical Center as a pharmacist (supervisor Dr. K. J. M. Schimmel). In 2008, she started her specialisation as a hospital pharmacist also at the Leiden University Medical Center (supervisor Prof. dr. H.-J. Guchelaar), which was completed in 2012. As part of her training she started working on the research presented in this PhD thesis in which she was supervised by Prof. dr. H.-J. Guchelaar and Dr. J. Zwaveling from the Department of Clinical Pharmacy & Toxicology and Dr. A. C. Lankester and Dr. R. G. M. Bredius from the Department of Pediatrics, both from the Leiden University Medical Center. As part of her research she visited the Human Genetics Deparment of the University of Chicago, Chicago, Il, USA for a period of three months (supervisors Prof. Dr. A. Di Rienzo and Dr. J.C. Maranville). After completion of her PhD thesis, Marloes will continue her career at the Leiden University Medical Center as a hospital pharmacist.

LIST OF PUBLICATIONS

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Nawoord

Dit is het dan, het laatste hoofdstuk van het proefschrift. Het begon allemaal ergens in 2008, toen ik de mogelijkheid kreeg om een onderzoek vanuit de apotheek van het LUMC in samenwerking met de afdeling Hematologie en Kindergeneeskunde op te pakken. Ik had nooit verwacht en durven dromen dat het begin in 2008 zou leiden tot dit proefschrift. Het is gelukt en daar hebben velen om mij heen aan bijgedragen.

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