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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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ABSTRACT

Busulfan is 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, Castres Cedex, France) intravenously (iv.) once daily in a 4-day course. The starting dose was 120 mg/m² or 80 mg/m² 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/m²/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/m² 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/m²/day. Both drugs could be combined with thiotepa iv. 8 mg/kg (day -8), melphalan iv. 140 mg/m² (day -1). When busulfan and fludarabine were combined with clofarabine iv. (30 mg/m²/day, also on day -7 until day -4), the fludarabine dose was 10 mg/m²/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.
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 precolumnnderivatization, liquid/liquid extraction and UV detection, previously described by our group.18 Briefly, the limit of quantification was 30 μg/L.
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). The calculated mean population PK parameters, clearance and half-life, were individualized according to the maximum a posteriori Bayesian fitting method.

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.

**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. 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 (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 Rh² was calculated using gPlink. Haplotypes with Rh² > 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/m² 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/m²) than in the patient older than 2 years (6.8 ± 1.7 L/h/m²; 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/m²) compared with the other indications; hematologic malignancies: 7.1 ± 1.6 L/h/m²; β-thalassemia: 6.9 ± 2.0 L/h/m² and other non-malignant hematologic diseases: 6.3 ± 1.3 L/h/m² (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;
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/m² vs 4.8 ± 1.1 L/h/m²). 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

<table>
<thead>
<tr>
<th>SNP/Haplotype</th>
<th>Genotype</th>
<th>n (%)</th>
<th>Busulfan clearance (L/h/m² mean ± sd)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTA1</td>
<td>*A/*A</td>
<td>32 (39.5%)</td>
<td>7.2 ± 1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*A/*B</td>
<td>38 (46.9%)</td>
<td>6.6 ± 1.7</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>*B/*B</td>
<td>11 (13.6%)</td>
<td>5.2 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>CYP39A1_TC</td>
<td>WT/WT</td>
<td>21 (25.3%)</td>
<td>7.2 ± 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT/TC</td>
<td>32 (38.6%)</td>
<td>6.3 ± 1.6</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>TC/TC</td>
<td>30 (36.1%)</td>
<td>6.0 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>CYP39A1</td>
<td>G/G</td>
<td>42 (50.0%)</td>
<td>6.7 ± 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>33 (39.3%)</td>
<td>6.7 ± 1.7</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>9 (10.7%)</td>
<td>5.7 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>C/C</td>
<td>52 (61.9%)</td>
<td>6.6 ± 2.1</td>
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<tr>
<td></td>
<td>C/T</td>
<td>27 (32.1%)</td>
<td>6.6 ± 1.5</td>
<td>0.81</td>
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<tr>
<td></td>
<td>T/T</td>
<td>5 (6.0%)</td>
<td>6.3 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>ABCB4_GAT</td>
<td>WT/WT</td>
<td>28 (34.1%)</td>
<td>6.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT/GAT</td>
<td>33 (40.2%)</td>
<td>6.6 ± 1.8</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>GAT/GAT</td>
<td>21 (25.6%)</td>
<td>6.8 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>SLC7A8</td>
<td>A/A</td>
<td>35 (41.7%)</td>
<td>6.7 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>35 (41.7%)</td>
<td>6.6 ± 2.0</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>14 (16.6%)</td>
<td>6.1 ± 1.8</td>
<td></td>
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<tr>
<td>SLC22A4</td>
<td>C/C</td>
<td>33 (39.8%)</td>
<td>6.4 ± 1.9</td>
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<tr>
<td></td>
<td>C/T</td>
<td>39 (47.0%)</td>
<td>6.7 ± 1.8</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>11 (13.3%)</td>
<td>6.8 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

Distribution of genotypes of each of the seven genetic markers and mean busulfan clearance per genotype. SD: Standard deviation, WT: wild-type.
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/m² in the GSTA1 wild-type group, 5.9 ± 2.2 L/h/m² in the heterozygous group (*A/*B) and 4.1 ± 0.5 L/h/m² 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/m²; this was 6.8 ± 1.4 L/h/m² in heterozygous patients and 5.9 ± 1.6 L/h/m² 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 R²) and p = 0.004.
**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. 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. 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 \textit{GSTA1} and \textit{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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