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Glutathione Transferase Gene Variants Influence Busulfan Pharmacokinetics and Outcome After Myeloablative Conditioning

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Background: Busulfan (Bu) and cyclophosphamide (Cy) are frequently included in conditioning regimens before hematopoietic stem cell transplantation (HSCT). Both drugs are detoxified by glutathione transferases (GST), and *GST* gene variants may explain some of the interindividual variability in pharmacokinetics and drug toxicity.

Methods: The study investigated adult patients (n = 114) receiving oral Bu pre-HSCT. Bu doses were adjusted to obtain an average steady-state concentration (C_{ss}) of 900 mcg/L.

Results: Median first dose Bu C_{ss} was 1000 mcg/L (600–1780 mcg/L). Patients carrying 1 and 2 *GSTA1*B* (rs3957357) alleles demonstrated median 12% and 16% higher Bu C_{ss} ($P \leq 0.05$). Bu exposure (average C_{ss}; odds ratio = 1.009, 95% confidence interval = 1.002–1.017, $P = 0.013$) and *GSTM1* gene copy number (odds ratio = 17.1, 95% confidence interval = 1.46–201, $P = 0.024$) were significant predictors of mortality ≤30 days. The mortality was 25% versus 2% among carriers of 2 versus no *GSTM1* copies ($P = 0.021$). Mortality ≤3 months was associated with higher first dose Bu exposure (1090 versus 980 mcg/L, $P = 0.021$). GSTM1 expression and high Bu exposure may increase Cy toxicity by reducing intracellular glutathione.

Conclusions: *GST* genotyping before HSCT may allow better prediction of Bu pharmacokinetics and drug toxicity, and thereby improve outcome after BuCy conditioning.

Key Words: busulfan, glutathione transferase, hematopoietic stem cell transplantation, pharmacogenetics, therapeutic drug monitoring

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INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) represents an important treatment option for patients with hematological malignancies or severe, nonmalignant conditions. A common conditioning regimen consists of busulfan (Bu) and cyclophosphamide (Cy). Both drugs display large pharmacokinetic (PK) variability and a narrow therapeutic range.[1] For Bu, drug exposure has been correlated to clinical outcome, and therapeutic drug monitoring (TDM) based on area under the concentration–time curve (AUC) estimates is reported to reduce rejection rates, nonrelapse mortality, and relapse after oral and intravenous (IV) Bu dosing.[2,3] In contrast, monitoring Cy or active metabolites remains to be established in routine clinical practice. Despite the significant improvements with TDM-guided Bu dosing, treatment-related toxicity after BuCy conditioning still remains a considerable problem.[2] Furthermore, Bu monitoring based on AUCs is time-consuming, laborious, and expensive. Altogether, this emphasizes the need for more efficient strategies to individualize conditioning therapy pre-HSCT.

The primary elimination route for Bu is conjugation with glutathione, catalyzed by glutathione transferases (GST). Bu is predominantly metabolized by the major hepatic isoenzyme GSTA1, whereas GSTM1 and GSTP1 have demonstrated 46% and 18% of the Bu conjugation activity, respectively.[4] Furthermore, GSTT1 has been associated with Bu clearance.[5] Cy is metabolized by cytochrome P450 enzymes to active and inactive metabolites. The subsequent detoxification of Cy metabolites involves glutathione conjugation by GST enzymes, including GSTA1, GSTM1, GSTP1, and GSTT1.[6]

The large interindividual variability in glutathione conjugation may be related to differences in GST function and glutathione availability. Several *GST* gene variants have been reported to impact GST activity, suggesting that the interindividual variability in Bu metabolism may partly be explained by *GST* gene variants. A group of linked single-nucleotide variants in the *GSTA1* promoter region define 2 haplotypes, *GSTA1*A* and *GSTA1*B*, where *GSTA1*B* has been associated with reduced hepatic gene transcription and GSTA1 expression.[7] Clinically, *GSTA1*B* carriers have demonstrated lower Bu clearance (CL) and higher dose-adjusted Bu concentrations.[5,8–10] *GSTA1*B* allele frequencies differ between populations, ranging 38%–42% among whites and 8%–13% in Asian populations (dbSNP).[7]

The activity of GSTM1 and GSTT1 largely depends on gene copy numbers.[11,12] Homozygous deletion (null genotype)

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of the *GSTM1* and *GSTT1* gene is observed among 45%–60% and 10%–30% of whites, respectively.[13] The *GSTM1* null genotype has been associated with altered Bu PK and increased risk of drug-related toxicity.[5,14–16] Other studies observed significant correlations with the combined *GSTM1* and *GSTT1* null genotype but not with the individual *GSTM1* or *GSTT1* genotypes.[5,17] In contrast to the reported increased risk of drug toxicity, the *GSTM1* null genotype has been associated with a lower risk of treatment-related mortality.[18] Furthermore, *GSTP1* p.Ile105Val has been associated with lower enzyme activity and increased Bu exposure.[16] The reported associations between *GST* gene variants, drug exposure, and clinical outcome suggest a potential for genotype-guided Bu dosing. However, the findings so far are not consistent, and further studies in larger populations are needed to confirm the results.[19,20]

The aim of the present study was to investigate the association between Bu PK, *GST* gene variants, and clinical outcome post-HSCT to further elucidate the potential of individualized Bu dosing based on *GST* genotyping.

MATERIALS AND METHODS

Study Population

The retrospective study included 114 consecutive adult patients receiving BuCy conditioning pre-HSCT at Oslo University Hospital from February 2008 to January 2012. The study was approved by the Research Ethics Board of the hospital, and all patients signed informed consent on data collection regarding details of the transplant procedure and posttransplant follow-up. Inclusion criteria were approval for HSCT, age ≥16 years, and planned oral Bu conditioning. Patients were excluded if Bu was given IV, samples were missing, or the HSCT was canceled.

Conditioning Regimen

Conditioning was initiated with oral Bu (Myleran; GlaxoSmithKline, Brentford, United Kingdom), given every 6 hours for 4 days, starting 7 days pre-HSCT. The 2 first doses were fixed (~1 mg/kg), followed by dose adjustments to obtain an average steady-state concentration (C_{ss}) of 900 mcg/L. Cy (Sendoxan; Baxter, Halle/Westfalen, Germany) 60 $mg \cdot kg^{-1} \cdot d^{-1}$ was administered IV for 2 consecutive days, starting ~20 hours after the last Bu dose.

Prophylaxis against acute graft-versus-host disease (aGVHD) consisted of methotrexate (MTX) on day 1, 3, 6, and 11, and cyclosporine. MTX was discontinued in cases of moderate-to-severe mucositis or organ toxicity. All patients received phenytoin as seizure prophylaxis.

Busulfan Monitoring

Blood samples were drawn before and 30, 60, 90, 120, 180, 240, and 300 minutes after the first, fifth, and ninth Bu doses. The sampling scheme is according to the standard clinical routine monitoring of oral busulfan pre-HSCT in adult patients at Oslo University Hospital, Rikshospitalet. Bu plasma concentrations were measured by liquid chromatography (modified after Rifai et al[21]). Intraassay and interassay variability was <10% for concentrations ranging 700–1100 mcg/L. The lower limit of quantification was 100 mcg/L.

The AUC from one dose was calculated from 0 to 5 hours using the linear trapezoidal rule and extrapolated to infinity by dividing the last measured concentration by the terminal elimination constant and subtracting the contribution from the previous dose. The average C_{ss} was calculated by estimating AUC at steady state and dividing by the dosing interval. Bu CL/bioavailability (F) was calculated by dividing dose by AUC, providing the basis for subsequent dose adjustments targeting an average C_{ss} of 900 mcg/L.

Genotyping

Genotyping was based on EDTA–blood samples collected pre-HSCT. The samples were excess materials from routine pre-HSCT samples stored in a diagnostic biobank. DNA was extracted using the DNA Isolation Kit I (Roche Diagnostics, Mannheim, Germany) on the MagNA Pure instrument (Roche Applied Science, Penzberg, Germany). *GSTA1* c.-69C>T (NM_145740.3:c.-135T>C; rs3957357, C = *A and T = *B) and *GSTP1* p.Ile105Val (NM_000852.3:c.313A>G; rs1695) variants were analyzed by melt curve genotyping. *GSTM1* and *GSTT1* gene copy numbers were determined by quantitative real-time PCR and normalization to *ALB* and *RPPH1*. All genotyping assays were performed on the LightCycler 480 instrument (Roche Applied Science) using the LightCycler 480 Probes Master kit. Primer and probe sequences are listed in **Supplemental Digital Content 1** (see **Table**, http://links.lww.com/TDM/A102) and PCR conditions are given in **Supplemental Digital Content 2** (see **Table**, http://links.lww.com/TDM/A103). Assay validation included BLAST (Basic Local Alignment Search Tool) homology searches, SYBR Green I (Roche) melt curve analysis, gel electrophoresis, and Sanger sequencing of samples with different genotypes.

Outcome

Regimen-related toxicity was evaluated using body weight gain, total serum bilirubin, and serum creatinine within 30 days posttransplant. Furthermore, discontinuation of MTX at day 6 or 11 was considered a surrogate marker indicating mucositis or organ toxicity. Neutrophil engraftment was defined as the first of 3 consecutive days with absolute neutrophil cell counts $\geq 0.2 \times 10^9$/L. The aGVHD was graded according to standard criteria, and chronic GVHD was defined as limited or extensive.[22–24] The mortality rate was determined at 30 days, 3 months, and 6 months posttransplant.

Statistics

Statistical analyses were performed using SPSS v.18.0 (SPSS Inc, Chicago, IL). Comparisons between groups were performed using the χ^2 test, Fisher exact test, or Mann–Whitney *U* test. Wilcoxon rank sum test was used for comparisons within groups. Binary logistic regression analysis was used to identify predictors of Bu C_{ss} ≥75th percentile and mortality ≤30 days and ≤3 months posttransplant. Linear relationships between continuous variables were characterized using Pearson R correlation test. Comparisons between groups were performed using χ^2 test or Fisher exact test for

categorical variables and Mann–Whitney *U* test for continuous variables. The Wilcoxon rank sum test was used for comparisons within groups. Binary logistic regression analysis was used to identify pretransplant predictors of Bu C_{ss} ≥75th percentile and mortality ≤30 days and ≤3 months posttransplant. Candidate variables for prediction of C_{ss} ≥75th percentile included demographic data, diagnosis, disease status, and *GST* genotypes. Analysis of predictive factors of mortality additionally included Bu PK and HSCT variables.

Results are presented as median (range), numbers and percentages, or odds ratios (ORs) with 95% confidence intervals (CIs). All statistical tests were 2 sided, and $P < 0.05$ was considered statistical significant.

RESULTS

Patient characteristics are presented in Table 1. All patients received conditioning therapy with oral Bu followed by IV Cy. One patient additionally received total body irradiation.

GST Genotypes

The *GST* genotype frequencies are presented in Table 2. All investigated variants were relatively common (>10%), with frequencies as expected in white populations (dbSNP).[7,13,25] *GSTA1* genotype frequencies deviated from the Hardy–Weinberg equilibrium, presenting a higher than expected proportion of heterozygotes ($P < 0.05$).

TABLE 1. Patient Characteristics

Parameter	Patients, n (%) or Median (Range)
Male/female	65 (57)/49 (43)
Age (yrs)	47 (16–65)
Age <18 yrs	3 (2.6)
Actual body weight (kg)	74 (46–120)
Diagnosis and disease status	
Acute myeloid leukemia	73 (64.0)
First CR/second CR	43/24
Relapse	6
Acute lymphoblastic leukemia	13 (11.4)
First CR/second CR/later CR	5/5/2
Unknown	1
Myelodysplastic syndrome	13 (11.4)
Chronic myeloid leukemia	7 (6.1)
First CP	2
Second or later CP	5
Myeloproliferative disease	6 (5.3)
Myelofibrosis	2 (1.8)
Source of donor hematopoietic stem cells	
Related donor	35 (30.7)
Matched unrelated donor	79 (69.3)
Bone marrow	17
Peripheral stem cells	96
Combination	1
Number of transplant	
First/second	113/1

CR, complete remission; CP, chronic phase.

TABLE 2. *GST* Genotype Frequencies

Gene	Genotype	Patients, n (%)
GSTA1	C/C	45 (39.5)*
c.-69C>T	C/T	40 (35.1)*
(C=*A and T=*B)	T/T	29 (25.4)*
GSTP1	A/A	39 (34.2)
c.313A>G	A/G	62 (54.4)
	A/A	13 (11.4)
GSTM1	n = 0	50 (43.9)
Gene copy number	n = 1	52 (45.6)
	n = 2	12 (10.5)
GSTT1	n = 0	18 (15.8)
Gene copy number	n = 1	52 (45.6)
	n = 2	44 (38.6)

*Deviation from Hardy–Weinberg equilibrium, χ^2 test $P < 0.01$.

Busulfan Pharmacokinetics

Bu PK parameters are listed in Table 3, demonstrating large interindividual variability with up to 3.7-fold differences in dose-adjusted Bu C_{ss}. Following the first dose, 36% of the patients obtained target C_{ss} of 900 mcg/L ± 10%, whereas 50% of the patients were exposed to higher levels. After 2 days of dose adjustments, Bu C_{ss} was reduced from median 1000 to 890 mcg/L ($P < 0.001$, Table 3) and 71% of the patients obtained drug exposure of 900 mcg/L ± 10%. The individual average exposure of the first, fifth, and ninth doses was 930 mcg/L (690–1190 mcg/L). CL/F increased during conditioning therapy and was 0.09 and 0.13 $mL \cdot min^{-1} \cdot kg^{-1}$ higher after the fifth and ninth dose versus the first dose ($P < 0.001$).

Predictors of Busulfan Exposure

Bu C_{ss} differed between *GSTA1* genotype groups. The C_{ss} after the first dose was 12% and 16% higher among patients with 1 and 2 *GSTA1*B* alleles, respectively ($P < 0.01$, Fig. 1A). Similar, dose-normalized C_{ss} after the fifth dose was 15% and 22% higher, and CL/F was 11% and 18% lower, among carriers of 1 and 2 *GSTA1*B* alleles. Following 2 days of dose adjustments, *GSTA1*A/*B* and **B/*B* patients received 8% and 14% lower Bu doses ($P < 0.034$, Fig. 1A), respectively, whereas there was no significant difference in C_{ss} between genotype groups (Fig. 1B). Nevertheless, despite dose adjustments, the average Bu C_{ss} of the 3 dosing intervals remained higher among carriers of 1 (4%, $P = 0.038$) and 2 *GSTA1*B* alleles (6%, $P = 0.006$). The distribution of gender and body weight was similar across *GSTA1* genotypes.

There was no association between Bu PK and individual *GSTM1, GSTT1*, or *GSTP1* genotypes. However, within the *GSTA1*B/*B* group (n = 29), patients with the *GSTM1* null genotype (n = 14) tended to have higher C_{ss} than patients with 1 or 2 *GSTM1* alleles (1195 versus 1010 mcg/L, $P = 0.172$). Furthermore, patients with a combined *GSTA1*B/*B*,

TABLE 3. Oral Bu PK in Dosing Intervals

Bu PK Parameter	First Dose, Median (Range)	Fifth Dose, Median (Range)	*P**	Ninth Dose, Median (Range)	*P**
Dose (mg/kg)	1.0 (0.80–1.1)	0.91 (0.5–1.5)	<0.001	0.92 (0.40–1.5)	<0.05
C_{ss} (mcg/L)	1000 (600–1780)	900 (450–1460)	<0.001	890 (660–1530)	<0.001
C_{max} (mcg/L)	1630 (659–2653)	1705 (709–2920)	0.057	1670 (798–2903)	0.301
CL/F ($mL \cdot min^{-1} \cdot kg^{-1}$)	2.76 (1.33–5.03)	2.74 (1.34–5.95)	<0.05†	2.86 (1.63–4.88)	<0.001†

*Difference from PK parameter at first Bu dose.
†Based on individual differences: see Statistics.
C_{max}, maximum concentration.

GSTM1 null, and *GSTT1* null genotype presented the highest Bu exposure of all genotype groups (Fig. 1C), with C_{ss} of 1670 mcg/L (1180–1740 mcg/L, n = 3) versus 1045 mcg/L (600–1540 mcg/L, n = 26) among *GSTA1*B/*B* patients with expression of GSTM1 and GSTT1 ($P = 0.022$).

Seven patients received Bu starting doses <0.9 mg/kg and were excluded from the analysis of predictors of high first dose Bu exposure. Binary logistic regression analysis identified *GSTA1* genotype as the only significant predictor of C_{ss} ≥75th percentile, where the *GSTA1*B/*B* and

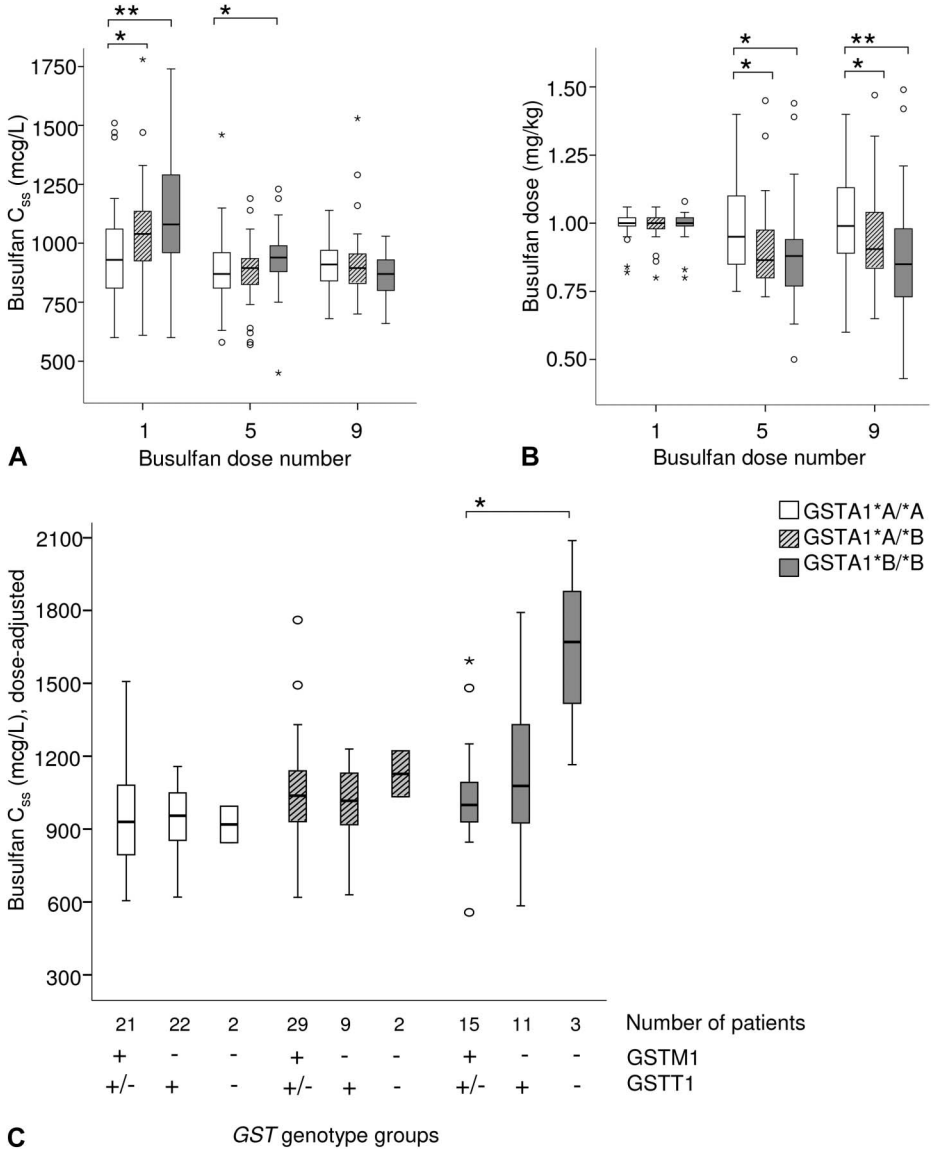

FIGURE 1. Estimated Bu average C_{ss} (A) and administered Bu doses (B) (in mg/kg) at the first, fifth, and ninth dosing interval among patients with *GSTA1*A/*A*, **A/*B*, and **B/*B* haplotypes. (C), Average Bu C_{ss} (dose-adjusted) after the first Bu dose are presented according to combined *GSTA1*, *GSTM1*, and *GSTT1* genotype. "+" indicates expression (1 or 2 gene copies) and "−" indicates no expression of the *GSTM1* or *GSTT1* gene.*$P <$ 0.05 and **$P < 0.01$, Mann–Whitney *U* test.

*GSTA1*A/*B* genotypes resulted in ORs of 4.072 (95% CI = 1.175–14.112, $P = 0.027$) and 3.539 (95% CI = 1.060–11.816, $P = 0.040$), respectively. No significant associations were observed between Bu exposure and age, diagnosis, or disease status at transplant.

Clinical Outcome

Clinical outcome parameters were evaluated >6 months after inclusion of the last patient, and these results are summarized in Table 4. The median follow-up time was 467 (9–1614) days, with an overall survival of 58%. The predominant cause of early mortality (≤3 months) was multi-organ failure, occurring in 89% (8/9) and 74% (17/23) of cases within 30 days and 3 months posttransplant, respectively. The major causes of death after 3 months were relapse (60%) and GVHD (20%; Table 5).

Mortality within the first 3 months was associated with higher levels of toxicity markers (Table 5). The highest levels were observed among patients dying within the first 30 days (n = 9), presenting higher maximum bilirubin (174 versus 64 μmol/L, $P < 0.01$), creatinine (260 versus 107 μmol/L, $P < 0.01$), gain of body weight (18% versus 5%, $P < 0.01$), and more frequent omission of MTX on day 6 (56% versus 7.6%, $P < 0.01$) than patients surviving 30 days post-HSCT.

Bu C_{ss} of the first dose was higher among patients who died within 3 months posttransplant compared with survivors (1090 versus 980 mcg/L, $P = 0.021$). A similar trend was observed considering mortality within 30 days [1090 (890–1740) versus 990 (600–1780) mcg/L, $P = 0.075$]. There was no difference in Bu exposure after the fifth and ninth doses. However, the individual average C_{ss} of the 3 dosing intervals was higher among patients who died within 30 days [970 (910–1190) versus 930 (690–1190) mcg/L, $P = 0.014$], but the difference did not reach statistical significance considering mortality at 3 months [950 (770–1190) versus 930 (690–1190) mcg/L, $P = 0.085$].

The mortality ≤30 days differed between *GSTM1* genotype groups ($P = 0.026$), as we observed 25% mortality among carriers of 2 *GSTM1* copies versus 2% among *GSTM1* null individuals ($P = 0.021$). Furthermore, discontinuation of MTX day 6 was more frequent among carriers of 1 or 2 *GSTM1* copies (17% versus 4%, $P = 0.037$), suggesting a higher level of early toxicity and mucositis in this group. Logistic regression analysis identified average Bu C_{ss} (OR = 1.009, 95% CI = 1.002–1.017, $P = 0.013$) and *GSTM1* gene copy number as significant predictors of mortality ≤30 days. Controlling for differences in C_{ss}, the presence of 2 *GSTM1* gene copies increased the risk of mortality ≤30 days by 17.1-fold (95% CI = 1.46–201, $P = 0.024$), whereas carriers of 1 *GSTM1* gene copy tended to have a higher risk of mortality versus *GSTM1* null individuals (OR = 5.1, 95% CI = 0.055–47, $P = 0.152$). In contrast, there was no significant association between *GSTM1* genotype and mortality after 3 months.

The most common causes of mortality >3 months were relapse and aGVHD, accounting for 60% and 20% of deaths, respectively (Table 5). There were no associations between relapse-related mortality and GST genotype ($P > 0.452$) or Bu exposure ($P > 0.752$). Furthermore, no significant associations were observed between *GST* genotype or Bu exposure and GVHD, engraftment, or infectious complications. Considering patients surviving >30 days, discontinuation of MTX on day 11 increased the risk of aGVHD grade III–IV (26% versus 8%, $P = 0.048$), which in turn was associated with an increased risk of mortality (80% versus 36%, $P < 0.01$).

TABLE 4. Clinical Outcome After HSCT

Outcome	Patients, n (%) or Median (Range)
Mortality	
30 d	9 (7.9)
3 mo	23 (20.2)
6 mo	33 (28.9)
Acute GVHD	
Grade I–II	26 (22.8)
Grade III–IV	19 (16.7)
Total	45 (39.5)
Chronic GVHD	
Limited	34 (29.8)
Extensive	17 (14.9)
Total	51 (44.7)
Mucositis	7 (6.1)
Transplant microangiopathy	14 (12.3)
Discontinuation methotrexate	
Day 6	13 (11.4)
Day 11	66 (57.9)
CMV reactivation/disease	34 (29.8)/1 (0.8)
Infectious complications	
Bacterial/fungal/viral/combination	29 (25.4)/7 (6.1)/5 (4.4)/2 (1.8)
Relapse	23 (19.8)
Time to relapse (d)	135 (41–844)
Time to engraftment (d) >0.2 neutrophils × 10^9/L	13 (9–19)*

*Seven patients died before engraftment.
CMV, cytomegalovirus; GVHD, graft-versus-host disease.

DISCUSSION

The large interindividual variability of Bu PK may partly be explained by *GST* gene variants, and analysis of these variants may provide a useful supplement to PK measurements to individualize Bu therapy. The present study investigated *GST* gene variants in relation to Bu PK and clinical outcome in a relatively large and homogenous population of 114 patients receiving BuCy conditioning pre-HSCT.

Similar to previous studies, Bu PK varied considerably between patients, with up to 3.7-fold differences in dose-adjusted C_{ss} after the first dose. The majority of patients were exposed to C_{ss} above the 900 mcg/L target, suggesting that the initial doses may be too high. Although TDM reduced Bu overexposure, the interindividual variability after 2 days of dose adjustments was still considerable, with C_{ss} ranging 660–1530 mcg/L (Table 3). Seizure prophylaxis with phenytoin has been reported to induce GST enzyme activity, and the observed increase in CL/F during Bu therapy is probably explained by an induction of GST enzymes and a reduction in bioavailability.[1]

TABLE 5. Clinical and Bu Exposure Parameters in Relation to Survival 3 Months After HSCT

Outcome	Mortality ≤3 mo (n = 23)	Alive >3 mo (n = 91)	*P*
Cause of mortality, n (% of deaths)			
Multi-organ failure	17 (73.9)	3 (12)	<0.001
Relapse	2 (8.7)	15 (60)	<0.001
GVHD	0 (0)	5 (20)	0.051
Bleeding	2 (8.7)	0 (0)	0.224
Other	2 (8.7)	2 (8)	—
Serum bilirubin (μmol/L), maximum ≤30 d	154 (8–480)	53 (8–220)	<0.001
Serum creatinine (μmol/L), maximum ≤30 d	159 (55–550)	102 (41–320)	<0.001
Gain of body weight (%), relative to baseline*	14 (0.0–31)	4.3 (0.0–23)	<0.001
Discontinuation methotrexate (% of patients)			
Day 6	39.1	4.4	<0.001
Day 11	95.7	48.4	<0.001
Busulfan C_{ss} (mcg/L)			
First dose	1090 (760–1740)	980 (600–1780)	0.021
Fifth dose†	900 (570–1060)	900 (450–1460)	0.876
Ninth dose†	900 (680–1530)	890 (660–1290)	0.742
Average first, fifth, and ninth dose	953 (773–1187)	927 (693–1187)	0.085

*Two missing values.
†C_{ss} after dose adjustments to obtain a target C_{ss} of 900 mcg/L.
GVHD, graft-versus-host disease.

The strongest predictor of Bu exposure was *GSTA1* genotype. Dose-adjusted C_{ss} was up to 15% and 22% higher, whereas CL/F was 11% and 18% lower, among patients with 1 and 2 *GSTA1*B* alleles, respectively. This corresponds to previous findings of 10%–40% lower oral and IV Bu clearance among *GSTA1*B* allele carriers.[8–10] In contrast, other studies have reported no association between *GST* genotype and Bu PK.[19] Abbasi et al[26] reported an association between oral Bu CL/F and *GSTA1* genotype but no significant differences in IV clearance between *GSTA1* genotype groups. The analyses were based on average clearance of 3 dosing intervals. Thus, changes in GSTA1 activity because of glutathione depletion or enzyme induction may have masked potential genotype effects on Bu metabolism after the first dose. Zwaveling et al[19] did not observe any effect of *GST* genotype on Bu PK in pediatric patients. This may be related to the relatively heterogeneous patient population, involving different dosing regimens and variability in age (0.2–23 years). Young children (1–3 years) have shown 77% higher oral Bu CL/F than older children and adults, probably related to an upregulation of *GSTA1* expression.[27] The present study investigated patients ≥16 years, and no significant associations between age and Bu PK were observed.

The accuracy of the *GSTA1* genotyping assay was confirmed by Sanger sequencing. This suggests that the observed deviation from the Hardy–Weinberg equilibrium (Table 2) most likely is related to the limited size of the study population or caused by processes like overdominant selection, outbreeding, or genetic drift.

In contrast to the *GSTA1* genotype effects, no significant associations were observed between individual *GSTM1*, *GSTT1*, or *GSTP1* genotypes and Bu PK. This is probably related to the minor impact of these enzymes on Bu metabolism.[26] Interestingly, within the *GSTA1*B/*B* genotype group, the combined *GSTM1* and *GSTT1* genotype was significantly associated with Bu exposure. The highest dose-adjusted Bu C_{ss} was observed among *GSTA1*B/*B* individuals with a combined *GSTM1* and *GSTT1* double-null genotype (Fig. 1C). Similar to our findings, Kim et al[5] reported lower Bu clearance and increased drug exposure among patients with a combined *GSTM1* null and *GSTT1* null genotype. Furthermore, Ansari et al[28] demonstrated an association between *GSTM1* copy number, but not *GSTA1* genotype, and IV Bu clearance in pediatric patients.

Conditioning therapy with BuCy has been associated with a considerable risk of treatment-related organ toxicity, particularly hepatic sinusoidal obstruction syndrome (SOS) and hepatorenal failure.[1] The most frequent cause of early mortality in the present study was multi-organ failure, accounting for 89% (n = 8) and 74% (n = 17) of cases of death within 30 days and 3 months posttransplant, respectively (Table 5). The early mortality was associated with significant gain of body weight and elevation of creatinine and bilirubin levels (Table 5), indicating hepatic and renal toxicity.

Both Cy and Bu are likely to cause organ toxicity. Regarding Bu, C_{ss} >1000 mcg/L has been associated with an increased risk of hepatic and neurological toxicity.[1] We observed that patients dying within 3 months presented significantly higher first dose exposure than patients surviving 3 months posttransplant (C_{ss} 1090 versus 980 mcg/L, P = 0.021). Despite dose adjustments and similar Bu exposure after the fifth and ninth doses between survival groups, the average drug exposure of the first, fifth, and ninth doses still tended to be higher among patients dying ≤3 months (median C_{ss} 953 versus 927 mcg/L, P = 0.085). Furthermore,

discontinuation of MTX on day 11 because of toxicity increased the risk of aGVHD grade III–IV (26% versus 8%, $P < 0.05$), which in turn increased the risk of mortality. Altogether, this indicates that high drug exposure, even after a single Bu dose, may be sufficient to increase the risk of organ toxicity and treatment-related mortality. However, low Bu exposure ($C_{ss} < 600$ mcg/L) increases the risk of graft failure and disease recurrence, discouraging a general reduction of the conditioning therapy.[1] The present study did not demonstrate any association between Bu PK and disease recurrence or graft failure. This may be related to the relative high Bu exposure and short follow-up of the study.

To date, reports of associations between *GST* genotype and outcome after HSCT are limited and findings are conflicting between studies. We observed that *GSTA1* genotype predicted Bu C_{ss} and that high Bu exposure was associated with an increased risk of toxicity and mortality. However, we did not observe any direct associations between *GSTA1* genotype and clinical outcome. Furthermore, Kim et al[29] reported a lower incidence of aGVHD, but not SOS, among patients with the *GSTA1*A/*A* haplotypes.

The increased risk of mortality following high Bu exposure may be related to Bu toxicity directly or indirectly by increasing the risk of Cy toxicity. Detoxification of Bu and reactive Cy metabolites both depend on GST enzymes and glutathione. Bu metabolism reduces intracellular glutathione levels, and this may in turn reduce the detoxification of cytotoxic Cy metabolites and thereby increase the risk of treatment-related mortality.[1] Animal studies have demonstrated that compounds causing SOS initially reduce intracellular glutathione in sinusoidal epithelial cells and that infusion of glutathione or the glutathione precursor N-acetyl-D-cystein reduces Bu toxicity and prevents SOS.[30] The importance of glutathione is further emphasized by clinical studies showing that shorter time intervals (7–15 hours) between Bu and Cy dosing, and dosing of Bu before Cy result in higher treatment-related toxicity compared with time intervals >24 hours and administration of Cy before Bu.[31–33]

Several studies have described associations between *GSTM1* genotype and drug response or toxicity. Patients with homozygous deletion of the *GSTM1* gene have shown reduced hepatic GST protein expression and an increased risk of SOS after BuCy conditioning. However, the association does not seem to involve reduced Bu clearance, and the mechanism remains to be elucidated.[15] Furthermore, the combined *GSTM1* and *GSTT1* null genotype has been identified as a significant predictor of drug-induced liver injury.[17] In contrast to these findings, the *GSTM1* null genotype has also been reported to reduce the risk of mortality. A study of 373 adult patients demonstrated higher treatment-related mortality among GSTM1 positive patients after treatment with different conditioning regimens.[18] This is similar to our findings of 25% versus 2% mortality ≤30 days among carriers of 2 versus no *GSTM1* gene copies, respectively ($P = 0.021$). The increased risk of treatment-related mortality among *GSTM1* carriers may be related to reactive intermediates or toxic metabolites generated by the GSTM1 enzyme.[18] Alternatively, expression of the GSTM1 enzyme may increase glutathione consumption during Bu metabolism and oxidative stress, which in turn increases the risk of Cy toxicity.

The variable findings between studies may be related to differences in study populations, sample size, investigated GST enzymes and gene variants, assays, oral versus IV Bu, concomitant medication, primary disease, and risk factors for liver toxicity. The results of the present study are limited by the retrospective study design, low number of subjects within combined genotype groups, short follow-up, and unknown Bu bioavailability. Furthermore, the mechanisms of the observed treatment-related toxicity should be characterized by measurement of glutathione and Cy metabolite concentrations.

CONCLUSIONS

The results support the use of *GST* genotyping as a supplementary tool to further optimize BuCy conditioning. Analysis of *GSTA1* gene variants, or preferably *GST* genotype combinations, may allow better prediction of Bu dose requirements before drug administration and thereby reduce Bu overexposure or underexposure, reduce the need for intensive PK monitoring, and finally improve clinical outcome. Furthermore, knowledge of *GSTM1* copy numbers may help identify patients at particular risk of treatment-related toxicity, who might benefit from alternative conditioning regimens. However, the value of *GST* genotyping needs to be determined in larger and prospective studies.

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