Using Novel Precision Medicine Tools to Identify Variability in Hematopoietic Cell Transplantation

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Lay-out & printing: ProefschriftMaken || www.proefschriftmaken.nl

ISBN 978-94-6423-868-6

Using Novel Precision Medicine Tools to Identify Variability in Hematopoietic Cell Transplantation

Nieuwe methoden gebruiken om variabiliteit in allogene hematopoëtische stamceltransplantatie te identificeren

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
op dinsdag 4 oktober 2022 des middags te 2.15 uur

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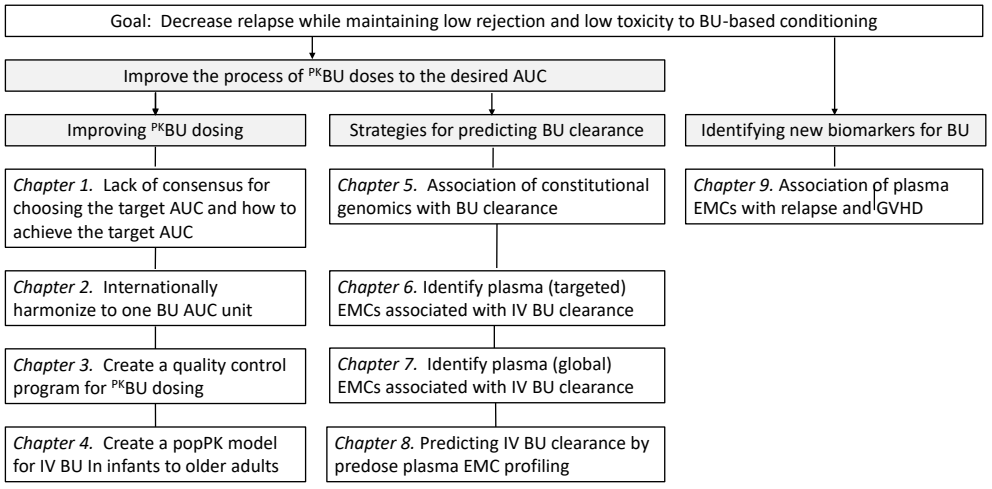
PREFACE

Cancer is a leading cause of death, accounting for one in six deaths worldwide.[1] Patients with cancer can be treated with one or a combination of the four pillars of cancer treatment: surgery, radiation, chemotherapy, and immunotherapy.[2] The alkylating agents were among the first anti-cancer drugs.[3] The earliest alkylating agents – busulfan, chlorambucil, cyclophosphamide – became available in the 1950s.[4] These alkylating agents are amongst the most commonly used chemotherapy drugs today.[3] Alkylating agents act directly on DNA, causing cross-linking of DNA strands, abnormal base pairing, or DNA strand breaks, preventing the cell from dividing. Generally, alkylating agents are considered cell cycle phase nonspecific (i.e., they kill the cell in various and multiple phases of the cell cycle).

Busulfan is a bi-functional alkylating agent that has been used since the 1950s (Table 1).[5] Over the past 70 years, its use has evolved from low doses of daily busulfan used to treat patients with chronic myeloid leukemia to, at present, high doses of short-4-day courses of busulfan used to condition patients before hematopoietic cell transplant (HCT). Busulfan was initially used in low doses (2 to 6 mg/day orally) to treat patients with chronic myelogenous leukemia (CML) because of busulfan's cytotoxicity to hematopoietic precursors and pluripotent stem cells.[6] Short courses of high-dose busulfan (e.g., 3.2 mg/kg/day intravenously (IV) for four days, termed busulfan hereafter) is a significant component of chemotherapy-based conditioning before HCT. Busulfan is currently used in many regimens for allogeneic HCT but to a much lesser extent when conditioning for autologous HCT.[7] Busulfan has a narrow therapeutic index, with small changes in the dose being associated with increased toxicity (historically liver toxicity) or decreased efficacy (rejection of the allograft or relapse of the underlying malignancy).[6] A specific busulfan area under the plasma concentration-time curve (AUC) has been associated with important clinical outcomes in HCT patients.[8] Thus, busulfan doses are often personalized to a specific plasma AUC using the individual patient's clearance.[9] This process was traditionally termed therapeutic drug monitoring (TDM), but more recently is referred to as targeted busulfan (^{T}BU) or pharmacokinetic (PK)-guided busulfan (^{PK}busulfan).

Over the past 30 years since the first publication showing the benefit of ^{PK}busulfan dosing,[10] the use of ^{PK}busulfan has increased. In addition, most allogeneic HCT patients have their busulfan doses personalized to a target AUC.[9] However, challenges have emerged over that long-time span with ^{PK}busulfan, which we sought to overcome with the long-range goal of decreasing relapse while maintaining low rejection and toxicity to busulfan-based conditioning regimens in allogeneic HCT. Broadly, the two challenges are 1. to improve the process of ^{PK}busulfan to the desired AUC and 2. to identify new biomarkers for busulfan (Figure 1).



Abbreviations: AUC – area under the plasma concentration-time curve; BU – busulfan; EMC – endogenous metabolomic compounds; GVHD – graft-versus-host-disease; IV – intravenous; popPK – population pharmacokinetic; PKBU – pharmacokinetic-directed busulfan dosing, also referred to as targeted BU (TBU)

Figure 1. Graphical summary of the thesis

Focusing on the first challenge, the process of PKbusulfan has minimally changed over the past 30 years. The resource- and time-intensive process is: 1. choosing the target AUC specific for that patient, their conditioning regimen, and their underlying disease; 2. administration of a busulfan dose based on weight or body surface area, 3. followed by intensive pharmacokinetic sampling, 4. quantitation of busulfan concentrations, 5. pharmacokinetic modeling of the resulting concentration-time data to estimate the patient-specific busulfan clearance, and 6. then using that clearance to personalize the dose to achieve the target AUC. The first four chapters focus on improving the process for PKbusulfan. **Chapter 1** describes how an international group of clinical pharmacologists and allogeneic HCT physicians could not reach a consensus for choosing the target busulfan AUC and how (i.e., the process) to achieve the target busulfan AUC. **Chapter 2** described how clinicians used various busulfan plasma exposure units (BPEU) to characterize busulfan AUC. This variety in BPEU is a potential source for misinterpretation of publications and protocols and is a barrier to data capture by HCT registry databases. We then conducted a Delphi process to choose one unit to express busulfan AUC, which will enable its inclusion in registry databases and hopefully large-scale studies of the association of busulfan AUC with clinical outcomes. **Chapter 3** used the unique resources within the Netherlands of the KKGT, specifically to conduct international proficiency exercises in busulfan quantitation, pharmacokinetic modeling, and dose recommendations. The lessons learned from Chapters 1 to 3 can provide insight into the process of PKbusulfan, which remains essentially unchanged. **Chapter 4** then describes how a population pharmacokinetic (popPK) model, created over the entire age continuum, can be used to estimate the initial busulfan dose (i.e. the "right-dose-first-time" paradigm[11]) and be used to enable model informed precision dosing of busulfan (MIPDbusulfan).

The second series of chapters seek to identify novel precision medicine tools (PMT) to estimate busulfan clearance. Although [PK]busulfan is feasible, the short 4-day duration of busulfan dosing makes it challenging to estimate the busulfan clearance quickly enough to personalize the dose in those HCT centers without an on-site method to quantitate concentrations. Clinically, using a test dose – i.e., before HCT conditioning starts, administering a low busulfan dose to estimate the busulfan clearance. However, its success has been limited. In **Chapter 5**, we used the candidate gene approach to evaluate whether busulfan clearance was associated with polymorphisms in the genes regulating the predominant metabolizing enzymes involved in busulfan conjugation, specifically glutathione S-transferase (GST) isoenzymes A1 (GSTA1) and M1 (GSTM1). We then evaluated the association of IV busulfan clearance with endogenous metabolomic compounds (EMCs), which reflect the influence of the patient's genotype and environmental factors (e.g., nutritional status).[12] Metabolomics is the profiling of a broad range of EMCs present in biological fluids.[12-18] Clayton et al. introduced the concept of personalized drug treatment using pre-dose EMC profiling to predict drug response in individual subjects, which the authors termed "pharmacometabonomics."[12,17] We evaluated if pre-busulfan EMC profiling can predict IV busulfan clearance using a targeted (**Chapter 7**) or untargeted (**Chapter 6 and 8**) approach.

In allogeneic HCT, grafting hematopoietic stem cells from one individual to another provokes immunologic reactions involved in engraftment of the donor cells, graft-versus-host disease (GVHD), control of a malignancy (relapse), the development of tolerance, and immune reconstitution.[19] These immunologic reactions are influenced by the conditioning regimen, the type and source of the donor graft, and the post-transplant immunosuppressive regimen, all of which are essential components of the HCT procedure. Busulfan-based conditioning is the most commonly used high-dose conditioning regimen for allogeneic HCT. Busulfan is hepatically metabolized through glutathione (GSH) conjugation by glutathione S-transferase (GST) enzymes; this process depletes hepatocyte GSH stores in murine hepatocytes in vitro.[20] Dysregulation of GSH and accumulation of cysteine, cystathionine, and cysteinylglycine are associated with GVHD in experimental murine models of HCT.[21] Relapse is reduced by PK busulfan;[5] however, relapse is a persistent challenge in allogeneic HCT patients. Thus, in **Chapter 9**, we used a global pharmacometabonomics approach to identify novel biomarkers of relapse or acute GVHD in 84 patients receiving [PK]busulfan before allogeneic HCT.

Table 1. Thesis chapters in the historical context of busulfan (BU) use

Year	Clinical use/ pharmacodynamics	Pharmacokinetics/ ^{PK}BU Dosing	BU Dose	
			Oral	IV
1954	FDA approves PO BU to treat CML[22]		2 mg Q24H	
1974–77	BU/CY in preclinical alloHCT models[23-25]			
1983	BU/CY in alloHCT patients[26]		1 mg/kg Q6H	
1989	↑AUC ↑hepatotoxicity – BU/CY adults[10]	^{PK}BU lowers hepatotoxicity[27]		
1995	↓AUC ↑rejection – BU/CY pediatrics[28]	^{PK}BU increases engraftment rates[29]		
	↓AUC ↑ relapse – BU/CY CML[30]	^{PK}BU lowers relapse rates[31]		
		Primary elimination route is glutathione conjugation[32-34]		
1999		Age-dependent metabolism of PO BU;[32] presumably due to upregulation of GST activity in enterocytes of children < 4 years[35]		
1999, 2003	Approval of IV BU in BU/CY by FDA & EMA, respectively			0.8 mg/kg Q6H
2007		IV BU clearance is similar between Q6H and Q24H BU dosing		3.2 mg/kg Q24H
2006–2022		Pharmacogenomic study of *GST* polymorphisms with oral or IV BU clearance[36,37] **Chapter 5[38]**		
2014		PopPK model for IV BU in infants to older adults. BU clearance, scaled to normal fat mass, is predicted to be 95% of the adult clearance at 2.5 years postnatal age. **Chapter 4[39]**		
2016	ASTCT could not write an evidence-based review about ^{PK}BU because published literature is of insufficient quality. **Chapter 1[5]**			
2016–2022		Identifying EMCs and pathways associated with IV BU clearance **Chapters 6[40], 7[41], 8**		
2019		Harmonize worldwide to one BU AUC unit (i.e., mg × h/L) **Chapter 2[42]**		
2021	Identifying EMC novel pathways associated with relapse or acute GVHD in ^{PK}BU patients. **Chapter 9[43]**	With KKGT, created an interlaboratory quality control program for ^{PK}BU dosing. **Chapter 3[44]**		

Abbreviations: alloHCT: allogeneic hematopoietic cell transplant; ASTCT: American Society of Transplantation and Cellular Therapy (formerly ASBMT or American Society for Blood and Marrow Transplantation); AUC: area under the plasma concentration-time curve; BU: busulfan; ^{PK}BU: pharmacokinetic-guided busulfan dosing to a target AUC using an individual's busulfan pharmacokinetic parameters; BU/CY: busulfan followed by cyclophosphamide; CML: chronic myeloid leukemia; CY: cyclophosphamide; EMA: European Medicines Association; EMC: endogenous metabolomic compounds; FDA: Food & Drug Administration; FLU: fludarabine; GST: glutathione S-transferase; GVHD: graft versus host disease; IV: intravenous; KKGT: Dutch organization - Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology; popPK: population pharmacokinetics; Q6H: every 6 hours; Q24H: every 24 hour administration; THT+: gamma -glutamyl-beta -(S-tetrahydrothiophenium)-alanyl-glycine; ^{T}BU targeted busulfan dosing, same as ^{PK}BU

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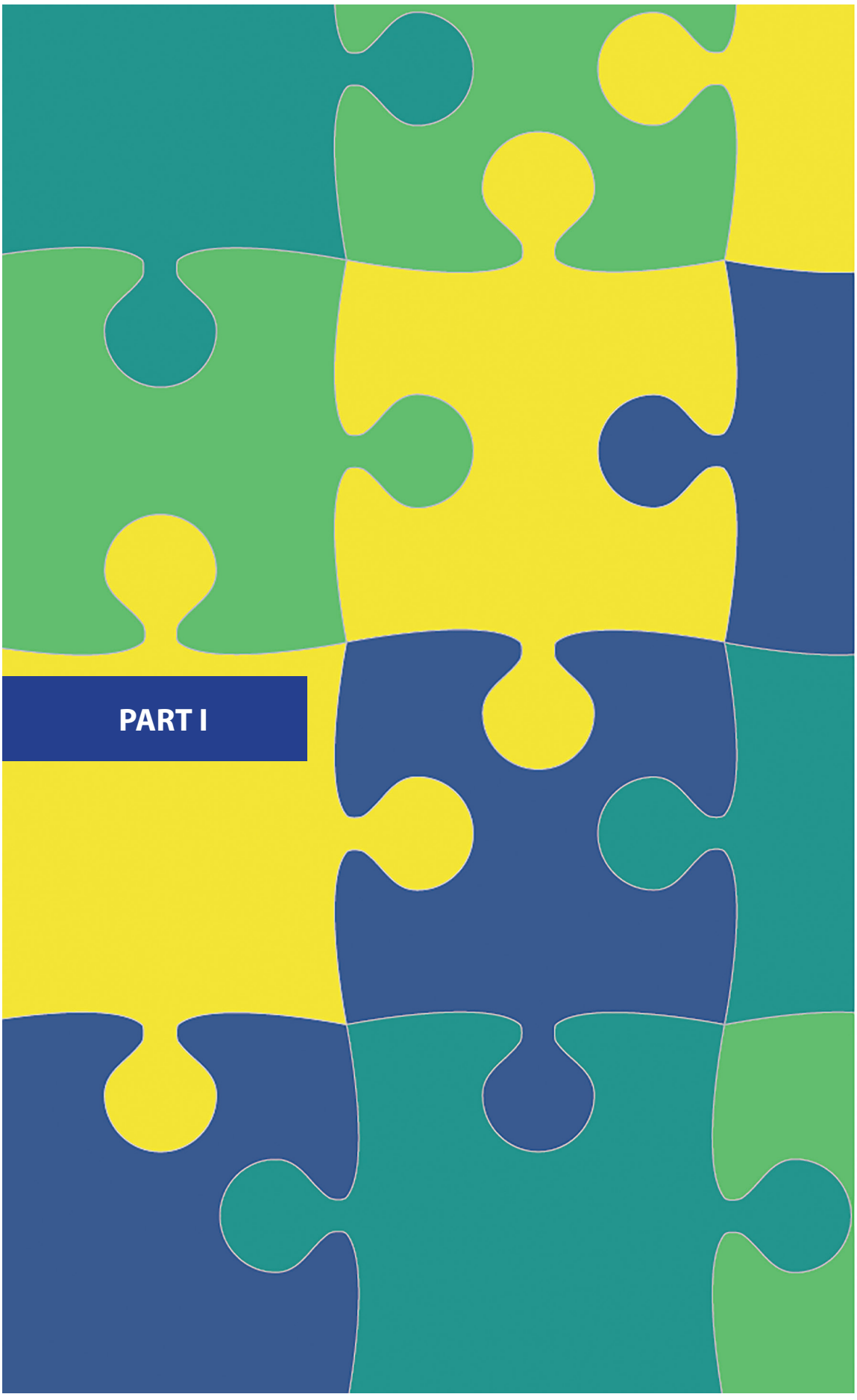
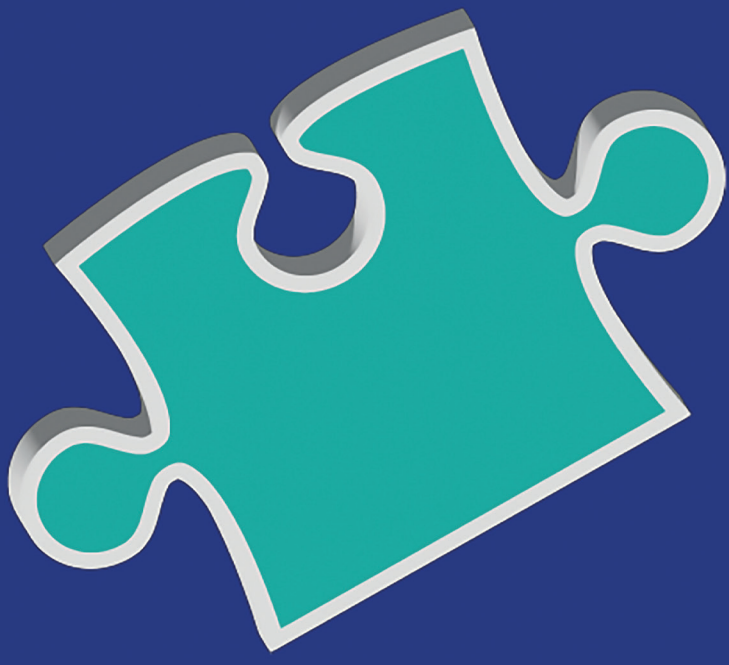

PART I

Improving Pharmacokinetic-guided Dosing of Busulfan



CHAPTER 1

Personalizing Busulfan-based Conditioning: Considerations from the American Society for Blood and Marrow Transplantation Practice Guidelines Committee

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Biol Blood Marrow Transplant. 2016 Nov;22(11):1915-1925

ABSTRACT

The Practice Guidelines Committee of the American Society of Blood or Marrow Transplantation (ASBMT) sought to develop an evidence-based review about personalizing busulfan-based conditioning. The Committee sought to grade the relevant published studies (June 1, 2008 through March 31, 2016) according to criteria set forth by the Steering Committee for Evidence Based Reviews from ASBMT. Unfortunately, the published literature was too heterogeneous and lacked adequately powered and sufficiently controlled studies for this to be feasible. Despite this observation, the continued interest in this topic led the Practice Guidelines Committee to develop a list of most frequently asked questions (FAQs) regarding personalized busulfan dosing. This "Considerations" document is a list of these FAQs and their responses, addressing topics of practical relevance to hematopoietic cell transplantation clinicians.

Abstract

INTRODUCTION

The bifunctional alkylating agent, busulfan (BU) has been used for approximately 40 years as a major component of chemotherapy-based conditioning before hematopoietic cell transplant (HCT). High-dose BU is currently still used in many regimens for allogeneic HCT but to a much lesser extent when conditioning for autologous HCT. Historically, low-dose BU was used (2-6 mg orally daily) to treat myeloproliferative neoplasms like chronic myelogenous leukemia (CML), polycythemia vera[1,2] and essential thrombocytosis[3] because BU is cytotoxic to hematopoietic precursors and pluripotent stem cells. Nowadays, high-dose BU-based conditioning is frequently used but, because BU causes limited lymphotoxicity, it is unable to provide adequate immunosuppression as standalone conditioning. As a result, BU-induced myelotoxicity has been complemented by adding lymphotoxic agents (e.g., cyclophosphamide or fludarabine) and sometimes also with agents that have additional activity against the tumor (e.g. thiotepa, melphalan or clofarabine). Seminal studies led by George Santos and Peter Tutschka[4-6] demonstrated that high-dose BU plus cyclophosphamide (i.e., BU/CY) was effective conditioning[6-8] for allogeneic HCT. However, sinusoidal obstruction syndrome (SOS) was quickly understood to be a dose-limiting toxicity.[9] This observation provided an early hint of the narrow therapeutic index of BU and subsequent data leading to the development of personalized BU dosing with the goal of improving patient outcomes.

Personalized dosing of BU using the patient-specific BU clearance, referred to as BU TDM hereafter, is conducted by personalizing the BU dose to a target exposure based on TDM. Target exposure is reflected in the measurement called AUC (area under the plasma concentration-time curve) or Css (concentration at steady state). The Css is simply the AUC divided by dose frequency. The Practice Guidelines Committee of the American Society of Blood or Marrow Transplantation (ASBMT) sought to develop an evidence-based review of this complex topic but found that the published literature was too heterogeneous and lacked the necessarily controlled studies for this to be feasible. This conclusion was reached after a comprehensive review of articles about the association of BU exposure with clinical outcomes, termed pharmacodynamic associations hereafter. Data published between June 1, 2008 and March 31, 2016 were reviewed, with earlier data included when deemed necessary. We searched the PubMed database using the terms busulfan and pharmacokinetic as well as topics relevant to each particular discussion section. Only finalized peer-reviewed publications were included for review. Initially, we sought to grade studies according to criteria set forth by the Steering Committee for Evidence-Based Reviews from ASBMT.[10] However, those criteria could not be used because of the heterogeneity of thepatient population, conditioning regimen, BU dosing and BU pharmacokinetic data from studies of typically fewer than 100 patients. As a result, the purpose of this manuscript is to present and then answer frequently asked questions (FAQs, see Table 1) regarding personalized BU dosing; the answers try to take into consideration what is most practically relevant for offering guidance to HCT clinicians.

Table 1. Frequently asked questions (FAQs).

FAQs	Summary of Answers
FAQ1. Why does personalized busulfan (BU) dosing need to be considered during hematopoietic cell transplantation (HCT)?	Personalized BU dosing is considered mainly because BU has a narrow therapeutic index and a specific BU exposure have been associated with important clinical outcomes in in HCT patients. Therefore, personalized BU dosing via therapeutic drug monitoring (TDM) needs to be considered to minimize sinusoidal obstruction syndrome, lower graft rejection rates, and lower relapse rates in certain situations.
FAQ2. Is personalized BU dosing always necessary?	No. BU TDM is currently considered to be unnecessary for reduced intensity conditioning (RIC) regimens where the balance of BU toxicity to BU efficacy is favorable. With RIC, data is needed to determine if lower BU doses or lower BU exposure compromise efficacy.
FAQ3. When should conditioning utilize BU TDM?	The first consideration to use BU TDM is when the specific BU exposure is associated with clinical outcome(s) in a homogenous patient population. BU TDM must be used in children receiving high-dose BU before allogeneic HCT to lower the risk of graft rejection. Another significant consideration for personalizing BU is when the regimen was developed with BU TDM.
FAQ4. Is oral or IV BU preferred?	Intravenous (IV) busulfan tends to be preferred on the basis of patient convenience and concerns about intrapatient pharmacokinetic variability because of unpredictable gastrointestinal absorption of oral BU and hepatic first-pass effects.
FAQ5. How should personalized BU dosing be achieved?	Personalized BU dosing should be achieved by using TDM after selecting and administering the initial dose of high-dose BU.
FAQ6. How is the initial BU dose best selected?	The initial IV BU dose should be based on the European Medicines Agency (EMA) nomogram for children with a target area under the curve (AUC) of 1125 μmolar×minute. For adults with the same target AUC, the initial IV BU dose should be 0.8 mg/kg every 6 hours or 3.2 mg/kg every 24 hours. The initial IV BU dose may need adjustment for lower or higher target AUC. Oral BU dosing always begins at 1 mg/kg.
FAQ7. What is the optimal dosing frequency of BU?	The available IV BU data for adults do not suggest a significant difference in outcomes between Q6H and daily dosing, likely because BU clearance, volume of distribution and half-life appear to be similar regardless of dosing frequency. In children relevant studies are ongoing. Oral BU should be administered Q6H.
FAQ8. What is the best method for predicting BU clearance?	BU clearance is calculated based on the administered BU dose and an estimate of post-dose BU exposure using validated pharmacokinetic modeling tools (see Technical Appendix). Test dose strategies are not currently recommended.
FAQ9. How do other medications affect BU pharmacokinetics?	Ideally, there would be no changes to medications given concomitantly with BU in order to minimize any drug-drug interactions that alter BU pharmacokinetics. The following medications have affected IV BU clearance: fludarabine, deferasirox, metronidazole; or oral BU clearance: fludarabine, metronidazole, ketobemidone, and itraconazole. Phenytoin affects oral BU clearance but its effect upon IV BU clearance is unclear. By extrapolation, voriconazole or posaconazole would likely decrease BU clearance and should be avoided during conditioning.
FAQ10. Should the initial BU dose be personalized based on genetic polymorphisms?	Pharmacogenomics-based dosing of BU, either IV or oral, is not recommended.

FAQ1. Why does personalized busulfan (BU) dosing need to be considered during hematopoietic cell transplantation (HCT)? Answer: *Personalized BU dosing is considered mainly because BU has a narrow therapeutic index and a specific BU exposure has been associated with important clinical outcomes in HCT patients. Therefore, personalized BU dosing via therapeutic drug monitoring (TDM) needs to be considered to minimize sinusoidal obstruction syndrome, lower graft rejection rates, and lower relapse rates in certain situations.*

In the original BU/cyclophosphamide (BU/CY) conditioning regimen, oral BU dosing was based on body weight (mg/kg). Shortly after that, Grochow et al reported that higher BU exposure is associated with more frequent hepatotoxicity in adults conditioned with BU/CY.[11] Over the next decade, case series of 50 or fewer patients confirmed this association[12,13] and Slattery et al[13] was the first to report in children that low BU exposure during BU/CY conditioning was associated with more frequent graft rejection.[13,14] The results of subsequent BU/CY case series found that compared to historically controlled weight-based dosing, the use of BU TDM was associated with a reduction in hepatotoxicity rates from 75% to 18%[15] and improved engraftment rates from 74% to 96%.[16] Higher BU exposure was associated with lower relapse rates[17] and targeting higher exposure through BU TDM did lower relapse rates[18] among patients conditioned with BU/CY with previously untreated chronic myeloid leukemia (CML) before the era of tyrosine kinase inhibitors. However, understanding the association of BU exposure with post-transplant relapse in children with acute myeloid leukemia (AML) has been difficult because of small sample sizes and heterogeneity of the AML population.[19,20] The difficulty with understanding the pharmacodynamic association presumably contributes to the variable target exposure chosen by clinicians for children with AML.[21] An association of BU exposure with GVHD was not found in BU/CY conditioned patients.[22-25] Thus, in the BU/CY regimen, BU TDM increases engraftment rates in children,[16] lowers hepatotoxicity rates in adults,[15] and lowers relapse rates in patients with previously untreated CML.[18] However, outside of these clinical situations, the benefit of BU TDM in BU/CY conditioned patients is less clear.

Furthermore, as explained in FAQ3, similar associations between BU exposure and outcomes were not found in patients receiving slightly different conditioning regimens.[26,27] Thus, there remains some controversy regarding the advantage of BU TDM for all conditioning regimens. It is unlikely that any randomized controlled trials will be conducted to understand the benefit of BU TDM. In the absence of such data, clinicians are left to consider whether the narrow therapeutic index of BU applies to their patient based on their conditioning regimen and whether pharmacodynamic associations might be relevant.

FAQ2. Is BU TDM always necessary? Answer: *No. BU TDM is currently considered unnecessary for reduced intensity conditioning (RIC) regimens where the balance of BU toxicity to BU efficacy is favorable. With RIC, data are needed to determine if lower BU doses or lower BU exposure compromise efficacy.*

BU TDM is only necessary for conditioning regimens that have a pharmacodynamic association of BU exposure with outcomes or, ideally, data showing that BU TDM improves outcomes, as explained in **FAQ1**. Notably, some studies have reported the unexpected

association that low BU exposure is associated with worse non-relapse mortality in the BU/FLU/thymoglobulin regimen.[28] In actuality, the usefulness of BU TDM in reduced intensity conditioning (BU < 9 mg/kg oral or intravenous [IV] equivalent) has not been systematically evaluated. There are also substantial logistical barriers to BU TDM with these regimens because BU is only administered for one or two days, necessitating on-site BU TDM. Therefore, it has not been feasible to identify a total BU dose that is unsafe when dosed based on body weight, or without TDM. A reduced intensity FLU/BU/ATG regimen has been successfully developed with BU TDM for infants with nonmalignant diseases. However, outside of this patient population, weight-based BU dosing without TDM has been predominantly used in reduced intensity conditioning.[29-32] In the interest of trying to determine whether BU dosing in the FLU/BU regimen had any effect on disease control, one group evaluated six different BU dose cohorts, ranging from 3.2 mg/kg to 12.8 mg/kg and found that the 11.2 mg/kg dose cohort, compared to all other predominantly lower dose cohorts, had improved OS and relapse-free survival. However, another group compared 3.2 to 6.4 mg/kg daily and found no difference in OS, DFS, GVHD or NRM. Only controlled trials will be able to answer adequately the question of whether there is the potential clinical benefit of BU TDM in reduced intensity conditioning.

FAQ3. When should conditioning utilize BU TDM? Answer: *The first consideration to use BU TDM is when the specific BU exposure is associated with clinical outcome(s) in a homogenous patient population. BU TDM must be used in children receiving high-dose BU before allogeneic HCT to lower the risk of graft rejection. Another significant consideration for personalizing BU is when the regimen was developed with BU TDM.*

The initial data showing that BU exposure was associated with clinical outcomes and was generated by BU TDM after orally administered BU (see **FAQ1)**. When Andersson et al[33] led the development of IV BU in the 1990s, it was hoped that improved interpatient variability in BU pharmacokinetics would obviate the need for BU TDM. Evidently this was not the case, as reflected by the product labeling for IV BU which clearly states: "Therapeutic drug monitoring and dose adjustment following the first dose of BUSULFEX is recommended" for pediatric HCT patients with CML conditioned with BU/CY.[34] The target IV BU exposure (AUC) is 1125 μmolar×minute with an acceptable range of 900-1350 ± 5% μmolar×minute after every 6 hour (Q6H) dosing (See **FAQ5** and Technical Appendix for further details).[34] Not surprisingly, the frequency of BU TDM increased shortly after the February 1999 FDA approval of IV BU (Supplemental Figure 1 of McCune et al[21]). Currently, BU TDM is considered only for high-dose conditioning (BU > 9 mg/kg oral or IV equivalent[35]) with pharmacodynamic associations or when the regimen was developed with BU TDM (e.g., BU/melphalan,[36] vorinostat/gemcitabine/BU/melphalan[37] or cyclophosphamide/BU[38]). Unfortunately, there cannot be one BU target exposure for all HCT conditioning regimens because each regimen was developed based on the maximum tolerated BU dose (or systemic exposure) within unique multicomponent regimens. This issue is compounded by the underlying disease type and risk for graft rejection (e.g. minimal pre-transplant therapy) which influences the optimal BU exposure. Ideally, the BU target exposure would be available for each high-dose regimen, but the published literature is often too heterogeneous with small case series. The section below describes confounding factors

and general considerations for choosing the target exposure, making recommendations for those regimens used most often.

Confounders

When choosing the target BU exposure to optimize clinical outcomes, one also needs to consider the impact of other conditioning regimen components and the baseline patient characteristics. The heterogeneity in the conditioning regimens and the baseline patient characteristics has confounded most retrospective outcomes analysis evaluating the association of BU exposure to clinical outcomes. Interpretation of GVHD analyses is particularly difficult because of age and GVHD practice variation across different centers.[39] The extent of HLA mismatch can also confound the risk for both graft rejection and GVHD. While rates of SOS after BU/CY can be minimized through personalized BU dosing,[16,18,40] there are alternative approaches to mitigating SOS. These approaches include replacing CY with fludarabine or administering the CY before BU as in the CY/BU regimen.[38] Delaying CY administration after BU might be beneficial based on data from a cohort of patients who received BU without phenytoin followed by CY at varying time intervals.[41] Specifically, a higher incidence of SOS was observed when the first CY dose was administered 7-15 hours versus 24-48 hours after the last BU dose.[41] In other studies BU exposure alone was not associated with SOS, but higher BU exposure was associated with SOS in patients receiving concomitant melphalan,[42] or in patients with neuroblastoma who were conditioned for autologous HCT with BU, melphalan and thiotepa.[43] Besides SOS, GVHD is a significant contributor to NRM in allogeneic HCT recipients. In general, busulfan exposure is not associated with GVHD although this association is confounded by the additional conditioning regimen components. Specifically, GVHD rates after BU/CY have not been influenced by BU exposure,[22-25] although two pediatric studies reported a higher incidence of GVHD when BU/CY was paired with MEL.[25,44]

General Considerations

In allogeneic HCT preceded by BU/CY conditioning, in general, the target BU concentration at steady state (Css, see **FAQ5** for further explanation) is 600-900 ng/mL when the underlying reason for HCT is hematologic malignancy other than previously untreated CML. More narrow target exposures (e.g., 800-900 ng/mL[38,45] or 900$\pm$100 ng/mL[46,47]) have been used in the BU/CY regimen. A large study by the Center for International Blood and Marrow Transplant Research (CIBMTR) showed that BU/CY conditioning is generally associated with superior outcomes compared with CY/total body irradiation (TBI) for first remission AML. Although a CIBMTR survey later found that 50-60% of reporting centers provide BU pharmacokinetic data, the AML study was unable to determine how BU TDM might have contributed to the reported outcomes.[48] Cautious interpretation is needed as these results based on retrospective analysis of registration data.

It should be recognized that when BU is combined with agents other than or in addition to CY (e.g., TBI, melphalan, or thiotepa), relationships between BU exposure and clinical outcomes are altered. This has been observed in BU/CY/TBI and in children receiving BU plus various alkylating agents (i.e., thiotepa alone, melphalan alone, CY/melphalan, CY/thiotepa). BU TDM should be conducted in children receiving BU-based conditioning for an allograft because personalizing doses reduces graft rejection (see **FAQ1**). In general, BU

TDM should be conducted in adults receiving BU/CY, fludarabine, BU, thymoglobulin±TBI, and any novel regimens developed using BU TDM (e.g., vorinostat/gemcitabine/busulfan/melphalan[49]). However, the use of BU TDM has varied with fludarabine/BU (FLU/BU),[50,51] clofarabine/BU,[32,52,53] and BU/cyclophosphamide/etoposide (BuCyE or BuCyVP) regimens mostly based on the magnitude of the busulfan dose.[54,55]

Of keen interest to the development of novel high-dose conditioning regimens is the replacement of CY with fludarabine (FLU), a purine nucleoside inhibitor that is potentially less toxic yet has similar immunosuppressive efficacy as CY.[30] Data from a recent Phase III trial in older patients with AML indicated that FLU/BU is associated with lower transplant-related mortality than BU/CY but retains anti-leukemic activity, suggesting FLU/BU should be the standard of care for such patients.[56] A large meta-analysis of 15 clinical trials including 1830 patients reported FLU/BU was associated with a lower risk for day 100 non-relapse mortality (NRM) at 100 days, no differences in all-cause mortality at 100 days, lower SOS and microbiologically documented infections compared to BU/CY.[30] Notably, engraftment kinetics, the risk of grade 3–4 mucositis, GvHD, relapse and NRM at the end of the study were all similar between FLU/BU and BU/CY. These findings led to the conclusion that both regimens have similar efficacy profiles, whereas toxicity is lower with FLU/BU regimen.[30] The replacement of CY with BU has allowed for higher BU target exposures without SOS. Keeping BU Css <900 ng/ml appears necessary for BU/CY conditioning,[13] but a BU Css of 800-1000 ng/ml is well tolerated in the FLU/BU regimen.[46] While centers developed the FLU/BU regimen with BU TDM, most others started with weight-based BU dosing which provided sufficient variability in the BU exposure to allow for discovery of associations between BU exposure and clinical outcomes.[46,50,57-60] The need to use BU TDM in the FLU/BU regimens is variable due to regimen permutations in the total BU dose, fludarabine dose, use of anti-thymocyte globulin, and/or type of post-grafting immunosuppression. When combined with a cumulative fludarabine dose of 120 mg/m^2, IV BU (dosed Q12H or daily) can be dosed based on body weight without TDM. Within this regimen, the cumulative BU doses range from 3.2[29] to 11.2[51] mg/kg in adults. With similar FLU/BU regimens, BU exposure is associated with outcomes. For adolescents through adults who received FLU/BU, a BU AUC >9,000 µmolar×minute/day led to SOS in all patients, whereas only two cases of SOS occurred among 69 patients with target AUC ≤ 7,500 µmolar×minute/day.[59] A study of FLU/BU/alemtuzumab demonstrated an increased risk of fatal SOS when maximum AUC exceeded 6800 µmolar×minute/day.[61] In the FLU/BU/ thymoglobulin±TBI regimen, a BU Css over 1026 ng/ml was associated with lower nonrelapse mortality (NRM), progression-free survival (PFS) and overall survival (OS).[28,62] Interestingly, Russell et al.,[28] found that for a heterogeneous study cohort of 158 patients who received FLU/BU/thymoglobulin±TBI, patients with BU exposure in the lowest quartile as well as the highest quartile had in increased risk of NRM. Specifically, amongst those patients with a BU Css <1026 ng/ml, the association of BU exposure with outcomes was evaluated over the four BU exposure quartiles. Those with a BU exposure of 759 to 854 ng/ml has the lowest risk of NRM, the lowest risk of acute GVHD, a better disease-free survival and better OS.[28] Engraftment did not differ between the BU exposure groups.[28] The data have been contradictory regarding the relationship between BU exposure and rates of GVHD; some studies showed higher rates of GVHD with low AUC,[28] yet others showed higher rates of GVHD with higher AUC.[42,63]

FAQ4. Is oral or intravenous BU preferred? Answer: *IV busulfan tends to be preferred on the basis of patient convenience and concerns about intrapatient pharmacokinetic variability because of unpredictable gastrointestinal absorption of oral BU and hepatic first-pass effects.*

The disadvantages of oral BU are the need for multiple tablets per dose (1 mg/kg dose with only 2 mg tablets available), delayed absorption that can confound BU TDM, the potential for emesis and the need for a standard practice around whether to replace oral BU doses after emesis, and greater intrapatient (between dose) pharmacokinetic variability. There is a debate regarding whether oral BU has greater interpatient (between patient) variability in BU clearance.

The effects of oral versus IV BU on efficacy, toxicity, and pharmacokinetics on the outcomes of allogeneic HCT have been analyzed retrospectively.[54,64-70] These studies are often confounded by: heterogeneous patient populations, the use of BU TDM for only one of the administration routes, differences in the other conditioning regimen components and BU exposure data not being available. Only those studies with the largest samples sizes – specifically the CIBMTR studies, either in allogeneic[64,69] or autologous HCT are described.[66] Differences between patients who had received IV versus oral BU were compared in the CIBMTR study in patients with AML in first remission transplanted following BU/CY conditioning and allogeneic HCT. Compared to patients receiving oral BU/CY, multivariate analysis found that patients who received IV BU had lower rates of relapse after one-year post transplant. As noted in **FAQ3**, whether patients in this study received BU TDM is unknown but a survey of centers done shortly after the study suggested 50-60% of the centers who reported to CIBMTR used BU TDM.[48] This analysis contrasts with results that have not noted any increased risk in relapse rates when comparing similar regimens that differed only by the route of BU administration.[68,70-72] The effect of the administration route on SOS has also not been consistent with some reports that found a significantly higher rate of SOS after oral BU when compared to IV BU,[64,65,67] while others found no major differences.[68,70-72] Additionally, there have been no reported differences in OS after allogeneic HCT between groups who received oral BU vs. IV BU regimens.[48,70,72] This lack of difference in OS[69,70] supports the continued use of the less expensive oral BU by some HCT centers.[38,70] In a retrospective Japan registry analysis of 460 children, just over half the study cohort had ALL and the remainder had AML, 262 had received oral BU, and 198 had received IV BU in combination with one or two of CY, melphalan or etoposide. The data showed no significant impact of route of BU administration on rates of SOS, non-relapse mortality, relapse or OS for both ALL and AML.[73] This led to an accompanying editorial questioning if the rapid adoption of IV BU occurred too quickly.[74]

Differences between oral and IV BU were compared in patients with non-Hodgkin lymphoma who were conditioned with BuCyE) for autologous HCT. Compared to patients receiving oral BU, IV BU was associated with lower relapse rates, superior relapse-free-survival and OS. Notably, BU TDM has not been consistently used in BuCyE conditioning for autologous HCT. Most publications show that weight-based BU dosing without BU TDM was used[55] although BU TDM can be used.[72,75] In patients with non-Hodgkin's lymphoma receiving BuCyE, improved OS with IV BU has been observed,[66,72] but again, contradictory data exist.[54]

One final point is that because engraftment is expected in the context of autologous HCT, the lower limit of the target BU exposure should not be based on data obtained from allogeneic HCT recipients showing low BU exposure is associated with poor engraftment (see **FAQ1**).

FAQ5. How should personalized BU dosing be achieved? Answer: *Personalized BU dosing should be achieved by using TDM after selecting and administering the initial dose of high-dose BU.*

Personalized BU dosing should be achieved using TDM, which is also referred to as targeted BU, target concentration intervention, or pharmacokinetic-guided dosing. The procedure for BU TDM follows general pharmacokinetic principles and has been previously published. Close attention to detail, a validated analytical method to quantitate BU plasma concentrations and expertise in pharmacokinetic modeling are necessary (see Technical Appendix).

For BU TDM, an initial dose of BU is chosen (see **FAQ6**) and administered. Next, sequential pharmacokinetic samples are drawn before the subsequent BU dose. Obtaining pharmacokinetic samples over an acceptable time period is critical for accurate estimation of a patient's BU exposure. An acceptable time period for BU pharmacokinetic sampling must balance the half-life of the drug (typically 2-3 hours), the dosing frequency (see **FAQ7**) and the practical logistical issue of obtaining the TDM results in a timely fashion to personalize the BU dose. For BU personalized dosing, an acceptable time period for BU pharmacokinetic sampling can be as short as 4 hours, which occurs with a 2 hour BU infusion and every 6 hour (Q6H) dosing, or as long as 8 hours, which occurs with a 3 hour BU infusion and every 24 hour (daily) dosing. However, the acceptable time period for BU pharmacokinetic sampling can be shortened if population pharmacokinetic (popPK) modeling is used instead of the traditional noncompartmental analysis.[76] The BU clearance is calculated from the administered BU dose and the resulting BU exposure (AUC).

Eq. 1: $CL = \frac{\text{Administered dose 1}}{AUC_{0-\infty}}$ **Eq. 2**: $\text{Personalized dose} = CL \times \text{target AUC}$ **Eq. 3**: $Css = \frac{AUC_{0-\infty}}{\text{dosing frequency}}$

Firm knowledge of the BU dose and accurate estimation of the BU exposure are essential for predicting BU clearance. After the initial BU dose, the estimated BU exposure is the area under the plasma concentration-time curve to time infinity ($AUC_{0-\infty}$). The actual AUC value is a complex derived value that uses the pharmacokinetic sample data as detailed in the **Technical Appendix**. Using Equation 1, the patient's BU clearance is estimated and used to estimate subsequent BU doses to achieve the desired patient target exposure as described in **FAQ3**. With the subsequent Equations 2 and 3, the personalized dose and Css are calculated, respectively.

In the United States, AUC is reported as (micromoles/liter)×minute (i.e., μmolar×minute or μM×minute) and C_{ss} is reported as ng/mL. A C_{ss} of 900 ng/mL = AUC of 1315 μmolar×minute with every six-hour dosing, or an AUC of 5260 μmolar×minute for daily dosing (Table 2). A more detailed table of the equivalents is included in the Technical

Appendix. Harmonization of the method for reporting BU exposure and clearance is needed to minimize confusion with interpreting studies from different institutions.

Table 2. BU AUC to Css Equivalency Table.

AUC	AUC	Css[a]	AUC[b]	AUC
µmolar×minute Q6H dosing	µmolar×minute daily dosing	ng/ml	mg/L×h Q6H dosing	mg/L×h daily dosing
877	3508	600	3.60	14.4
900	3800	650	3.90	15.6
1125	4500	770	4.62	18.5
1316	5262	900	5.40	21.6
1500	6000	1026	6.16	24.6

All BU plasma exposures are presented in this manuscript using the units within the original manuscript and, if needed, converted to BU concentration at steady state (Css). The Technical Appendix and Equations 1 to 3 in FAQ5 explain how to convert between the various BU exposure units. [a]Css = AUC divided by the dosing frequency. [b]When the AUC is expressed in micromolar (micromoles/L) units, then the BU molecular weight (246.3 g/mol) must be used to calculate the AUC in mg/L units.

FAQ6. How is the initial BU dose best selected? Answer: *The initial IV BU dose should be based on the European Medicines Agency (EMA) nomogram[77] for children with a target AUC of 1125 µmolar×minute. For adults with the same target AUC, the initial IV BU dose should be 0.8 mg/kg every 6 hours or 3.2 mg/kg every 24 hours. The initial IV BU dose may need adjustment for lower or higher target AUC. Oral BU dosing always begins at 1 mg/kg.*

Children

When given orally, BU is given at 1 mg/kg every 6 hours for four days (a total of 16 mg/kg). Nowadays, children rarely receive oral BU, presumably due to ease of IV administration over oral administration (see **FAQ4**), although recent data has suggested caution regarding the replacement of oral BU with IV BU in children.[73,74]

An IV BU dose of 0.8 mg/kg results in similar BU exposure to 1 mg/kg of oral BU; a 2-hour IV infusion duration was chosen to mimic the time to the maximum plasma concentration after oral administration. Because the initial dose occasionally does not achieve the desired target BU exposure, dose adjustment is required during the conditioning regimen. In fact, over the course of a four-day BU regimen, the daily BU exposure may fluctuate greatly. It is unclear whether these fluctuations over the entire course of the conditioning regimen will significantly impact outcomes, although achieving target exposure late in the four-day course has been associated with worse hepatotoxicity in the context of the BU/CY regimen.[12]

To identify the optimal pediatric dose, both the EMA and the U.S. Food & Drug Administration (FDA) created separate recommendations for weight-based dosing of IV BU with Q6H dosing frequency to achieve a target AUC of 1125 µmolar×minute, which equates to a BU Css of 770 ng/ml. The FDA labeling advises that the initial dose is based on ideal or actual body weight (whichever is lower) and that 1.1 mg/kg be

used for ≤12 kg and 0.8 mg/kg for >12 kg with an acceptable BU AUC being 900-1350 ± 5% μmolar×minute[34,78] (Css=650-924 ng/mL). The EMA algorithm is more complicated because it has five dose cohorts (Table 3) that are based on actual body weight to achieve a BU AUC of 900-1500 μmolar×minute (Css = 650–1026 ng/mL). *These two different dosing recommendations were based on popPK modeling* (see **FAQ8** for description). Alternative dosing recommendations exist for children, many also based on popPK models. A greater proportion of patients achieve the target exposure when using EMA dosing (70%) than when using the FDA dosing (57%), based on simulations using a popPK model built using data from 1,610 HCT recipients (92% of which were children).[79] Thus, the EMA dosing is recommended (Table 3).

Table 3. European Medicines Agency's IV Busulfan Dose to Achieve a Plasma Busulfan AUC of 1125 (900-1500) Micromolar × Minute after Q6H Dosing (Css = 770 [650 – 1026] ng/ml).

Patient's actual body weight	EMA dosing with Q6H	Corresponding dosing with Q24H
<9 kg	1 mg/kg/dose	4 mg/kg/dose
9 to <16 kg	1.2 mg/kg/dose	4.8 mg/kg/dose
16 to <23 kg	1.1 mg/kg/dose	4.4 mg/kg/dose
23 to 34 kg	0.95 mg/kg/dose	3.8 mg/kg/dose
>34 kg	0.8 mg/kg/dose	3.2 mg/kg/dose

Please note only Q6H dosing was evaluated by Nguyen[77] and only Q6H dosing is approved by the EMA and the Food and Drug Administration (FDA).

When the initial dose is based on EMA nomogram,[77] the initial dose has achieved BU target exposures in 59% to 81%[80] of children.[77] In the US, there has been substantive variability in the initial BU dose prescribed for children with only a minority having received the FDA-approved dose. Unfortunately, in current clinical practice, the initial IV BU dosing has achieved desired target exposure in only 24.3% of children and improved approaches to selecting the preferred initial IV BU dose are desirable (e.g., test dose and pharmacogenetics). *These observations show that carefully selected initial BU dosing does not obviate a need for BU TDM* (see **FAQ5**), *especially if a narrow target exposure is desired*.

Adults

Both the FDA and EMA recommend an initial IV Q6H BU dose of 0.8 mg/kg for adults, specifically stating this as patients >12 kg per the FDA or >34 kg per the EMA. With once daily IV BU, the initial adult dose has been 3.2 mg/kg, 4 mg/kg,[38,81] or 130 mg/m^2.[63,82] BU target exposure influences the initial dose selection because a 4 mg/kg initial dose achieved target Css of 800-1000 ng/ml in a higher percentage of patients than the traditional 3.2 mg/kg dose. For obese adults, current ASBMT guidelines recommend dosing IV BU based on adjusted ideal body weight as calculated using Equation 4.[83] This equation should be used in adults receiving mg/kg dosing or mg/m^2 dosing with body surface area estimated using actual body weight.[83]

Eq. 4: Adjusted ideal body weight = ideal body weight + 0.25 (actual body weight-ideal body weight)

For oral BU, the initial dose of 1 mg/kg Q6H continues to be appropriate for adults. In obese patients, oral BU should be dosed based adjusted ideal body weight (Equation 4). Hemodialysis has been shown to enhance BU clearance after oral BU administration; a similar effect would be expected after IV BU administration as well. The costs of BU TDM, compared with the medication costs, are described in Table 4.

Table 4. Costs of BU-based conditioning and therapeutic drug monitoring.

Drug	Cost per mg	Typical dose in high-dose HCT	Cumulative dose (mg)	Cumulative ASP[a][84]
Oral busulfan	$11.73[84]	1 mg/kg PO Q6H × 16 doses	1,280	$15,008
IV busulfan	$35.21[84]	0.8 mg/kg IV Q6H × 16 doses[b]	1,024	$36,055
IV cyclophosphamide	$0.42[84]	60 mg/kg IV Q24H × 2 doses	9,600	$4,219
IV fludarabine	$1.29[84]	40 mg/m^2 IV Q24H × 4 doses	320	$4,124

	Cost per sample	Typical number of samples per AUC	Number of AUCs	
Busulfan TDM	$25.22[85]	6	1	$151.32[c]

Center for Medicare & Medicare Services average sale price (ASP) for BU-based conditioning and fee schedule for BU TDM in a hypothetical patient who weighs 80 kg and 6 feet tall, with a body surface area of 2.02 m^2. [a]Reimbursement amount was based on the available dosage formulations, rounded to the nearest pill or vial size that is commercially available in the US. [b]FDA approved dose; [c]The charge per sample ranges from $125 to $225 in the United States and therefore, the charge per AUC ranges from $750 to $1,350.

Liver Disease

For patients with liver disease, it is unclear what is the preferred initial and whether this matters if dose adjustment will be made using BU TDM. In general, high-dose BU-based conditioning is relatively contraindicated in patients with severe liver dysfunction. However, for patients with known liver fibrosis, hepatitis, or significant iron overload who are cleared for HCT, some regimens suggest lower initial doses and dose adjustment using BU TDM to avoid liver toxicity. An example would be the IV BU initial dose algorithm suggesting reduced initial BU doses for children > 8 years old with hepatomegaly or serum ferritin > 5,000 µg/L undergoing HCT conditioning with BU/CY/FLU/thiotepa for beta-thalassemia major.[86]

FAQ7. What is the optimal dosing frequency of BU? Answer: *The available IV BU data for adults do not suggest a significant difference in outcomes between Q6H and daily dosing likely because BU clearance, the volume of distribution and half-life appear to be similar regardless of dosing frequency. In children relevant studies are ongoing. Oral BU should be administered Q6H.*

BU dosing frequency has ranged from the traditional Q6H to every 24 hours (Q24H or daily),[63,81] or as a continuous infusion.[87] For the first 20 years, BU was only available as 2 mg tablet that was administered Q6H. At least in adults, this dosing frequency allowed for a manageable number of pills per dose (e.g., 80 mg dose would be forty 2 mg tablets). In infants and small children, a nasogastric tube is necessary to administer oral BU. Alternative

dosing frequencies were obviously desired and, after the subsequent development of IV BU with Q6H dosing, the focus subsequently turned towards daily IV BU dosing. Daily administration is also more convenient and less resource-intensive than the Q6H dosing.

In adults, a comparison of different dose frequencies has mostly been addressed only retrospectively in case series or as a subset analysis of phase I/II trials.[51] Not unexpectedly, the maximum plasma concentration (C_{max}) is proportionally higher in adults receiving once-daily IV BU, but BU clearance, the volume of distribution and half-life appear to be similar regardless of dosing frequency. In general, clinical outcomes in adults have not differed after traditional Q6H versus once daily BU dosing. In particular, the rates of SOS,[42,88,89] OS,[42,89] and relapse[42] have not differed significantly between Q6H and Q24H dosing but definitive conclusions were impossible given the heterogeneity in the patient population, concomitant medications, and inconsistent use of BU TDM. In children, it has clearly been demonstrated that once daily IV BU administration is safe.[90-92] Most recently, in a prospective cohort study of children and adults with myeloid malignancies, the CIBMTR reported similar outcomes in HCT conditioning regimens using IV BU Q6H (n=586) or daily (n=427) in combination with CY or FLU.[93]

FAQ8. What is the best method for estimating BU clearance? Answer: *BU clearance is calculated based on the administered BU dose and an estimate of post-dose BU exposure using validated pharmacokinetic modeling tools (see Technical Appendix). Test dose strategies are not currently recommended.*

As explained in **FAQ5**, accurate estimation of a patient's individual BU clearance is essential to determine the personalized dose that is necessary to achieve target BU exposure for that patient (see Technical Appendix for details). BU TDM follows general pharmacokinetic principles, by conducting pharmacokinetic analysis of one patient's concentration-time data at a time. The techniques for BU TDM have remained essentially unchanged for the past twenty years

Population pharmacokinetic (popPK) models have great potential to improve the estimation of the preferred initial BU dose and an individual's BU clearance An established method to improve BU TDM is popPK modeling, which can characterize patient factors (covariates) such as weight and age that can be used to predict the initial (i.e., before TDM results are available) dose. Between-subject variability (BSV) and between occasion variability (BOV, i.e., between dose) of a drug's pharmacokinetics can be defined, and these are useful for Bayesian dose adjustment.[79] After 1999, the FDA guidance states an expectation that initial dose recommendations be based on popPK models.[94] Both FDA and EMA dosing strategies for initial IV BU (see **FAQ6**) were based on two different popPK models that led to the different dosing strategies in children. There have been many popPK models characterizing IV BU pharmacokinetics in children (reviewed in Supplemental Table 1 of McCune et al.).[79] PopPK models of IV BU have indicated that age and body size – either normal fat mass, actual body weight, body weight (not specified further) or body surface area – are associated with IV BU clearance in children.

Beyond initial dose estimation, popPK models can also be used to estimate an individual patient's BU clearance but the latter currently still requires pharmacokinetic samples and concentration-time data from the patient. PopPK-based approaches have already been applied to the TDM of oral BU and IV cyclophosphamide in HCT recipients. PopPK models also facilitate the development of limited blood sampling schedules (LSS). For example, the use of an individual patient's concentration-time data with a popPK model – term maximum *a posteriori* dose personalization – could allow in the case of once daily IV BU, for BU clearance to be accurately estimated using a pharmacokinetic sampling duration of less than 8 hours which might make TDM feasible in the outpatient clinic. However, the rate-limiting step for adopting this strategy is the creation of dashboards or clinical decision support tools for clinicians to use popPK models for BU dose adjustment.

Another method to estimate an individual patient's BU clearance is to utilize a pre-HCT test dose. Most test dose strategies evaluate BU clearance following a single small dose of BU, ranging from 0.25 mg/kg to 0.8 mg/kg. While the use of a test dose has been able to minimize subsequent dose adjustments during the actual conditioning, the test dose strategy does not predict clearance well enough to replace BU TDM.

FAQ9. How do other medications affect BU pharmacokinetics? Answer: *Ideally, there would be no changes to medications given concomitantly with BU to minimize any drug-drug interactions that alter BU pharmacokinetics. The following medications have affected IV BU clearance: fludarabine, deferasirox, metronidazole, or oral BU clearance: phenytoin, fludarabine, metronidazole, ketobemidone, and itraconazole. Phenytoin affects oral BU clearance, but its effect upon IV BU clearance is unclear. By extrapolation voriconazole and posaconazole would likely affect BU clearance.*

BU is hepatically metabolized through glutathione (GSH) conjugation by glutathione S-transferase (GST) enzymes; this process depletes hepatocyte GSH stores. Conjugation with GSH forms an unstable S-glutathione sulfonium conjugate (GS+THT). Recent data indicate that GS+THT undergoes b-elimination to form g-glutamyldehydroalanylglycine (EdAG), which may contribute to the narrow therapeutic index of BU through various mechanisms.[95,96] Plasma tetrahydrothiophenium ion (THT^+),[97] THT 1-oxide, sulfolane, and 3-OH-sulfolane[98,99] have also been reported in HCT recipients. GSTA1-1 is the most active human form of GST for BU conjugation; GSTM1-1 and GSTP1-1 also mediate IV BU conjugation, but their estimated *in vivo* contributions to IV BU conjugation are ~5% and 0.2%, respectively, after accounting for their lower activity for BU conjugation and lower hepatic expression relative to GSTA1.[100,101] Various cytochrome P450 (CYP) enzymes may be involved in the metabolism of THT to sulfolane.

When evaluating a potential drug interaction with BU, the clinician should ideally complete BU administration before starting the potentially interacting drug. For example, many centers defer azole antifungal medications until after graft infusion when conditioning has been completed to avoid harmful drug interactions.[102] If BU must be administered with a potentially interacting drug, the interacting drug should not begin or stop during BU administration to minimize intra-patient (i.e., between dose) changes in BU clearance. It logically follows that BU dose changes for a potential drug interaction

are not advised without BU TDM due to the narrow therapeutic index of BU. Notably, drug interactions that occur with oral BU cannot also be assumed to occur with IV BU because IV BU predominantly undergoes hepatic metabolism, while oral BU can also have drug interactions at the level of the gastrointestinal tract.

There are several medications that have a documented or theoretical risk of BU interaction, the most notable of which are the anti-epileptic drugs to prevent BU-induced seizures (Table 5). After recognition of its neurotoxicity, seizure prophylaxis concomitant with BU began shortly after that.[103] Characteristics of the ideal seizure prophylaxis include: 1) Can load to therapeutic dose within 8 hours; 2) No overlapping toxicity with conditioning regimen; 3) Does not interfere with donor cell engraftment; 4) Toxicity cannot obscure a diagnosis of skin graft versus host disease (i.e., no to minimal dermatologic toxicity); 5) Safe for outpatient administration; and 6) No to minimal pharmacokinetic interactions with BU.[104] Various anti-epileptic drugs have been used as seizure prophylaxis for BU-induced seizures. Phenytoin has been the preferred medication to treat BU-induced seizures, but many HCT centers have replaced phenytoin with newer antiepileptic drugs (typically levetiracetam).[104,105] Phenytoin is well-known as a potent inducer of hepatic drug-metabolizing enzymes such as cytochrome P450 (CYP) CYP2B6, 2C and 3A and UDP glucuronosyltransferases (UGT).[104] Hassan et al. reported that patients receiving phenytoin had a higher clearance of oral BU as compared to diazepam.[106] CYP2C9 may also play a role in the oxidation reactions of THT.[107] The effect of phenytoin upon IV BU clearance is unclear; the package insert states that phenytoin increases IV BU clearance by 15% or more possibly due to induction of GST.[34] However, phenytoin administration has had either a slight effect[108] to no effect[63,109,110] on IV BU clearance. The clinical relevance of phenytoin's potential drug interaction is part of an ongoing CIBMTR study evaluating the association of seizure prophylaxis with clinical outcomes.[111]

Table 5. Drugs that affect busulfan clearance.

Interacts with IV busulfan[a]	Interacts with oral busulfan	Hypothetical or presumed interaction
Deferasirox, Fludarabine Metronidazole	Fludarabine[112] Ketobemidone[113] Itraconazole[114] Metronidazole[115] Phenytoin[106]	Acetaminophen Posaconazole Voriconazole

[a]The effect of phenytoin upon IV BU clearance is unclear; the package insert states that phenytoin increases IV BU clearance by at least 15%[34] However, phenytoin administration has had either a slight effect[108] to no effect[63,109,110] on IV BU clearance

Beyond potential interactions with anti-epileptics, IV BU clearance decreased by an average of 9.7% during concomitant fludarabine administration,[59,81] however this has not been observed by others.[82,116] Other medications reported having drug-drug interactions with IV BU include metronidazole,[117] and deferasirox.[118] Medications that have reported drug-drug interactions with oral BU include fludarabine (~30% increase in BU AUC),[112] itraconazole,[114] ketobemidone,[113] and metronidazole.[115] The underlying causes of these BU-

drug interactions are known, making it difficult to extrapolate these interactions to other medications. Notably, interactions with BU and the newer azoles, including voriconazole, posaconazole, and isavuconazole, have not been reported. The presumed mechanism of itraconazole interacting with oral BU is CYP3A. All azoles inhibit CYP3A4 but with various potencies, with their potencies decreasing as follows: itraconazole, voriconazole, posaconazole (potent inhibitors), fluconazole and isavuconazole (moderate inhibitors). To error on the side of caution, it is assumed that voriconazole and posaconazole also interact with BU.[119]

Use of acetaminophen in combination with or within 72 hours before BU administration may cause a decrease in BU clearance by reducing glutathione concentrations in the blood and tissues. While the clinical significance of this interaction is not yet known, acetaminophen use should be avoided or minimized less than 72 hours before and avoided during BU administration, and for 24 hours afterward.

FAQ10. Should the initial BU dose be personalized based on genetic polymorphisms?
Answer: *Pharmacogenomics-based dosing of BU, either IV or oral, is not recommended.*

There has been substantial interest in whether constitutional genetic polymorphisms are associated with BU-associated clinical outcomes in HCT. So far, the main focus of pharmacogenomics studies has been on the different glutathione S-transferases. To date, none of the genes associated with GSTs have demonstrated a consistent effect on the efficacy, toxicity or pharmacokinetics of BU. However, as with the pharmacodynamic studies, many of the observations came from single institution case series; few studies had an *a priori* power calculation. Currently, personalizing BU doses based on genetic polymorphisms is not recommended for routine clinical practice. Meta-analyses of the existing BU pharmacogenomics data (Supplemental Table 1) remains of interest, with the hope that the larger sample size could discover a genotype associated with outcomes of interest.

CONCLUSIONS

Although there have been multiple publications outlining some of the issues with BU and appropriate dosing, more work needs to be done. To optimize the use of BU in allogeneic HCT, there are several steps we could take. First, the collection of data by CIBMTR, Children's Oncology Group and other organizations relevant to BU dosing and BU TDM would be of benefit for retrospective studies that seek to evaluate the association of BU exposure with post-transplant outcomes. A collection of such data could mitigate the need for prospective multicenter studies that aim to evaluate different dosing strategies. Second, new conditioning regimens should be developed to identify the maximally tolerated systemic exposure in which cohorts are defined by their target BU exposure and the BU exposure is sequentially increased to identify the exposure associated with maximum efficacy and least toxicity while simultaneously assessing the impact of other concomitant chemotherapy. This study design can provide greater clarity regarding the maximal exposure associated with the optimal outcomes. Third, advances in the methods

of BU TDM are needed, including the use of popK and identifying novel predictors of BU clearance like metabolomics. At present, harmonization of BU exposure units and how BU pharmacokinetic data are interpreted should be explored. In any case, all advances must be made with the intent of improving the efficacy of BU-based HCT conditioning.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Cancer Institute (CA182963).

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Supplemental Materials: https://www.astctjournal.org/article/S1083-8791(16)30249-X/fulltext#SupplementalMaterial

CHAPTER 2

Harmonization of Busulfan Plasma Exposure Unit (BPEU): A Community-Initiated Consensus Statement

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Biol Blood Marrow Transplant 25 (2019) 1890-1897

ABSTRACT

Busulfan therapeutic drug monitoring (TDM) is often used to achieve target plasma exposures. Variability in busulfan plasma exposure units (BPEU) is a potential source for misinterpretation of publications and protocols and is a barrier to data capture by hematopoietic cell transplantation (HCT) registry databases. We sought to harmonize to one BPEU for international use. Using Delphi consensus methodology, iterative surveys were sent to an increasing number of relevant clinical stakeholders. In Survey 1, 14 respondents were asked to identify ideal properties of a BPEU. In Survey 2, 53 stakeholders were asked: 1) to evaluate BPEU candidates according to: a) ideal BPEU properties established by Survey 1, b) local position statements for TDM and 2) to identify potential facilitators and barriers to adoption of the harmonized BPEU. The most frequently used BPEUs identified in descending order were: area under the curve (AUC) in μM×min, mg×h/L, concentration at steady state (Css) in ng/mL, AUC in μM×h, and mg×h/L. All respondents conceptually agreed on the ideal properties of a BPEU and to adopt a harmonized BPEU. Respondents were equally divided between selecting AUC in μM×min versus mg×h/L for harmonization. AUC in mg×h/L was finally selected as the BPEU, because it satisfied most of the survey-determined ideal properties for the harmonized BPEU and is easily understood in a clinical practice environment. Further, nine major professional societies have endorsed AUC in mg×h/L as the harmonized unit for reporting to HCT registry databases and for use in future protocols and publications.

Abstract

INTRODUCTION

Hematopoietic cell transplantation (HCT) offers curative treatment for malignant and nonmalignant diseases.[1] Recent data from the Center for International Blood and Marrow Transplant Research (CIBMTR) show that conditioning regimens for HCT frequently incorporate busulfan: 58% of allogeneic myeloablative regimens and 32% of allogeneic reduced-intensity conditioning.[2] For these regimens, busulfan plasma exposure has been associated with important post-transplant outcomes.[3-5] While low busulfan plasma exposure (under treatment) is associated with higher rates of graft rejection[6-8] or relapse,[9] the converse is associated with increased risks for hepatotoxicity[6,10-14] and non-relapse mortality.[13] By achieving the optimal plasma exposure, each of these outcomes is improved.[10,15-17]

Currently, multiple busulfan plasma exposure units (BPEUs) are used clinically and reported in publications. Lack of BPEU harmonization raises several concerns. First, when clinicians interpret publications or implement a protocol, they must often convert the BPEU to the BPEU used by their institution via a complicated and error-prone process. Second, the use of multiple different BPEUs has precluded busulfan plasma exposure being included as a data element in international registries such as CIBMTR. As a result, these large databases cannot be leveraged to answer scientific questions regarding busulfan plasma exposure and HCT outcomes. This is exemplified by the recent experience of the American Society for Blood and Marrow Transplant (ASBMT, now ASTCT) Committee on Practice Guidelines which was unable to create an evidence-based guideline for busulfan TDM due, in part, to heterogeneity in reported BPEUs.[5]

The overarching goal of this project was to minimize the risk for busulfan dosing errors and to facilitate the future use of multicenter databases to evaluate relationships between busulfan plasma exposures and HCT outcomes. Given that international harmonization to a single BPEU would likely resolve barriers and create opportunities for safer and more effective use of busulfan, we sought international harmonization to one BPEU. Using Delphi consensus methodology, we administered iterative surveys to relevant stakeholders.[18,19] The results of the BPEU harmonization project are presented here.

METHODS

Needs Assessment and Formation of Steering Committee

Shortly after the Autumn 2016 publication of the ASTCT Practice Guidelines Committee's Busulfan Considerations,[5] 23 busulfan TDM laboratories[5] or HCT centers worldwide known to perform busulfan TDM were invited to participate in discussion of solutions to the evidence gaps[5] highlighted in that ASTCT publication. From this group, a Steering Committee (LLD, EM, JSM, JR, RFY) was formed. Twenty-eight respondents responded to this invitation and identified 33 concerns. From these, the Steering Committee prioritized BPEU harmonization. Before beginning this project, support was obtained from leaders of seven relevant professional societies (see Acknowledgements).

Delphi process

The BPEU harmonization project comprised a series of web-based surveys completed by an increasingly larger circle of stakeholders involved in busulfan TDM during HCT. All survey responses were anonymous, and stakeholders were not aware of individual's responses. The study was approved by the Institutional Review Board at The City of Hope.

Survey participants

Invited survey participants included: a) HCT physicians who prescribe busulfan and choose the target busulfan plasma exposure b) analytic chemists who quantitate busulfan plasma concentrations and c) clinical pharmacists or pharmacologists who conduct pharmacokinetic modeling and use those results to personalize busulfan doses. These BPEU stakeholders were organized into three groups of increasing diversity of expertise and size (Figure 1, Supplemental Tables 1 and 2): the aforementioned Steering Committee (n=5), an Expert Panel (n=9) and a Task Force (n=38). The Steering Committee included experts in busulfan quantification, pharmacokinetic modeling and dose individualization. The Expert Panel, formed in August 2017, included Steering Committee members, the physician Chair of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) Chemotherapy Dosing Committee, plus HCT physician leaders from across the globe. The Task Force, formed in February 2018, added members recruited via the needs assessment responders and collegial networks of Steering Committee and the Expert Panel.

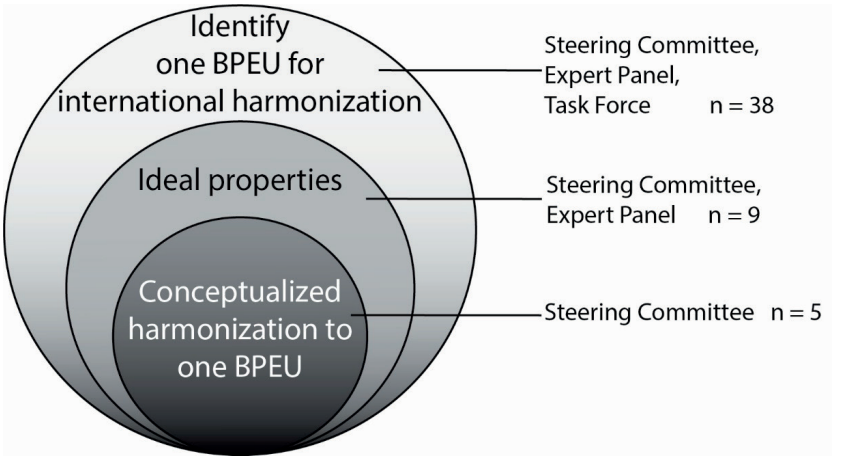


Figure 1. Conceptual schema to identify one busulfan plasma exposure unit (BPEU) for international harmonization.

Table 1. Survey responses to BPEU used in clinical practice.[a]

BPEU	Survey 1 Round 1	Survey 2 Round 1	Survey 2 Round 4
Respondents:	Steering Committee and Expert Panel	Steering Committee, Expert Panel, and Task Force	
Number responded	13	38	32[c]
AUC in mg×h/L[b]	0	1 (3%)	2 (6%)
AUC in μM×h[b]	0	0	2 (6%)
AUC in μM×min	7 (54%)	22 (56%)	17 (53%)
AUC in mg×h/L	4 (31%)	9 (23%)	5 (16%)
Css in ng/mL	2 (15%)	7 (18%)	5 (16%)

[a]This question asked on Survey 1 Round 1 (Steering Committee and Expert Panel), Survey 2 Round 1 (Steering Committee, Expert panel, and Task Force) and Survey 2 Round 4 (Steering Committee, Expert panel, and Task Force). Handwritten responses were counted. [b]NA: not asked in Survey 1 Round 1 or Survey 2 Round 1. AUC in mg×h/L was handwritten under "Other" category in Survey 2 Round 1. [C]one respondent purposely stated 'other' and typed micromole×min, which is missing a volume term.

Table 2. Rank order of the properties of an ideal BPEU identified from the Delphi Process of Survey 1[a, b.] 1.Participants were asked to rank the importance of the five ideal properties with 1 being most important: Ranking based on number of respondents reporting the specified format is important or most important.

	1. The relationship between the plasma exposure unit and the busulfan dose unit (e.g., milligrams) is clear.	2. The plasma exposure unit allows busulfan exposure to be expressed as total exposure.	3. The plasma exposure unit is included in the pharmacokinetic software platforms available for busulfan therapeutic drug monitoring.	4. The plasma exposure unit avoids small decimals (defined as 0.01 or smaller).	5. The plasma exposure unit is independent of the frequency of busulfan administration.
1, Most Important	14	7	4	4	3
2	2	11	7	5	7
3	3	9	12	2	6
4	4	5	4	9	10
5, Least Important	9	0	5	12	6

[a]≥70% of the Expert Panel stated each of these properties was "moderately important" or "very important" on two survey rounds.
[b]Rank order of properties of ideal BPEU was based on results from the broader group Survey 2 Round 4 that also included the Task Force.
[c]The term busulfan (plasma) exposure unit was used in each Round of the surveys.

Purposive sampling to obtain maximum variation in demographics, professional experiences, and health care professional roles, as well as snowballing strategies (where respondents can nominate or extend an invitation to other relevant stakeholders to participate), were used to select respondents.

Surveys

Each survey was developed by two co-authors (JSM and CMQ) and reviewed by two other members of the Steering Committee (LLD and JR) for content and face validity. Surveys are available upon request (JSM). Consistent with Delphi methodology,[20] Steering Committee and Expert Panel members received a summary of the project's goal and were invited to complete Survey 1 (Round 1) which had the goal to identify properties of the ideal BPEU. It included several BPEUs in current use and an initial list of three ideal BPEU properties: a) relationship between BPEU and busulfan dose unit is clearly understood, b) BPEU can be clearly understood regardless of the frequency of busulfan administration, and c) BPEU is used in the available pharmacokinetic software platforms for busulfan TDM. Iterative rounds of Survey 1 were developed after analysis of responses to the prior round; new questions could be added based on responses to the survey immediately prior. Revised rounds of Survey 1 were sent, together with the aggregated responses of the previous round, until no new information was provided or until consensus was achieved.

For each round, respondents were asked to rate their level of agreement with each statement using a four-point Likert scale: "Not at all important", "Slightly important", "Moderately important", "Very important". Offering a finite number of response options encouraged respondents to commit on a particular item.[20] To assist in making clear calculations on agreement and disagreement, a neutral middle point was excluded to

compel respondents to choose a particular option.[21] Consensus was defined, *a priori*, as having been achieved when ³70% of respondents stated that a property was "moderately important" or "very important." Each survey round ended by inviting respondents to provide general free-text feedback.

The goals of Survey 2 were to: a) evaluate each BPEU against the properties of the ideal BPEU as established via Survey 1, b) evaluate each BPEU against local position statements for TDM, and c) identify facilitators and barriers to international harmonization to one BPEU. Survey 2 participation was broader than Survey 1 and included Steering Committee and both the Expert Panel and Task Force. Similar to Survey 1, iterative rounds of progressively refined surveys were planned until no new information was gathered or consensus was reached regarding the one harmonized BPEU. Consensus was defined, *a priori*, as having been reached when ³70% of respondents ranked a BPEU as "very likely" or "extremely likely" to be adopted for international harmonization. The performance of each candidate BPEU was also evaluated with consensus defined, *a priori*, as occurring when ³70% or more respondents "agreed" or "strongly agreed" that a BPEU possessed a property of the ideal BPEU.

This project used Research Electronic Data Capture (REDCap), a secure, HIPPA and, FISMA compliant web application for building and managing online surveys and databases, hosted at City of Hope. Server security and application compliance is jointly managed by administrators in Information Technology Services and Research Informatics. Where feasible, validation rules (e.g., logic checks, format restrictions, min/max range, etc.) were added to ensure valid and accurate data entry. Users were able to complete surveys on any computer with internet access or a compatible mobile application.[22]

Statistics

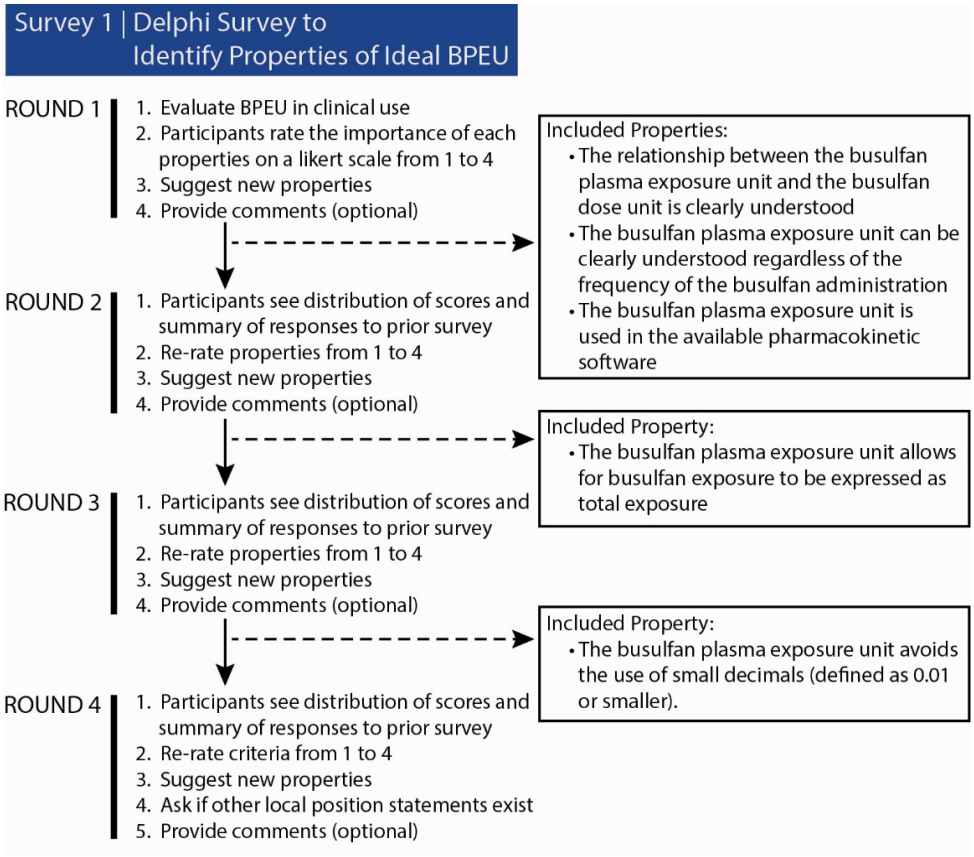
There is no universal agreement on the "minimum" or appropriate sample size for a Delphi process. Reliable outcomes have been generated by relatively small Delphi panels where members are selected carefully based on expertise and background[23] (e.g., the chronic GVHD Delphi process invited 64 participants).[24] *A priori*, for Survey 1 we assumed 100% response rate from the Steering Committee and Expert Panel (N=14). For Survey 2, we assumed a 75% response rate from the larger group of stakeholders (N=an additional 38 invited for total of 52) based on the response rate to a recent survey by the International Chronic GVHD Special Interest Group, which is a voluntary group of investigators who are interested in chronic GVHD research.

Descriptive statistics of survey responses were used to provide a summary of the group's view on each item with percentage scores for each statement providing the level of agreement amongst respondents.[21] SQL exports from the REDCap web-enabled survey data capture system and MS Excel (Redmond, WA) were used for analysis.

RESULTS

Survey 1: Steering Committee and Expert Panel identify properties of ideal BPEU

Thirteen of 14 (92%) invited participants responded to the first round of Survey 1 (Supplemental Table 3) and identified commonly used BPEUs (Table 1): area under the curve (AUC) in μM×min ($\frac{\text{micromole}}{\text{liter}} \times \text{minute}$) by 54%; AUC in mg×h/L ($\frac{\text{milligram}}{\text{liter}} \times \text{hour}$) by 31% and concentration at steady state (Css) in ng/mL ($\frac{\text{nanogram}}{\text{milliLiter}}$) by 15% (Figure 2A). Round 1 identified a new ideal BPEU property that was included with the second round, namely, that an ideal BPEU allows for busulfan exposure to be expressed as total exposure. Likewise, after Round 2, the third round added that an ideal BPEU avoids the use of decimals £0.01. Free text comments from Round 3 revealed a position statement of the Royal Academy of Pathologists in Australia that is relevant to international BPEU harmonization. Therefore, in the Round 4 participants were asked to describe any additional relevant local position statements; none were identified. Free text comments from Round 4 revealed a fourth BPEU (AUC in μM×h) in use. Survey 1 concluded after Round 4 with [3]70% of respondents agreeing on the properties of the ideal BPEU (Figure 2A; Table 2).



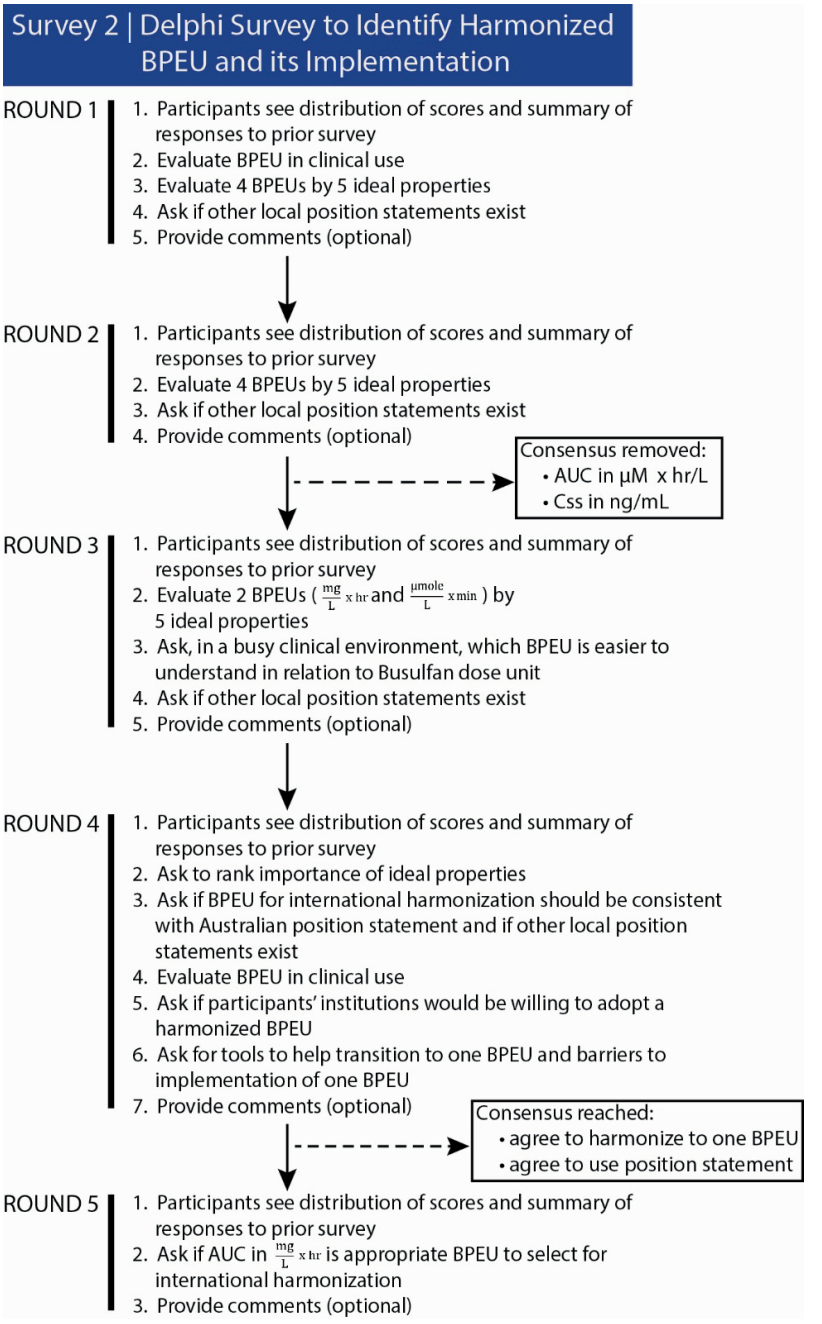


Figure 2A and 2B. Summary of each Round of Surveys.[a]
[a]Consensus of ≥70% of respondents stating a property was "moderately important" or "very important"

Table 3. Percent of Survey 2's respondents who "Agree" or "Strongly Agree" that the BPEU has the property listed. Those numbers > 70% are bolded.

	Round 1		Round 2		Round 3	
Property of the ideal BPEU	**μM×min**	**mg×h/L**	**μM×min**	**mg×h/L**	**μM×min**	**mg×h/L**
The relationship between it (BPEU) and the busulfan dose unit (e.g., milligrams) is clear.	46%	**82%**	53%	**78%**	50%	**92%**
It (BPEU) allows busulfan exposure to be expressed as cumulative exposure.	92%	90%	92%	92%	89%	89%
It (BPEU) is included in the pharmacokinetic software platforms available for busulfan therapeutic drug monitoring.	**77%**	**74%**	**81%**	69%	**84%**	**76%**
It (BPEU) avoids small decimals (defined as 0.01 or smaller).	**100%**	67%	**97%**	56%	**95%**	68%
It (BPEU) is independent of the frequency of busulfan administration.	56%	59%	58%	58%	66%	63%

Survey 2: Steering Committee, Expert Panel, and Task Force agrees to international harmonization

In the first round of Survey 2 (Figure 2B), 39 respondents indicated that four BPEUs were used globally (Table 1): AUC in μM×min (56%); AUC in mg×h/L (23%), Css in ng/mL (18%) and AUC in mgxh/L (3%). Free text comments to Survey 2 Round 1 revealed that a fifth BPEU was in current use: AUC in mg×h/L. The four BPEUs identified from Survey 1 were evaluated for properties of the ideal BPEU. Consensus was reached that two AUC units (mg×h/L and μM×min) each met three of five ideal properties, whereas AUC in μM×h met only two properties, and Css in ng/mL met none of the desired properties (Supplemental Table 4). No additional local position statements were identified.

Table 4. Application of 2010 Position Statement of the Royal College of Pathologists of Australia.[25a]

Recommendations	**Application to BPEUs**	**Compliance with Recommendation**	
		μM×min	**mg×h/L**
mass units should be used for reporting therapeutic drug concentrations in Australia and New Zealand;	Busulfan plasma concentrations should be reported in units of ng/mL or μg/L. To avoid conversion to micromolar, only $\frac{\mu\text{icrogram}}{\text{L}} \times \text{h}$ and $\frac{\text{mg}}{\text{L}} \times \text{h}$ could be used to report BPEU.	Non-compliant	Compliant
the litre (L) should be used as the denominator when expressing concentration. Examples of these units are mg/L and μg/L.	Busulfan plasma concentrations should be reported in units of μg/L.	Compliant	Compliant
Exceptions relevant to busulfan		Not applicable	Not applicable

Recommendations	Application to BPEUs	Compliance with Recommendation	
		μM×min	mg×h/L
drugs for which there is current uniformity of reporting and supporting information using molar units, notably lithium (mmol/L) and methotrexate (μmol/L);	Table 2 shows that there is no current uniformity of reporting and supporting information using molar units	Not applicable	Not applicable

[a]This position statement was written by a working party from the Australasian Association of Clinical Biochemists, Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists, Royal College of Pathologists of Australasia and Royal Australasian College of Physicians. Thus, the Australian spelling of liter (i.e., litre) is used.

In Round 2, iterative reevaluation of the four BPEUs occurred and consensus was reached that AUC in μM×min met three of five ideal BPEU properties and AUC in mg×h/L met two of five properties. AUC in μM×h unit and Css in ng/mL unit each met only one of five ideal properties (Supplemental Table 4). Round 2 was the first to evaluate different BPEUs against the qualities stipulated by the only local position statement identified: the 2010 Position Statement of the Royal College of Pathologists of Australia. The following question was asked. "We also draw to your attention the 2010 Position Statement of the Royal College of Pathologists of Australia (Table 4) that states: "....It is recommended that mass units be used routinely for reporting results of therapeutic drug concentrations measured by pathology laboratories in Australia and New Zealand. It is also recommended that the litre (liter in American spelling, L) be used as the denominator when expressing the concentration. Examples of these units are mg/L and μg/L. These recommendations relate to drugs which are normally given therapeutically, whether measured for therapeutic drug monitoring purposes or assessment of overdose."" Thirty-six Round 2 respondents were asked which BPEU is in most agreement with the above position statement and 53% selected AUC in mg×h/L, 33% selected AUC in μM×min, 14% chose Css in ng/mL and none chose AUC in μM×h. Based on the results of the first and second rounds, AUC in μM×min and AUC in mg×h/L remained under consideration as the future harmonized BPEU.

In the third round of Survey 2, respondents were asked to evaluate these two BPEUs against the properties of the ideal BPEU. Consensus was reached that each possessed three of the five ideal BPEU properties (Table 3). Respondents were also asked which BPEU most agreed with the 2010 Position Statement of the Royal College of Pathologists of Australia.[25,26] AUC in mg×h/L was chosen by 33 (87%) respondents as being in agreement with the Position Statement while 5 (13%) respondents chose AUC in μM×min. An additional question was also asked: "In a busy clinical environment, which BPEU is easier to understand in relation to the busulfan dose unit (e.g., milligrams)?". In response, 22 (63%) respondents chose AUC in mg×h/L and 13 (37%) respondents chose AUC in μM×min.

In the fourth round of Survey 2, 32 respondents indicated that five BPEUs were used globally (Table 1): AUC in μM×min (53%); AUC in mg×h/L (16%), Css in ng/mL (16%), AUC in μgxh/L (6%) and AUC in μM×h (6%). They were also asked to rank the importance of the five ideal properties and whether they supported harmonization to a single harmonized BPEU (Table 2). All 32 respondents stated their willingness to harmonize to a single BPEU

and 87% (28/32) reported that their respective institution/program would be willing to do so. Consensus was reached that the BPEU chosen for harmonization should be consistent with the 2010 Position statement of the Royal College of Pathologists of Australia and easy to understand in relation to the busulfan dose unit (e.g., milligrams) in a busy clinical environment (i.e., μM×min requires converting with busulfan's molecular weight while mg×h/L does not).[27]

A final question in Round 4 asked participants (n=32 respondents) to identify facilitators and barriers to implementation of a harmonized BPEU. An identified facilitator was "step-by-step instructions" in the following formats: web-based app (most preferred), PDF available, smartphone app, video tutorial and one-on-one personal training (least preferred). Among key barriers to a harmonized BPEU, 78% identified potential lack of familiarity with the chosen BPEU and 31% identified a lack of perceived benefit of making the change. Other barriers in free text were noted (Supplemental Table 5). In the fifth (final) round iteration, 34 respondents were asked to indicate their level of agreement with the statement: "AUC in mg×h/L is the appropriate unit of BPEU to select for international harmonization". Fifty percent selected: "Strongly Agree" or "Agree" while 50% chose "Strongly Disagree" or "Disagree".

Table 5. Professional Societies in support of mg×h/L as the single BPEU.[a]

Society[a]	Representative	Date
ACCP Hematology/ Oncology Practice and Research Network	Marco Martino	March 2019
ASTCT Executive Committee	Miguel-Angel Perales Navneet Majhail	April 2019
ASTCT Practice Guideline Committee	Paul Carpenter Bipin Savani	February 2019
BMT CTN	Marcelo Pasquini Miguel-Angel Perales	February 2019
Brazil BMT Society	Nelson Hamerschlak	January 2019
CIBMTR	Marcelo Pasquini	February 2019
EBMT & EMBT Pharmacy[c]	Mohamed Mohty Erik van Maarseveen	January 2019
HOPA	Susanne Liewer	June 2019
IATDMCT – Chemotherapy Group	Erik van Maarseveen	January 2019
KSBMT	Hyoung Jin Kang	April 2019
PBMTC	Michael A. Pulsipher	February 2019

[a]Abbreviations: ACCP= American College of Clinical Pharmacy; ASTCT= American Society for Transplantation and Cellular Therapy; BMT CTN= Blood and Marrow Transplant Clinical Trials Network; CIBMTR= Center for International Blood and Marrow Transplant Research; HOPA=Hematology/Oncology Pharmacists Association; IATDMCT=International Association of Therapeutic Drug Monitoring and Clinical Toxicology; KSBMT= Korean Society of Blood and Marrow Transplantation. [b]The IATDMCT approved mg×h/L; the other societies endorsed mg×h/L.[c]The EMBT Pharmacy committee is responsible for such medication related decision; their committee decision is supported by Dr. Mohty, President of EBMT at the time of the decision.

Choice of one BPEU for international harmonization

Although Survey 2 respondents were evenly split in the final round with respect to their choice for the harmonized BPEU, the Steering Committee believed that selection of AUC

in mg×h/L as the harmonized BPEU was most in alignment with guiding principles and aggregate survey responses. This decision was supported by nine professional societies which are listed in Table 5.

DISCUSSION

By iterative surveys of international stakeholders in busulfan dose individualization we have: 1) found that five BPEUs are currently used clinically; 2) reached consensus regarding the properties of the ideal BPEU; and 3) reached consensus regarding willingness to harmonize to a single BPEU. Since respondents were evenly split regarding the choice of one BPEU for harmonization, the Steering Committee, and ASTCT Practice Guideline Committee made a decision that reflected the consensus reached among stakeholders regarding the most important properties of the ideal BPEU: AUC mg×h/L was selected as the harmonized BPEU. Although we did not meet our threshold for consensus among stakeholders with respect to the selection of the single harmonized BPEU, we believe that the Steering Committee's choice reflects the philosophy expressed by the survey respondents.

The Steering Committee and Expert Panel (Figure 1) came to a consensus regarding the ideal properties of a BPEU (Table 3), which predominantly focused on ease of use and understanding in the busy clinical setting. Survey 2 respondents reached consensus that the single BPEU selected for international harmonization should be consistent with the 2010 Position statement of the Royal College of Pathologists of Australia.[25,26] This position statement recommended that mass units be used routinely for reporting results of drug concentrations measured by pathology laboratories in Australia and New Zealand. Thus, conversion of the concentration-time points from mg/mL to µM is avoided. Survey 2 respondents also reached consensus that the BPEU should be easy to understand in relation to the busulfan dose unit (e.g., milligrams). On this point, the majority (63%) chose AUC in mg×h/L while a minority (37%) chose AUC in µM×min.

When interpreting busulfan pharmacodynamic data, conversion between doses of busulfan (mg) and the various BPEUs is difficult. Variation in dose frequency, with busulfan being given every 6, 12 or 24 hours, and total duration of therapy often ranging from 2-4 days, also adds complexity. It follows that converting BPEUs is error-prone, but the incidence of near misses resulting from mathematical conversion errors is unknown. Sadly, this is not surprising because only a few studies to date have explored chemotherapy safety and chemotherapy errors.[28-30]

There are various harmonization efforts within laboratory medicine, including harmonization[31] of cancer biomarkers by pathologists. The University of California Athena Breast Health Network demonstrated variation between expert observers and that technical and interpretive harmonization between expert observers is possible.[32] Another notable example is clinical sequence variant interpretation from the vast amounts of genome-scale sequencing. Supported by NIH, the Clinical Genome Resource (ClinGen) is forming multidisciplinary expert groups to systematically evaluate variants in clinically

relevant genes.[33] These examples established the precedence for multidisciplinary collaboration with the aim of harmonization to improve biomarker testing, documentation, and minimization of interlaboratory variation. Our BPEU harmonization project is another example of such efforts; here, we seek to standardize documentation and facilitate safer and more accurate interpretation of patient results by improving procedures and processes at the laboratory-clinical interface.

We recognize that global BPEU harmonization will require a carefully planned change management strategy in order to roll out the relevant changes, educate clinicians, and gain acceptance of these processes by all stakeholders.[31] We therefore have developed an implementation strategy, which includes the Steering Committee and multiple Expert Panel members working together to develop a plan for educating clinicians. After the final survey, the optimal next steps were discussed and agreed between the Steering Committee, the ASTCT Practice Guideline Committee, BMT CTN Chemotherapy Dosing Committee, Brazilian Bone Marrow Transplant Society, the EBMT Pharmacy Committee, and the IATDMCT Oncology Scientific Committee. The timeline for implementing AUC in mg×h/L as the harmonized BPEU was developed after a series of verbal and email communications and 18 months from publication of this consensus statement, AUC in mg×h/L will be used to express plasma busulfan exposure. To facilitate the transition to the BPEU, an updated Technical Appendix and a Microsoft Excel spreadsheet converting between the most common BPEUs are also available.[27] The technical appendix and MS Excel spreadsheet were reviewed by the Steering Committee, Expert Panel and pharmacists with leadership positions in the relevant HCT societies. Select members of the Expert Panel have initiated discussions to develop a web-based or smartphone-based busulfan calculator to convert between the commonly used busulfan concentrations and exposures. These various processes were designed to maximize the acceptance of the harmonized BPEU.

A strength of our study is the use of Delphi methods to create consensus among international stakeholders. The controlled communication of the Delphi process minimizes direct confrontation and allows individual respondents to express independent thought and enables equitable contribution from all respondents. It has been used successfully in many settings including solid organ transplant. Specifically, the Standardized Outcomes in Nephrology-Transplantation initiative developed a core outcome set for trials in kidney transplantation that is based on the shared priorities of all stakeholders.[19] A further strength of this project is the endorsement of AUC in mg×h/L as the harmonized BPEU by leading international organizations (Table 5) and its adoption by journals in this field. Thus, we believe the validity of our process and the likelihood of stakeholder acceptance are increased.

In conclusion, with international input, we have identified one BPEU for harmonization: AUC in mg×h/L. This choice is endorsed by nine professional societies (Table 5). In order to promote the safe clinical use of busulfan and to facilitate future multicenter research regarding busulfan plasma exposure and HCT outcomes, we strongly suggest that individual centers convert to the harmonized BPEU and that future publications and research protocols use it exclusively.

ACKNOWLEDGMENTS

We are grateful to Dr. Paula D. Robinson, Guideline Methodologist, Pediatric Oncology Group of Ontario for guidance regarding Delphi methods and to the Task Force members who responded to the various Rounds of Survey 2.

We are also grateful to the following professional societies who supported this project at its inception. Their leaders in Autumn 2016 are as follows: Leland B. Baskin, MD, MS, FCAP, FACB representing the Therapeutic drug monitoring/toxicology division of the American Association for Clinical Chemistry (AACC); Steven Devine, MD, Co-Principal Investigator, Blood and Marrow Transplant Clinical Trials Network (BMT CTN); Nelson Hamerschlak, MD, President, Brazilian Bone Marrow Transplant Society; Mary Horowitz, MD, MS, Chief Scientific Director, Center for International Blood & Marrow Transplantation (CIBMTR), Krishna Komanduri, MD, President, American Society for Blood and Marrow Transplantation (then ASBMT, now ASTCT), Mohamed Mohty, MD, President, European Society for Blood and Marrow Transplantation (EBMT), Miguel-Angel Perales, MD, Chair, BMT CTN Chemotherapy Dosing Committee, Michael A. Pulsipher, MD, Chair, Pediatric Blood and Marrow Transplant Consortium.

We are grateful to the following pharmacists, with leadership positions in the relevant HCT societies, who reviewed the BPEU results summary and reviewed the BPEU conversion worksheet: Olga Militano (COG), Kamakshi Rao (ASTCT). Steering Committee member and coauthor Erik van Maarseveen represented EBMT Pharmacy committee and IATDMCT Oncology Scientific Committee.

This work was supported by National Institutes of Health, National Cancer Institute (NCI) grant CA182963, P30 CA033572, and Dr. McCune's startup funds provided by City of Hope.

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Supplemental Materials: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6755045/

CHAPTER 3

Quality Control of Busulfan Plasma Quantitation, Modeling and Dosing: An Interlaboratory Proficiency Testing Program

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Ther Drug Monit. 2021 Oct 1;43(5):657-663

ABSTRACT

Background: Personalizing busulfan doses to target a narrow plasma exposure has improved the efficacy and lowered the toxicity of busulfan-based conditioning regimens used in hematopoietic cell transplant. Regional regulations guide interlaboratory proficiency testing for busulfan concentration quantification and monitoring. To date, there have been no comparisons of the busulfan pharmacokinetic modeling and dose recommendation protocols used in these laboratories. Here, in collaboration with the Dutch Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology, a novel interlaboratory proficiency program for the quantitation in plasma, pharmacokinetic modeling, and dosing of busulfan was designed. The methods and results of the first 2 rounds of this proficiency testing are described herein.

Methods: A novel method was developed to stabilize busulfan in N,N-dimethylacetamide, which allowed shipping of the proficiency samples without dry ice. In each round, participating laboratories reported their results for 2 proficiency samples (one low and one high busulfan concentrations) and a theoretical case assessing their pharmacokinetic modeling and dose recommendations. All participants were blinded to the answers; descriptive statistics were used to evaluate their overall performance. The guidelines suggested that answers within $\pm$15% for busulfan concentrations and $\pm$10% for busulfan plasma exposure and dose recommendation were to be considered accurate.

Results: Of the 4 proficiency samples evaluated, between 67% and 85% of the busulfan quantitation results were accurate (ie, within 85%–115% of the reference value). The majority (88% round #1; 71% round #2) of the dose recommendation answers were correct.

Conclusions: A proficiency testing program by which laboratories are alerted to inaccuracies in their quantitation, pharmacokinetic modeling, and dose recommendations for busulfan in hematopoietic cell transplant recipients was developed. These rounds of proficiency testing suggests that additional educational efforts and proficiency rounds are needed to ensure appropriate busulfan dosing.

Abstract

INTRODUCTION

High-dose busulfan is frequently used in allogeneic hematopoietic cell transplantation (HCT) conditioning regimens. Typical HCT busulfan doses range from 2–4 mg/kg/day for 1–4 days, resulting in a total dose of 3.2–16 mg/kg.[1] The busulfan area under the plasma concentration-time curve (AUC) value has been associated with important post-transplant outcomes in different conditioning regimens[2-5], with higher rates of graft rejection[6-8] or relapse[9] being closely associated with low busulfan AUC values. High busulfan AUC values (over treatment) have been linked to higher rates of hepatotoxicity[6,10-14] and non-relapse mortality.[13] In busulfan followed by cyclophosphamide (BU/CY) conditioning, personalized busulfan regimens developed using patient-specific busulfan clearance rates, often referred to as busulfan therapeutic drug monitoring (TDM), has been linked to reduced hepatotoxicity rates (from 75% to 18%[15]) and reduced graft rejection (from 26% to 4%[16]). Since busulfan is administered over a short period of time (i.e., 1–4 days), busulfan TDM is time-sensitive, which forces most HCT centers to evaluate their busulfan samples using local laboratories.

Recently, the American Society for Blood and Marrow Transplant's (now the American Society for Cellular Therapy and Transplantation (ASTCT)) Committee on Practice Guidelines sought to produce an evidence-based guideline for personalizing busulfan-based conditioning.[4] Unfortunately, they could not update or create new target AUCs because the published data is too heterogeneous and lacks adequately powered and sufficiently controlled studies.[4] To overcome this challenge, we invited numerous busulfan TDM laboratories to discuss solutions to resolve these evidence gaps.[4] These discussions identified several concerns within the industry and two projects were prioritized: 1) busulfan plasma exposure unit harmonization[17] and 2) unified busulfan quantitation, pharmacokinetic modeling, and dose recommendations (BuQMD), which is reported here.

At present, the evaluation of busulfan quantitation is overseen by regional and national regulators. To the best of our knowledge, there have been no interinstitutional comparisons of busulfan pharmacokinetic modeling and dose recommendations completed to date. The busulfan proficiency testing program described here includes assessing each task involved in busulfan TDM, including: 1) the quantitation of busulfan plasma concentrations; 2) the pharmacokinetic modeling of these concentration-time variables; and 3) busulfan dose recommendations based on these evaluations

The aim of this program was to minimize the risk of busulfan dosing errors and facilitate the production of multicenter databases to evaluate the relationships between busulfan AUC and HCT outcomes. Here, we report on the program's development and the results of the first two rounds of proficiency testing.

MATERIALS AND METHODS

This BuQMD project was developed as an external proficiency testing program and designed to facilitate the validation of busulfan TDM result accuracy. The Drug Analysis

and Toxicology division (KKGT) of the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML, www.kkgt.nl)[18] has existing infrastructure designed to facilitate proficiency testing for drug quantitation and dosage recommendations.

Participating Laboratories

The co-authors of this paper extended invitations to various laboratories within the HCT scientific community to participate in the BuQMD proficiency testing program. These invitations were sent electronically and snowballing strategies (where respondents could nominate or extend an invitation to other relevant stakeholders) were used to identify participating laboratories.

Proficiency Test Kit Development

We aimed to develop an affordable method for sending busulfan proficiency samples to international sites while maintaining their stability. Aqueous busulfan solutions exhibit temperature-dependent stability, and since busulfan solutions degrade more rapidly at higher temperatures[19], samples are typically shipped on dry ice. However, international dry-ice shipping is cost-prohibitive. To address these technical challenges, we developed a method to stabilize busulfan in plasma samples so that we could produce a test kit that could be shipped on ice packs and eliminate the need for dry ice.

Each proficiency test kit was prepared using a multi-step process. Each kit contained two busulfan proficiency samples (one low and one high busulfan sample). First, a research staff member (AP) from Utrecht University Medical Center would produce the two proficiency samples from a 1000 mg/L busulfan stock solution prepared using N,N-dimethylacetamide. This stock solution was stored in 1 mL aliquots at -80 °C and shown to be stable (recovery within 95%) for 4.5 years. For round #1 testing, the stock solution was diluted to either 3.2 mg/L (low concentration) or 28 mg/L (high concentration) in N,N-dimethylacetamide. For round #2, the stock solution was diluted to 5 mg/L (low concentration) or 16 mg/L (high concentration) in N,N-dimethylacetamide. Then, 90 mL of each sample was diluted in 1 mL of blank calf serum to produce sample kits with the following concentrations: 0.264 mg/L (low) and 2.312 mg/L (high) (Round #1) and 0.413 mg/L (low) and 1.321 mg/L (high) (Round #2). These theoretical concentrations were used as the reference values when evaluating the participating laboratory's quantitation of these samples. To assess the accuracy of the sample kit dilutions, we went on to quantify the proficiency samples using liquid chromatography-mass spectrometry (LC-MS) in a European co-operation for Accreditation (EA) ISO15189-accredited laboratory (see Supplemental Method 1). These values were shown to be within 15% (i.e., 85-115%) of the theoretical value and, therefore, considered acceptable. After this evaluation, the proficiency samples were sent to KKGT for distribution, with the time between preparation and shipping to KKGT not exceeding 3 weeks.

These proficiency samples were stored for 1 week in a -80 °C freezer before shipping as part of the proficiency test kits to the participating laboratories. These kits included: 1) busulfan samples in N,N-dimethylacetamide; 2) polypropylene micro tubes (1.5 mL, Brand, Wertheim, Germany) with blank plasma; 3) instructions for preparation of the busulfan proficiency samples; and 4) an internal temperature sensor to monitor the kit's contents.

The recipient laboratories evaluated their temperature recordings on receipt but did not report them to KKGT.

Assessment of Busulfan Quantitation (Q of BuQMD)
For each round of evaluation the participating laboratories were asked to quantitate the proficiency samples. All participating laboratories were blinded to the concentrations of these samples, and their results were considered accurate if they fell within 15% of the reference value. This 15% was found to be consistent with the guidelines described by the Food and Drug Administration and the European Medicines Association for bioanalytical validation.[20,21]

Assessment of Pharmacokinetic Modeling and Dose Recommendations (MD of BuQMD)
We developed a set of theoretical clinical cases to assess the participant laboratories' busulfan pharmacokinetic modeling and dose recommendations. These cases were revised iteratively and following completion were solved by four of the manuscript's co-authors. These four answers were averaged, and this average was used as the reference value.

The participating laboratories were asked to answer the questions in one case per proficiency round. Proficiency round #1's case had four questions and proficiency round #2's case had two questions (Supplemental Method 2). In proficiency round #1, the three most common methods for calculating busulfan plasma exposure (AUC in mg×h/L ($\frac{\text{milligram}}{\text{liter}} \times \text{hour}$), AUC in µMol×min ($\frac{\text{micromole}}{\text{liter}} \times \text{minute}$) or concentration at steady state (Css) in ng/ml ($\frac{\text{nanogram}}{\text{milliLiter}}$) – were accepted as units of measurement.[17] After the completion of this evaluation, AUC expressed as mg×h/L was adopted as the preferred harmonized unit for busulfan plasma exposure,[17] which meant that all answers from proficiency round #2 were only accepted in this format. Answering these questions was optional. Each question was open-ended and each answer was entered as free-text and not restricted to a specific numerical range. These cases were answered in a blinded manner ensuring that no two laboratories knew each other's answers.

For each question, answers within ±10% of the reference value were defined as accurate. This 10% reference value was chosen because busulfan exposure can be targeted to a single exposure value[22] or a narrow range (e.g. a Css of 800–900 ng/ml[23], which equates to a daily AUC of 19.2– 21.6 mg×h/L using the harmonized unit for busulfan plasma exposure[17]).

Data Analysis
R Studio (Version 1.3.1073) and R (version 4.0.2) were used for all data analysis, with all the descriptive statistical evaluations described in the results. The results of each participating laboratory were anonymized in accordance with the relevant KKGT privacy policies.

RESULTS

To date, two rounds of busulfan proficiency testing have been completed (Figure 1). Proficiency testing round #1 was completed between May 31, 2019, and September 22, 2019, and involved 27 laboratories while proficiency testing round #2 was completed between December 11, 2019, and January 21, 2020, and involved 25 laboratories. Most of the participating laboratories used LC-MS to quantitate their busulfan samples (Table 1), but any valid method was accepted.

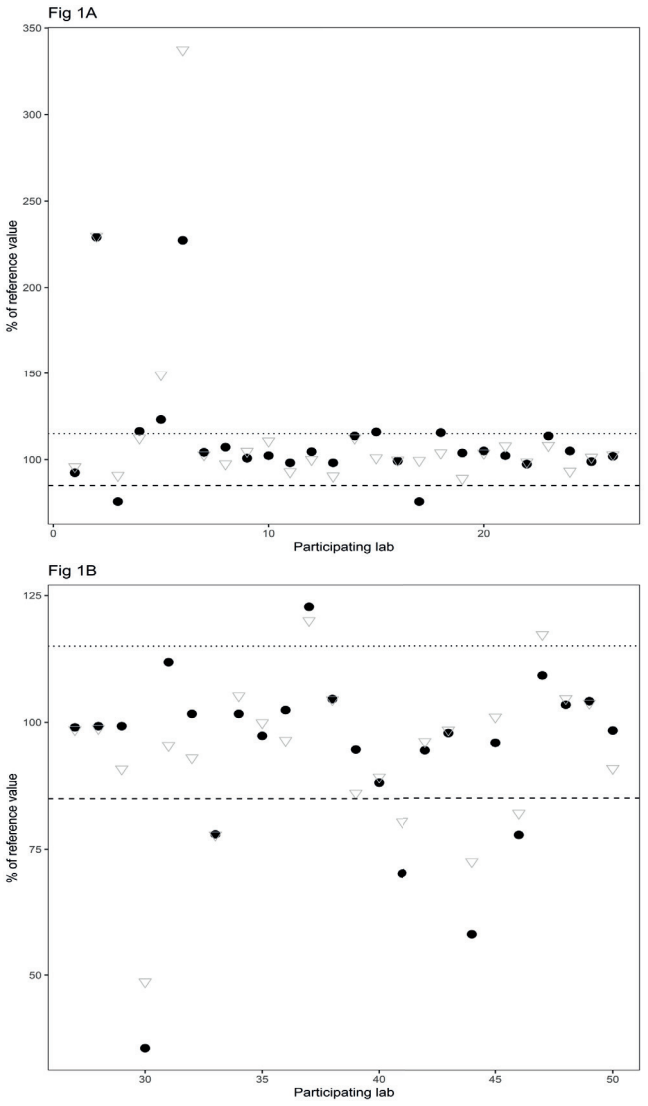

Figure 1. Busulfan quantitation results for proficiency round #1 (Fig 1A) and #2 (Fig 1B). The x-axis represents the participating laboratories (lab) and the y-axis represents the percentage of the reference value. The black dots represent the low busulfan concentration sample and the white triangles represent the high busulfan concentration sample. The dashed line represents the 85th percentile and the dotted line represents the 115th percentile. Outliers > 250 mg/L removed.

Table 1. Analytical methods used to quantitate busulfan concentrations by self-report of participating laboratories.

Analytical method	Proficiency round #1[a]	Proficiency round #2[a]
Immunoassay	0	0
Gas chromatography–mass spectrometry	4 (15%)	4 (16%)
High-performance liquid chromatography	1 (4%)	1 (4%)
Liquid chromatography–mass spectrometry	22 (81%)	20 (80%)
Total number of participating laboratories	27	25

[a]Shown as number (%)

Quantitation of Plasma Busulfan Concentrations (Q of BuQMD)

In round #1, one laboratory (3.7%) appeared to have a typographical error, with its values being 1000 times higher than any of the other laboratories, while in round #2, one laboratory (4%) reported a value 1000 times higher than the other laboratories. While their original answers were recorded in the analysis they are not included in Figure 1.

In proficiency round #1, 18 out of 27 laboratories (67%) reported a busulfan value within 15% of the reference value for the low concentration samples while 23 out of the 27 laboratories (85%) where within 15% of the reference value for the high concentration samples. For proficiency round #2, 18 out of 25 sites (72%) reported a value for the low concentration samples within 15% of the reference value while 17 out of these 25 laboratories (68%) reported a concentration within 15% of the reference value for the high concentration samples.

Pharmacokinetic Modeling and Dose Recommendations (MD Of BuQMD)

The reference values and answers used in both rounds of proficiency testing are described in Table 2. Test laboratories were not required to answer the pharmacokinetic modeling and dose recommendation questions.

Table 2. Answers to the pharmacokinetic modeling and dose recommendation questions provided by the participating laboratories.

Proficiency round	Questions (Qu)	Reference value	Answer median (range)	% of accurate[a] answers
#1	Qu 1. What is the AUC with Dose 1 in mg h/L?	24.0	24.1 mg×h/L (18.6–24,279)[b]	80% (12 of 15)[b]
	Qu 2. What is the AUC with Dose 1 in µmol/min?	5847	5868 µmol/min (4518–6631)	88% (14 of 16)
	Qu 3. The concentration at steady state (Css) in ng/mL is another commonly used descriptor in busulfan measurements. If the dosing frequency is measured over a 24-hour period (every 1440 minutes), what is the Css for a dose of 1 ng/mL?	993	1007 ng/mL (0.98–1133)[c]	75% (12 of 16)[c]
	Qu 4. Based on your calculated dose 1 exposure (AUC and/or Css), what dose (in mg) would you recommend for dose 2 in order to achieve the desired targeted exposure over the course of the busulfan conditioning?	260	267 mg (240–1080)	88% (14 of 16)

Proficiency round	Questions (Qu)	Reference value	Answer median (range)	% of accurate[a] answers
#2	Qu 1. What is the AUC (mg×h/L) for day 1 with dose 1?	15.2	15.1 mg×h/L (10.2–110)	82% (13 of 17)
	Qu 2. Based on your calculated dose for 1 AUC, what dose (in mg) do you recommend for dose 2 in order to achieve the desired targeted exposure over the course of busulfan conditioning?	285	290 mg (167–895)	71% (12 of 17)

[a] within 10% of the reference value; [b] if the answer of 24, 279 mgxh/L is assumed to be a typographical error with the intended answer being 24.279 mgxh/L, then the range is (18.6–27.2) and 87% (13 of 15) of the answers are accurate. [c] If the answer of 0.98 ng/mL is assumed to be a typographical error with the intended answer being 980 ng/mL, then the range is (750–1133) and 81% (13 of 16) of the answers are accurate.

Proficiency round #1 included three questions related to pharmacokinetic modeling, requiring laboratories to estimate the plasma busulfan exposure in the three most commonly used units of measurement and then to make a dose recommendation based on these values. In proficiency round #1, 15 (55%) and 16 (59%) out of the 27 laboratories answered the plasma exposure and dose questions, respectively. Two of these laboratories (11.7%) appeared to have made a typographical error in their answers with one laboratory recording an AUC of mg×h/L of 24,279 for question one and another recording a Css of 0.98 ng/mL for question three. Their original answers were retained in the analysis. Supplemental Figure 1 shows the distribution of the answers provided and indicates the reference value for each question. The proportion of laboratories supplying accurate answers to the questions regarding busulfan plasma exposure ranged from 75% (Css in ng/mL) to 88% (AUC in µmol×min) (Table 2), with the majority (88%) of the dose recommendations shown to be accurate.

Proficiency round #2 had two questions: one designed to estimate the plasma busulfan concentration using the harmonized unit of measurement, mg×h/L, and the other one designed to evaluate the dose recommendation capacity of these laboratories. In proficiency round #2, 17 out of the 25 (68%) laboratories completed these questions. A total of two laboratories (11.7%) seemed to have made a mistake in their answer with the total AUC in mg×h/L; however, their original answers were retained in the analysis. Specifically, one laboratory answering an AUC of 72.8 mg×h/L and another laboratory answered an AUC of 110 mg×h/L.

However, their original answers were retained in the analysis. Although their original answers were used in the analysis, the typographic errors were "adjusted" to assumed answers in Supplemental Table 2. The majority (82%) of the pharmacokinetic modeling results, AUC (mg×h/L), and the majority (71%) of the dose recommendation answers were accurate (Table 2). Supplemental Figure 2 shows the distribution of these answers and highlights the reference value for each.

DISCUSSION

Our main findings from the two rounds of busulfan proficiency testing completed to date were as follows: 1) the majority of laboratories could quantify the busulfan concentrations using our sample kits with results within 15% of the reference value; 2) between 75% and 88% of the calculated busulfan plasma exposure values were within 10% of the reference value; and 3) 88% (round #1) and 71% (round #2) of the laboratories recommended a busulfan dose within 10% of the reference value. This proficiency testing program provides a framework for comparing busulfan quantitation, modeling, and dose recommendations between laboratories used by HCT centers for busulfan TDM. If continued, this program can help to assure the accuracy of busulfan TDM for both patient care and research.

Many drugs that undergo TDM, including busulfan[4], do not have high-quality evidence for their target exposure or plasma concentration.[24] The ASTCT Clinical Practice Guideline Committee could not establish these target AUCs because the published data is too heterogeneous and lacks adequately powered and sufficiently controlled studies.[4] Proficiency testing could facilitate more rigorous, multicenter studies evaluating the association between busulfan TDM and clinical outcome. The inclusion of busulfan exposure data from the Center for International Blood and Marrow Transplant Research (CIBMTR) database is likely expand this even further. Collecting this type of data is important, especially as a survey suggested that 50% to 60% of HCT centers who report to CIBMTR used busulfan TDM.[25] Such studies could facilitate the development of evidence-based guidelines for target busulfan AUC values in different HCT conditioning regimens designed for specific disease settings. The association between busulfan AUC and outcome may differ based on the HCT conditioning regimen, the patient's age, and their underlying disease.[4] Ideally, these studies would help to improve overall survival by maximizing efficacy and minimizing toxicity. Target busulfan AUC values can be refined using expanded studies designed to produce sufficient power and using appropriate controls.

This proficiency testing program overcame the rapid degradation of busulfan in aqueous solutions at higher temperatures[19], by resuspending samples in N,N-dimethylacetamide, reducing the need to transport these samples on dry ice. The first two rounds of proficiency testing used two busulfan reference samples, which meets the ISO criteria[26] but may not meet regional regulatory standards for between-laboratory comparisons.

To our knowledge, this is the first proficiency testing program evaluating busulfan pharmacokinetic modeling and dose recommendations. The accuracy of the participants' answers, defined as those answers falling within 10% of the reference value, for the pharmacokinetic modeling and dose recommendation evaluations were disappointing. For the pharmacokinetic modeling task, 12% to 25% of the busulfan exposure estimation answers were inaccurate (Table 2). For the busulfan dose recommendation, 12% or 29% were inaccurate (Table 2). Proficiency round #1 had two outliers that were possibly typographical errors, but these types of errors can be difficult to recognize if an HCT center typically uses a different unit of measurement for their busulfan plasma exposure values. For example, one answer in proficiency round #1 included a Css value of 0.98 ng/mL, which might be difficult to recognize as an error if the HCT center typically uses mg×h/L, where an AUC

range of 4.8.–5.4 mg×h/L after one dose of IV busulfan administered every 6 hours would be considered normal. This data suggests that there is an urgent need for more educational and regulatory intervention at these laboratories in order to improve the accuracy of their busulfan pharmacokinetic modeling and dose recommendations. Asynchronous online certifications in busulfan TDM may be beneficial.

In the absence of a community standard, we decided to use a 10% margin around the reference value as a cut off for accuracy based on our experience and understanding of the currently available evidence; however, this margin may need to be addressed in the future. In addition, our proficiency sample testing procedures have three limitations. First, errors could have been made in the preparation of the kits. Second, we relied on the participating laboratories to assess the temperature readings following shipment. Finally, we allowed an extended time period between sample receipt and busulfan quantitation reporting. Thus, some proficiency samples may have been stored in unknown conditions for an extended period. It is unknown how sample loss/degradation contributes to the inaccuracy of these results. Future proficiency testing will need to collect data around the temperature of the test kits during shipping and the storage conditions before quantitation. We may also consider reducing the timeframe for evaluation to prevent storage induced variation.

Our results suggest that additional work within the busulfan dose individualization for HCT is needed to improve accuracy. Education, certification, and mandatory participation in busulfan proficiency testing, in partnership with various organizations, such as the KKGT, the ASTCT and the European Blood and Marrow Transplant (EBMT) groups – may be valuable tools in improving these outcomes and harmonizing busulfan evaluation.

CONCLUSIONS

In conclusion, we have described the results of two rounds of busulfan proficiency testing using a novel method for maintaining busulfan stability when shipped on cold packs. While most participating laboratories were shown to be fairly accurate in each individual task (busulfan concentration quantitation, pharmacokinetic modeling, and dose recommendations) there is room for improvement. Certification of busulfan TDM proficiency and mandated participation in busulfan proficiency testing for each of the tasks in busulfan TDM may improve the accuracy of busulfan dose individualization and potentially improve both patient and research outcomes.

ACKNOWLEDGEMENTS

Sources of support: We acknowledge those laboratories who participated in these proficiency evaluations. Please email office@skml.nl to inquire about any future participation in the busulfan proficiency testing program. We also thank Dr. Chris Gandhi for his helpful comments on an earlier draft of this manuscript.

Disclosure of funding: This work was supported by the 2018 KKGT Pilot Grant Funding Program.

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SUPPLEMENTAL MATERIALS:

Because these Supplemental materials are behind a paywall, they are included in this thesis.

SUPPLEMENTAL METHOD 1:

Each proficiency sample was evaluated by an EA ISO 15189 accredited laboratory before its dispatch to the participating laboratories. The busulfan assays were then validated in accordance with the EMA guidelines.[21] The staff of this laboratory were blinded to the actual busulfan concentration, and samples were prepared by pipetting 100 µL of each plasma sample into an Eppendorf microcentrifuge tube; this was followed by adding 250 µL internal standard (busulfan-D8) at a concentration of 1 µg/mL (in methanol: acetonitrile 20:80) and vortexing for 60 s. Samples were centrifuged for 3 min at 13150 × *g*, and the supernatant was transferred into a vial containing 250 µL formic acid (2%) and vortexed for another 60 s. These samples were then ready for injection. The calibration curve was constructed using four standards in a concentration range of 400–6400 µg/L. Samples were then analyzed using a Vanquish ultra-high-performance liquid chromatography (UHPLC) instrument coupled to a triple quadrupole TSQ Quantum ACCESS MAX, Thermo Fisher Scientific (Waltham, MA, USA). A total of 10 µL per sample was injected onto a Dr. Maisch GmbH, GreatSmart RP18, 3 µm, 100 × 2.1 mm (Ammerbuch, Germany) analytical column (column temperature 30 °C) run using water with 0.1% ammonium acetate (eluent A) and acetonitrile with 0.1 % formic acid (eluent B). Eluent conditions were set to 0 to 5 min with a linear gradient from 5% B to 95% B; at 0.5 to 1 min, isocratic 95% B; from 1 to 1.1 min, a linear gradient from 95% B to 5% B; and from 1.1 to 3 min, isocratic 5% B, with a flow rate of 0.3 mL/min. Busulfan was analyzed via electrospray ionization (ESI) using selected reaction monitoring with a transition of 263.80 > 151.11 and busulfan-D8 271.8 > 159.11 in positive mode with collision energy 12 and tube lens 86 (both arbitrary units). Mass spectrometry conditions were as follows: spray voltage of 3000 V, ion transfer tube temperature of 175 °C, and vaporizer temperature of 30 °C.

SUPPLEMENTAL METHOD 2:

Theoretical case description used in proficiency round #2.

SA is a 63 y.o. female who is admitted for a matched unrelated peripheral blood stem cell transplant for acute myeloid leukemia. Her conditioning regimen is targeted busulfan IV every 24 hours on days -7, -6, -5, and -4, and cyclophosphamide 60 mg/kg IV every 24 hours on days -3 and -2.

Per her transplant care team, the desired busulfan exposure target range is 4650 to 5250 uMol*min per day over the 4 days of busulfan administration conditioning. There are no known significant drug interactions.

Only one dose adjustment will be made based on Dose 1 pharmacokinetic analysis. The new dose will start with Dose 2 and continue through Dose 4.

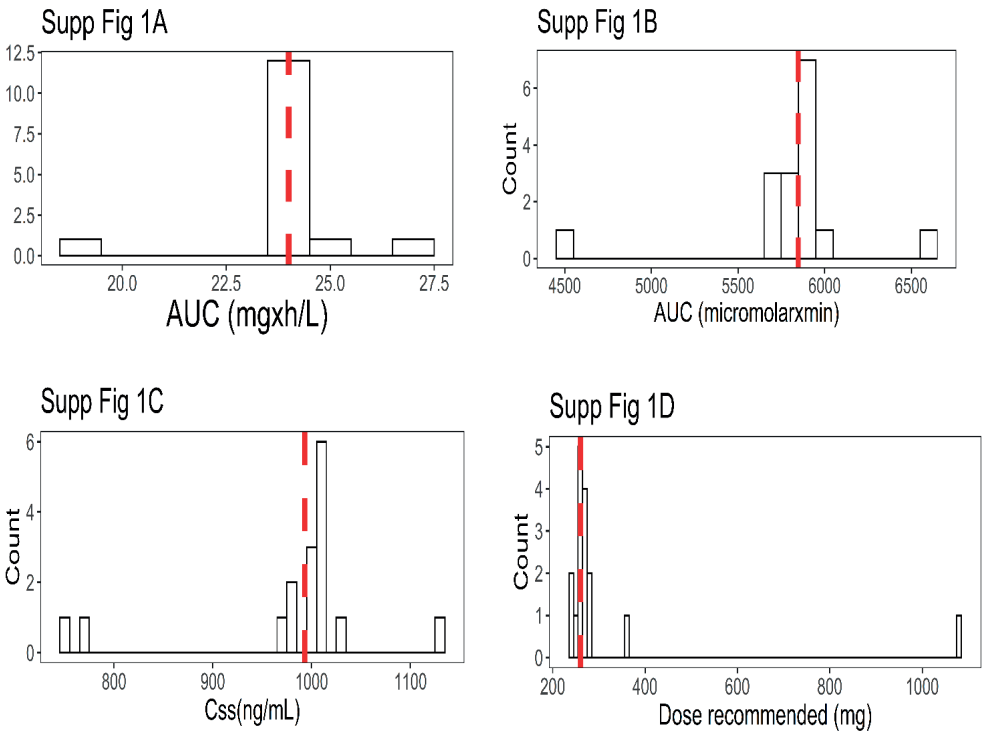
- Actual body weight: 65.7 kg
- Adjusted ideal body weight: 64.4 kg
- Height: 171.6 cm

Busulfan Dose 1 administered: 206 mg Infusion start time: 5:02 Infusion stop time: 8:03	
Actual sample collection times (24:00)	**Measured plasma concentrations (ng/mL)**
1. 8:03	3083
2. 8:18	2697
3. 9:33	1875
4. 11:04	1181
5. 13:03	727

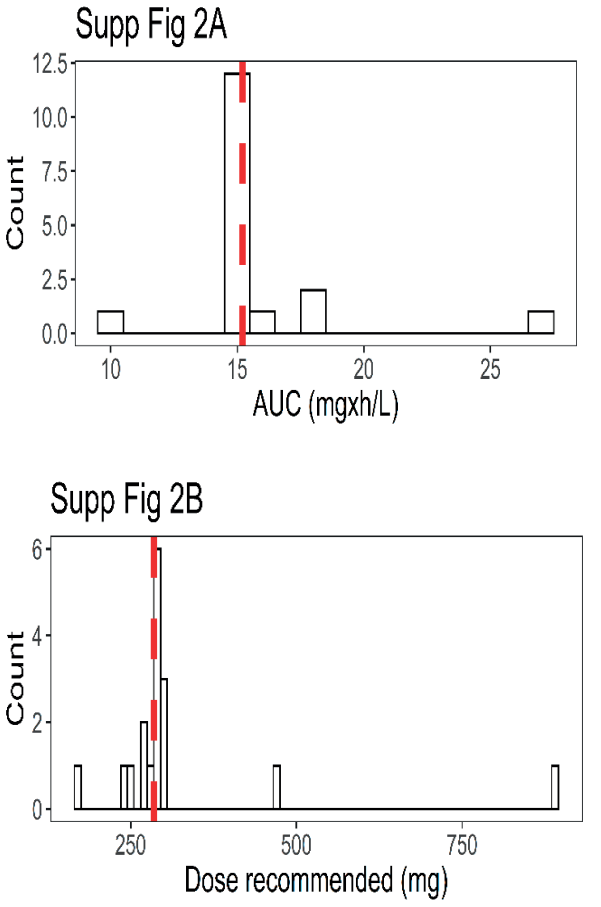
Questions:

1) What is the AUC (mg*h/L) for day 1 with Dose 1?

2) Based on your calculated Dose 1 AUC, what dose do you recommend for Dose 2 in order to achieve the desired targeted exposure?



Supplemental Figure 1.
Histograms describing the answers to the pharmacokinetic modeling and dose recommendation questions in proficiency round #1. The number of labs is reflected on the y-axis and the exposure (A, B, C) or dose recommended (D) units are reflected on the x-axis. The dashed red line indicates the reference value. While the original answers were used in the analysis, the typographic errors were 'adjusted' to the assumed answers in this figure. Specifically, for question one, one lab answered 24, 279 mgxh/L, which is assumed to be 24.279 mgxh/L. For question three, another lab answered 0.98 ng/mL, which is assumed to be 980 ng/mL.



Supplemental Figure 2.
Histograms describing the answers to the pharmacokinetic modeling and dose recommendation questions in proficiency round #2. The number of labs per response is recorded on the y-axis with the busulfan exposure (A) or dose recommend (B) on the x-axis. The dashed red line indicates the reference value. While the original answers were used in the analysis, the typographic errors were 'adjusted' to the assumed answers in this figure. Specifically, for question one, one laboratory answered 72.8 mg×h/L, which is assumed to be 18.2 mg×h/L and another laboratory answered an AUC of mg×h/L of 110 mg×h/L, which is assumed to be 27.5 mg×h/L.

CHAPTER 4

Busulfan in Infant to Adult Hematopoietic Cell Transplant Recipients: A Population Pharmacokinetic Model for Initial and Bayesian Dose Personalization

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Clin Cancer Res; 20(3) February 1. 2014

ABSTRACT

Purpose: Personalizing intravenous (IV) busulfan doses to a target plasma concentration at steady state (Css) is an essential component of hematopoietic cell transplantation (HCT). We sought to develop a population pharmacokinetic model to predict IV busulfan doses over a wide age spectrum (0.1 – 66 years) that accounts for differences in age and body size.

Experimental design: A population pharmacokinetic model based on normal fat mass and maturation based on post-menstrual age was built from 12,380 busulfan concentration-time points obtained after IV busulfan administration in 1,610 HCT recipients. Subsequently, simulation results of the initial dose necessary to achieve a target Css with this model were compared with pediatric-only models.

Results: A two-compartment model with first-order elimination best fit the data. The population busulfan clearance was 12.4 L/h for an adult male with 62kg normal fat mass (equivalent to 70kg total body weight). Busulfan clearance is predicted to be 95% of the adult clearance at 2.5 years post-natal age. With a target Css of 770 ng/mL, a higher proportion of initial doses achieved the therapeutic window with this age- and size-dependent model (72%) compared to dosing recommended by the Food and Drug Administration (57%) or the European Medicines Agency (70%).

Conclusion: This is the first population pharmacokinetic model developed to predict initial IV busulfan doses and personalize to a target Css over a wide age spectrum, ranging from infants to adults.

Abstract

INTRODUCTION

Allogeneic hematopoietic cell transplant (HCT) has curative potential for patients with either malignant or nonmalignant diseases.[1] Busulfan is the most common chemotherapy agent used in HCT conditioning regimens that do not include total body irradiation. Considerable interpatient variability exists in the effectiveness and toxicity of busulfan-containing conditioning regimens when dosed based on either body weight (mg/kg) or body surface area (BSA, mg/m^2).[2] The variability in clinical outcomes is due, in part, to between-patient differences in busulfan pharmacokinetics and the narrow therapeutic window of busulfan systemic exposure.[2] Rejection, relapse, and toxicity in HCT recipients are associated with busulfan plasma exposure, measured as area under the plasma-concentration time curve (AUC) or average steady state concentration (Css, calculated as Css=AUC/dosing frequency).[2] Personalizing busulfan doses to a target plasma Css improves each of these clinical outcomes (as previously reviewed[2]) and is clinically accepted in the context of the often-used intravenous (IV) administration route.[3,4] Because clinical practice is moving from every 6 hour (Q6h) to daily (Q24h) dosing frequency,[2,5,6] the target exposure expressed using Css is preferable to AUC because Css (i.e., Css=AUC/dosing frequency) incorporates the dosing frequency.

More efficient methods of personalizing IV busulfan therapy are desirable for numerous reasons. First, relapse and nonrelapse mortality continue to be problematic even in the context of therapeutic drug monitoring (TDM) of IV busulfan.[4,5,7] Second, the time delay to deliver a personalized busulfan dose recommendation with TDM presents a growing challenge with the increasing use of shorter IV busulfan courses, often administered as part of reduced-intensity conditioning prior to HCT[1,6] or gene therapy.[8] An established method to improve TDM of IV busulfan is population pharmacokinetic modeling, which can characterize patient factors (covariates) such as weight and age that can be used to predict the initial (i.e., before TDM results are available) dose. Between subject variability (BSV) and between occasion variability (BOV, i.e., between dose) of a drug's pharmacokinetic disposition can be defined and these are useful for Bayesian dose adjustment.[9-12] Population pharmacokinetic-based approaches have already been applied to TDM with oral busulfan[9] and IV cyclophosphamide[13] in HCT recipients. There is a clear need for improved initial IV busulfan dosing because current initial dosing practices have substantive variability and achieve the patient-specific therapeutic window of busulfan exposure in only 24.3% of children.[3] Although various groups have created population pharmacokinetic models in children (Supplemental Table 1), most of the studies have been small, which makes identification of covariates problematic.[14] Studies have typically focused on either pediatric or adult populations, requiring separate models for children and adults and limiting the generalizability of these models across the age continuum.[10,12] Our long-range goal is to improve outcomes in HCT recipients through more precise initial IV busulfan dosing and more effective TDM by more efficiently achieving the desired therapeutic window of busulfan exposure. Using the largest population of HCT recipients to date, we developed a population pharmacokinetic model over a wide age range to define busulfan pharmacokinetics regardless of age or body size with dosing guidance applicable from infants to adults. Subsequently, we compared initial IV busulfan

dosing predictions with the age- and size-dependent model to predictions from recent IV busulfan population pharmacokinetic models developed from pediatric populations.[15-17]

METHODS

Study population

Between June 1999 and September 2011, 1,610 HCT recipients aged 0.1 to 66 years underwent pharmacokinetic blood sampling to personalize IV busulfan doses (Table 1 and Supplemental Table 2) at the Fred Hutchinson Cancer Research Center (FHCRC) Pharmacokinetics Laboratory (1999-2001) or the Seattle Cancer Care Alliance (SCCA) Busulfan Pharmacokinetics Laboratory (2002-present). Approval of the FHCRC Institutional Review Board and Children's Oncology Group (COG, because AAML03P1 and AAML0531 participants were included) was obtained prior to analysis of anonymized data.

Table 1. Description of patient population[a]

	ABW-available only	Overall
Number (No.) of patients	133	1610
Age, in years	42.9±20.7 (0.4 - 65.8)	9.8 ±13.0 (0.1 to 65.8)
No. ≤ 4 years	16 (12%)	701 (43%)
Dosing weight (DWT, kg)	58.9±22.3	30.2 ± 24.1
No. DWT ≤ 12 kg[b]	13 (10%)	466 (29%)[b]
Sex		
Male	72 (54%)	904 (56%)
Female	61 (46%)	689 (43%)
Not reported	0	17 (1%)
Diagnosis[c]		
Malignant	100 (75%)	978 (61%)
Not malignant	33 (25%)	632 (39%)
Dosing frequency[d]		
Q6h	39 (29%)	1387 (88%)
Q8h	0	9 (1%)
Q12h	0	8 (1%)
Q24h (daily)	94 (71%)	166 (11%)
No. of Css per patient[e]		
1	13 (10%)	1401 (87%)
2	3 (2%)	89 (6%)
3	117 (88%)	120 (7%)

[a]Data presented as number (%) or mean ± standard deviation, percentages may not total 100 because of rounding; [b]per FDA-approved package labeling[4545]; [c]Supplemental Table 2 details disease classifications; [d]Unknown for 40 patients who only had TDM after a test dose; percentages calculated from the remaining 1,570 patients; [e]Css used to express busulfan exposure because of the different dosing frequencies.

For clinical TDM purposes, demographic data (i.e., age, sex, height, weight), and clinical data (i.e., disease, which was subsequently categorized as malignant or not malignant as described in Supplemental Table 2) were requested from the treating institutions (Supplemental Table 3). For the 133 patients (108 adults and 25 children) treated under the auspices of a FHCRC protocol at a Seattle-based institution, the actual body weight (ABW), dosing weight (calculated as previously described [18]), and ideal body weight (IBW) were available (i.e., "ABW-available cohort"). Institutions outside Seattle reliably provided only the busulfan dosing weight (DWT), which was calculated using their own institutional practices.

The initial busulfan dose, the dosing frequency, when the pharmacokinetic blood samples were obtained, and the acceptable therapeutic window of busulfan Css were chosen by the treating physician. Busulfan concentrations were determined by gas chromatography with mass spectrometry detection as previously described.[3] The laboratory participated in routine cross-validation exercises between laboratories. The assay dynamic range was from 25 to 4500 ng/mL, and the inter-day coefficient of variation was less than 8%. Ninety-one of 12,380 (0.7%) concentration-time points were lower than the lower limit of quantitation (62 ng/mL); these measurements were included in the data set.

Population pharmacokinetic analysis

Busulfan administration was assumed to be zero-order, with the infusion duration described by the treating institution. Both one- and two-compartment models were examined. A two-compartment model best fit the data with the lowest objective function value (OFV) and was used for all subsequent model construction.

Group parameter model

To characterize busulfan pharmacokinetics over the entire age continuum, all clearance (CL,Q) and volume (V1,V2) parameters were scaled for body size and composition using allometric theory and predicted fat free mass (FFM).[19-21] The ABW-available cohort (N=133) was used to estimate the fraction of fat mass (F_{fat}) contributing normal fat mass (NFM) for busulfan. F_{fat} is a drug- and pharmacokinetic parameter-specific quantity; the value of F_{fat} was estimated for each pharmacokinetic parameter.[21] Because ABW was not available for the remaining patients, these F_{fat} parameters were fixed in a second step when the overall cohort was used with an estimated value for total body weight (TBW) based on DWT (Supplemental Figure 1).[22,23] FFM was predicted using equation (A):

$$FFM = WHS_{max} \times HT^2 \times \left(\frac{TBW}{WHS_{50} \times HT^2 + TBW}\right) \quad \textbf{(A)}$$

where WHS_{max} is the maximum FFM for any given height (HT, m) and WHS_{50} is the TBW value when FFM is half of WHS_{max}. WHS_{max} is 42.92 kg/m^2 and 37.99 kg/m^2 and WHS_{50} is 30.93 kg/m^2 and 35.98 kg/m^2 for males and females, respectively.[19] The NFM was predicted using equation B:

$$NFM = FFM + F_{fat} \times (TBW - FFM) \quad \textbf{(B)}$$

ABW was used for total body weight (TBW) when it was available, but for patients whose ABW was not available, TBW was predicted using equation C:

$$TBW = DWT \times FDW \times FFEM_{DW} \quad \textbf{(C)}$$

where DWT is dosing weight provided by the treating institution, FDW is the fraction of DWT contributing to TBW, and $FFEM_{DW}$ is the fraction of DWT that predicts the difference in TBW in women compared to men.

Size differences were described using equation D. Following theory-based allometry,[24] the allometric (Pwr) exponent in equation D was fixed to ¾ for CL and Q and 1 for V1 and V2.

$$F_{size} = \left(\frac{NFM}{70}\right)^{Pwr} \quad \textbf{(D)}$$

F_{size} is the fractional difference in allometrically-scaled size compared to a 70 kg NFM individual. The NFM of 62 kg for CL and 59 kg for V correspond to an allometrically-scaled TBW of 70 kg. A sigmoid E_{max} model was used to describe the maturation of busulfan CL based on post-menstrual age (PMA) using equation E:

$$F_{mat} = \left(\frac{1}{1+\left(\frac{PMA}{TM_{50}}\right)^{-Hill}}\right) \quad \textbf{(E)}$$

where F_{mat} is the fraction of the adult busulfan clearance value, TM_{50} is the PMA at which maturation is 50% of the adult value, and Hill defines the steepness of the change with PMA.[20,21] PMA was estimated by adding a gestational age of 40 weeks to post-natal age.[25]

Differences associated with binary covariates (e.g., sex (F_{sex}) and disease ($F_{disease}$)) were described based on the fractional difference of pharmacokinetic parameter between the two groups. Once all the covariates were defined, covariate factors were combined to predict busulfan clearance for that specific group (CL_{GRP}). Group clearance includes those covariates identified in the model to characterize that specific population's pharmacokinetic parameters (equation F):

$$CL_{GRP} = CL_{pop} \times F_{mat} \times F_{size} \times F_{sex} \quad \textbf{(F)}$$

where CL_{pop} is the overall population value of parameter. A similar model was used for intercompartmental clearance (Q), with F_{mat} and F_{sex} fixed to 1, and for V1 and V2, with F_{mat} fixed to 1.

Random effects

Individual Parameter Model

Population parameter variability (PPV) was described using an exponential model for the random effects (equation G):

$$P_{ij} = P_{pop} \times exp(\eta_i + \kappa_j) \quad \textbf{(G)}$$

where P_{ij} is the parameter value for the i^{th} individual on the j^{th} occasion, and P_{pop} is the population value for the population parameter P (e.g., CL). The random effect model for BSV on TBW was proportional to TBW (equation H):

$$TBW_{ij} = TBW_{pop} \times (1 + \eta_i) \quad \textbf{(H)}$$

Only BSV was estimated for TBW under the assumption that the same DWT method was used on all occasions for an individual patient.

Observation Model

Residual unidentified variability (RUV) was described by assuming a combined model with proportional and additive normal distributions of random differences of the observed concentration-time data from the predicted concentration-time data. BSV in the residual error model was estimated for each observation by obtaining estimates of proportional (θ_{RUV_CV}) and additive (θ_{RUV_SD}) residual error parameters. The BSV of the RUV random effect (η_{PPV_RUV}) was estimated.[22] The ε random effect was fixed with a unit variance (equation I): J):

$$SD_{i,j} = sqrt\left(\left(C_{i,j} \times \theta_{RUV\_CV}\right)^2 + {\theta_{RUV\_SD}}^2\right) \times e^{\eta_{PPV_{RUV,i}}} \quad \textbf{(I)}$$

$$Y = C_{i,j} + SD_{i,j} \times \varepsilon \quad \textbf{(J)}$$

where $C_{i,j}$ is the predicted concentration in the i^{th} individual at the j^{th} measurement time.

Model Selection and Evaluation

Model selection was based on bootstrap parameter confidence intervals, OFV, and the plausibility of visual predictive check (VPC) plots. Measures of parameter imprecision were computed using bootstrap methods.[26,27] VPCs were used to evaluate the overall predictive performance of the model for concentrations.[28] Prediction-corrected VPCs were used to account for differences in covariates and dose adjustments based on previous concentrations.[29]

Initial Dosing Prediction

The initial busulfan dose for Q6h dosing frequency is provided by both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) product labels, as described in Supplemental Table 1. The FDA dosing is based upon the modeling of Booth *et al.*,[30] which recommends busulfan dosing based on ABW in children. In adults, the FDA model recommends using either ABW or ideal body weight, or adjusted ideal body weight (AIBW; equation K) for obese patients.

$$AIBW = IBW + 0.25 \times (TBW - IBW) \quad \textbf{(K)}$$

This differs from the EMA dosing, which is based upon the modeling of Nguyen *et al.*,[31] and has five dosing increments based on either ABW (for children who are not obese) or AIBW in obese adults. We evaluated the EMA dosing, using TBW for obese children and AIBW for

obese adults. A body mass index greater than 28 kg/m^2 was used to define obesity, and IBW or AIBW was applied only for age>16 years (adult) because the IBW calculation may give negative values in children. Using the EMA dosing, we also evaluated the clearance prediction models from three recently published studies [15-17] to calculate initial dose.

After final model construction, dosing simulations were conducted to estimate the initial IV busulfan dose using a daily (i.e., Q24h) dosing frequency. Linear pharmacokinetics after Q6h and Q24h dosing frequency have been reported with IV busulfan.[32] Thus, the initial dosing can be adjusted for any dosing frequency (e.g., dividing by 4 to obtain the intial dose with Q6h dosing frequency). Busulfan target exposure is expressed as Css, which is preferable to AUC because Css incorporates dosing frequency (i.e., Css=AUC/dosing frequency). The FDA and EMA dosing simulation had a busulfan target AUC of 1125 μM×min with a Q6h dosing frequency,[30,31] which equates to a Css of 770 ng/mL. Therefore, a target Css of 770 ng/mL was used for dosing simulations. To determine those within an acceptable range, the therapeutic window for bioequivalence - widely used for drugs with a narrow therapeutic index - was used. It was set as no greater than 25% higher and no less than 20% lower than the target. Therefore, the acceptable therapeutic window equals 592-963 ng/mL.

Computation

Non-linear models were developed using NONMEM (Version 7, Level 2.0) [33] and Wings for NONMEM.[34] The first-order conditional estimate method with the interaction option was used with PREDPP library models. A convergence criterion of three significant digits was used to identify successful minimization. Computation was performed using Intel Xeon, Pentium, Core or Athlon MP2000 processors with Microsoft Windows 2003, Windows XP, or Windows 7. The Intel Visual Fortran compiler (Version 11 or later) with compiler options of /nologo /nbs /w /4Yportlib /Gs /Ob1gyti /Qprec_div was used to compile NONMEM.

RESULTS

Patient characteristics

Patient pre-transplant demographics and HCT characteristics are described in Table 1 with a more detailed description in Supplemental Table 2. For the overall patient population, the mean age was 9.8 years (range: 0.1 to 65.8); the majority (92%) were <20 years old. The majority (904 of 1,610, 56%) of the patients were male. The gestational age and post-natal age were not available; the gestational age was calculated assuming that all infants were of 40 weeks gestation. Of the 466 infants (<2 years old), 256 were less than 1 year and 25 were less than 3 months post-natal age. There were 701 patients (44%) less than four years old, which is the dosing threshold for COG studies, and 451 patients (28%) weighed less than 12 kg, at which weight higher initial IV busulfan doses are recommended per the FDA package insert.[30]

Structural model

The final model consisted of two-compartments for distribution with first-order elimination. There was no evidence for mixed order elimination. Bootstrap population

parameter estimates from the age- and size-dependent model using theory-based allometry are summarized in Table 2. The shrinkage of the random effects for the structural parameters was CL= 17%, V1=31%, Q=31%, and V2=38%. Regarding the distribution process, a sample drawn exactly at the end of the infusion may be too soon to reflect the distribution process predicted from the model. A subset of the data excluding concentration-time points drawn within 5 minutes of the end of the infusion was used with the model developed from all of the data. The parameter estimates were very similar, suggesting that there was no important bias introduced from including the end of infusion concentration-time points.

Table 2. Population pharmacokinetic parameters estimates with theory-based allometric exponents (100 bootstrap replications)

Parameter	Description	Units[a]	Bootstrap Estimate (RSE%)
CL	Clearance	L/h/62kg NFM CL	11.4 (1.1)
V1	Central volume of distribution	L/59kg NFM V	13.9 (6.6)
Q	Inter-compartmental clearance	L/h/62kg NFM CL	135.2 (7.2)
V2	Peripheral volume of distribution	L/59kg NFM V	29.9 (3.0)
$FFAT_{CL}$[b]	Fat fraction for clearance	.	0.509 (42.8)
$FFAT_{V}$[b]	Fat fraction for volume	.	0.203 (51.6)
$TM50_{CL}$	PMA at 50% maturation	.	45.7 (4.3)
$HILL_{CL}$	Hill coefficient for maturation	.	2.3 (9.7)
$FFEM_{V}$	Fractional difference in total volume (V1+V2) in females	.	1.07 (1.2)
$FFEM_{DW}$	Fractional difference in dosing weight in females	.	1.08 (1.7)
FT1_CL	Fraction of 0-6 h clearance >6 and <36 h	.	0.932 (1.2)
FT2_CL	Fraction of 0-6 h clearance ≥36 h	.	0.919 (1.4)
Between Subject Variability (BSV)[a]			
TBW			0.166 (7.8)
CL			0.215 (4.7)
V1			0.410 (10.8)
Q			0.922 (9.1)
V2			0.120 (23.8)
Between Occasion Variability (BOV)[b]			
CL			0.113 (14.8)
V1			0.244 (20.0)
Q			0.577 (24.6)
V2			0.212 (12.4)
RUV_{ADD}[c]	Additive residual unidentified variability	ng/mL	26.2 (13.7)
RUV_{PROP}[c]	Proportional residual unidentified variability	.	0.0387 (12.8)

[a]The NFM of 62 kg for CL and 59 kg for V correspond to allometrically scaled total body weights of 70 kg.
[b]Bootstrap estimates for F_{FATCL} and $FFAT_{V}$ from ABW available data only.
[c]Random effects are expressed as the square root of the estimated variance. BSV and BOV estimates are the apparent coefficient of variation of the variability.

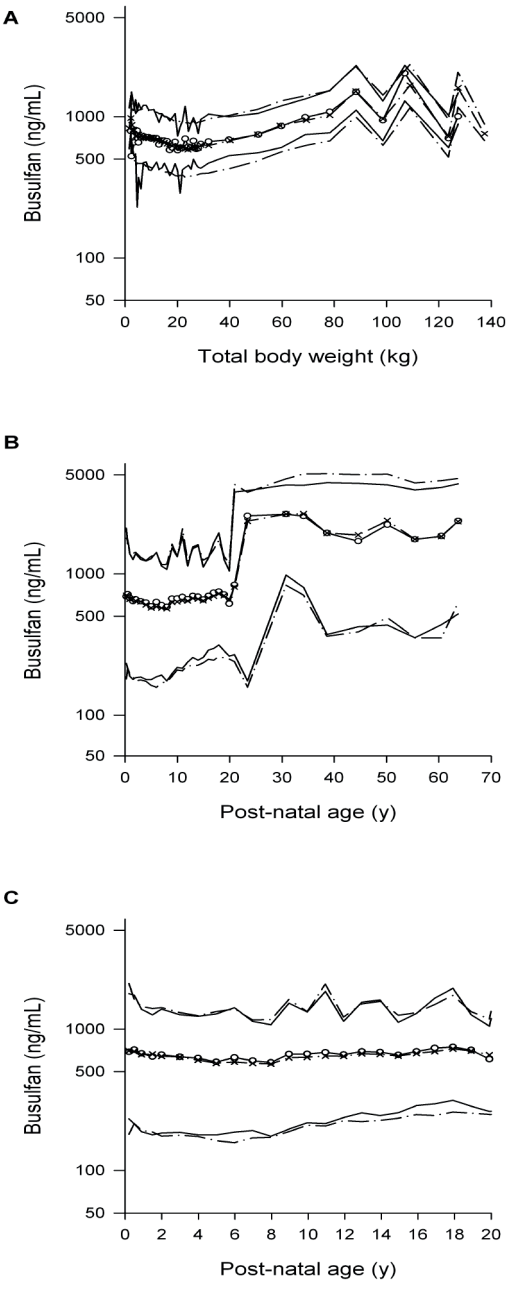

Figure 1. Visual predictive checks (VPCs) by total body weight for overall cohort (A), by post-natal age (PNA) for the overall cohort (B) and by PNA for children (C). Dashed lines represent the 5th and 95th percentiles of the observed data. Solid lines represent the 5th and 95th percentiles of simulated data. Open circles and crosses represent 50th percentile of observed and simulated data. Please note that most adults received daily IV busulfan as per the clinical protocol at the time.

Group parameter model

Because of the large number of patients and the wide spread of body sizes we estimated the allometric exponents for each of the four main pharmacokinetic parameters (Supplemental Table 4). Initial estimates of 2/3 and 1.25 were used for the clearance and volume exponents. Theory-based exponents were confirmed for CL (¾), V1 and V2 (1) (Table 2). The confidence interval for the intercompartmental clearance between V1 and V2 (Q) included the theory-based values for both clearances and volumes, so no conclusion could be drawn about the use of a theory-based value of ¾. When ABW was not available, it was estimated from DWT and sex (equation C). The fraction of dosing weight (FDW) that predicted TBW was indistinguishable from 1 and was fixed to 1, but predicted TBW was 8% higher in women (FFEMDW). The predicted distribution of TBW was similar to that of ABW in children with DWT < 40 kg; however, the TBW was higher than DWT in adults as expected if DWT is based on AIBW (Supplemental Figure 1). The estimates of the size-dependent parameters are expressed per 62 kg of NFM in an adult. Based on the ABW-available data set, the F_{fat} for clearance is 0.509 and for the volume of the central compartment (V1) is 0.203. These values indicate that the biologically effective body size determining clearance is proportional to FFM plus 51% of fat mass, while for volume it is proportional to FFM plus 20% of fat mass. As shown by the VPC (Figure 1), Supplemental Figure 2), this age- and size- model accurately described busulfan pharmacokinetics over the entire age continuum (0.1 – 65.8 years). The maturation of busulfan clearance reaches 50% of adult values at 46 weeks PMA, i.e. 6 weeks after birth assuming a full term gestational age of 40 weeks. Size standardized clearance reaches 95% of adult values at 2.5 post-natal years (Figure 2). In addition, busulfan clearance decreases over time. Compared to the clearance from 0-6 h, the clearance from 6-36h was 6.8% lower and from 36h-83h was 8.1% lower.

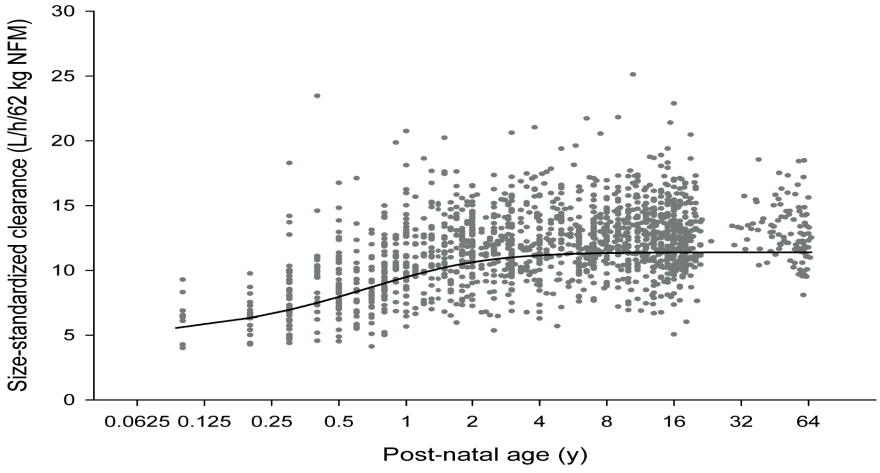

Figure 2. Maturation of size standardized IV busulfan clearance, as L/h/62 kg normal fat mass (NFM). Symbols are empirical Bayes estimates scaled to 62kg NFM. The solid line is the predicted maturation function for IV busulfan clearance.

Female sex was associated with a 7% higher volume of distribution (central and peripheral volume; 11.9 units smaller OFV in ABW-available data set and 35.4 units smaller with the full data set) but there was no effect of sex on clearance. There was no difference in clearance or volume of distribution in patients who had malignancy as their primary diagnosis (N=978) compared with patients with non-malignant diseases (N=632).

Random effects model

Both BSV and BOV were included to account for the potential influence of various factors on busulfan pharmacokinetics, including sex and disease (malignant vs. non-malignant) (Supplemental Table 5). The BSV was moderate for clearance, with greater BSV for the volumes of distribution (V1 and V2) and the intercompartmental clearance between V1 and V2 (Q). A similar trend was observed for the BOV. The BSV and BOV for clearance had apparent coefficients of variation of 21.5% and 11.3% respectively. The BSV around TBW had an apparent coefficient of variation of 16.0%.

Comparison with recent IV busulfan population pharmacokinetic models

By creating this model using data from such a wide age range, we sought to define busulfan pharmacokinetics regardless of age or body size to guide IV busulfan dosing and TDM for any patient with just one model. Our age- and size-dependent model accounted for physiologically-based differences in body composition and ontology over the age range. To evaluate the prediction accuracy in children, we examined recently published busulfan pharmacokinetic models created from pediatric datasets.[15-17] These models were tested by re-estimating the model parameters using our data set (Supplemental Table 6). The Paci and Bartelink models used empirical allometric models for clearance to account for size and maturation, while the Trame model used theory-based allometry without accounting for maturation (Supplemental Table 1). The current data set was more accurately described with the age- and size-dependent model using NFM. Specifically, the Paci model had a worse OFV by 1594 units with the ABW-available data set and by 6,446 units when the full dataset was used. The Bartelink model had a worse OFV by 911 units with the ABW-available data set and by 4097 units when the full dataset was used. Similarly, the Trame model had a worse OFV by 1025 units with ABW-available data set and by 5488 units when the full dataset was used.

Comparison of initial dosing predictions

The empirical Bayes estimate of clearance from our age- and size-dependent model was used to calculate the daily dose of busulfan to maintain the target Css of 770 ng/mL. These individual estimates are expected to be quite precise because the Bayesian shrinkage was only 17%. The initial IV busulfan dose predictions using our age- and size-dependent model (detailed in Methods and Supplemental Table 7) were compared to the FDA and EMA dosing (Table 3). Our age- and size-dependent model led to a higher percentage of patients achieving the therapeutic window compared to the FDA dosing in the entire population ($p<0.0001$), with the differences lying in children <10y. A similar percentage of patients would achieve the therapeutic window using the EMA dosing compared to our age- and size-dependent model in the entire population ($p=0.214$), with a statistically – but most likely not clinically – significant difference in children between 10 to < 15

years. Our age- and size-dependent model had comparable performance to the recent IV busulfan population pharmacokinetic models (Supplemental Table 6).

Table 3. Comparison of accuracy of model-based IV busulfan dose predictions by model and age group to achieve the therapeutic window for busulfan Css of 592 – 963 ng/mL.

Age (years)	% in therapeutic window by method (p-value[a])		
	Age- and size- dependent model	FDA[b]	EMA
All (N=1610[c])	72%	57% (<.0001)	70% (0.214)
≥ 20 (N=128)	82%	83% (0.87)	84% (0.616)
15 to <20 (N=224)	76%	78% (0.575)	80% (0.305)
10 to <15 (N=238)	77%	68% (0.031)	65% (0.005)
5 to <10 (N=249)	78%	49% (<.0001)	71% (0.081)
2 to <5 (N=304)	70%	33% (<.0001)	71% (0.79)
1 to <2 (N=210)	69%	54% (0.001)	72% (0.521)
< 1 (N=256)	62%	54% (0.060)	61% (0.785)

[a]p-value from chi-squared analysis of the number of patients within the therapeutic window by dosing method compared to age- and size-dependent model . [b]The product labeling doses for Q6h dosing frequency are as follows: FDA dosing is 1.1 mg/kg for ≤ 12 kg and 0.8 mg/kg for >12 kg. EMA dosing is 1 mg/kg for <9kg, 1.2 mg/kg for 9 to <16kg, 1.1 mg/kg for 16 to 23kg, 0.95 mg/kg for >23 to 34 kg, and 0.8 mg/kg for >34 kg. COG trials AAML03P1 and AAML0531 recommended initial busulfan doses for Q6h dosing frequency: 0.8mg/kg for <10kg, 1 mg/kg for ≥10 kg and ≤4 years old, 0.8 mg/kg for >4 years old. [c]Age was unavailable for one patient.

DISCUSSION

We sought to create an IV busulfan pharmacokinetic model that is generalizable to all patients, which was achieved by using this age- and size-dependent model (Table 2). Our main findings are: 1) this age- and size-dependent model accurately predicts IV busulfan concentrations over a wide range of body weights and ages (Figure 1); 2) IV busulfan clearance reaches 95% of adult values at 2.5 post-natal years (Figure 2); 3) the model yields similar pharmacokinetic parameters compared to recently reported population pharmacokinetic models from smaller, exclusively pediatric populations; 4) initial dosing predictions indicate that our age- and size-dependent model performs well compared to other methods, especially FDA dosing guidelines (Table 3).

This study has provided the first adequately-powered test confirming theory-based allometry for clearance and volume parameters. The maturation of clearance in infants has been described for many drugs using a sigmoid function of PMA.[21] Although the function is empirical, it has physiological limits. Specifically, these limits predict a clearance of zero

at conception and approach adult values as maturation is completed. We have applied the same maturation function to busulfan and find that maturation reaches 50% of adult values at 46 weeks PMA. Busulfan clearance reaches 95% of adult values at 2.5 post-natal years. An earlier analysis of a subset of the current data that found children less than four years of age had lower busulfan clearance than adults using BSA scaling without considering body composition.[3] Using physiologically-based descriptions of body composition and theory-based allometric principles, we have shown that busulfan clearance and volume are predicted neither by TBW nor by FFM, but by a size that lies between the two. We recognize that our dataset is limited because ABW was available in only 133 patients. However, DWT was available for all 1610 patients; many of the previously published busulfan population pharmacokinetic models were created with only DWT (Supplemental Table 1). There are few population pharmacokinetic models of IV busulfan from adults (N=37[12] to 127[10]). It should be appreciated that our age- and size-dependent model was constructed using data from one of the largest studies in adults (N=128). Likewise, the age- and size-dependent model may also improve IV busulfan dosing in the obese. The paucity of pharmacokinetic data for chemotherapy dosing in obese patients is gaining attention, and pooling data from previous studies to test chemotherapy dosing recommendations for obese patients has recently been encouraged.[35] Validation of the model in adult populations – particularly the obese – is needed, as our results clearly show our age- and size-dependent model predicts busulfan pharmacokinetics as well as the existing models generated from pediatric data (Supplemental Table 6). Notably, busulfan pharmacokinetic parameters were not influenced by disease (malignant vs. non-malignant), which is consistent with previous data.[3,36] Also consistent with previous data[37,38] was our observation of a slight decrease in busulfan clearance over time.

This data set was obtained from 51 institutions that were targeting IV busulfan doses for clinical purposes, thus providing an accurate assessment of the challenges of personalizing doses based on pharmacokinetics (i.e., TDM). Only a minority of concentrations (367 of 12,747, see Supplemental Methods) were considered problematic, proving that TDM is feasible in a clinical setting. Our recent analysis of prescribing patterns in 729 pediatric HCT recipients revealed that the initial busulfan dose achieved the target exposure in only 24.3% of children.[3] Appreciable debate regarding the optimal initial IV busulfan dose has resulted from the Trame report.[15,39] The FDA dosing guidance was based on simulations using a pediatric population pharmacokinetic model that indicated that ~60% of children would achieve a busulfan Css between 615 and 925 ng/mL.[30] Nguyen *et al.* had developed a 5-category dosing guidelines (i.e., EMA dosing) that was expected to achieve a mean busulfan Css of 770 ng/mL based on a different pediatric population pharmacokinetic model.[31] The success of the EMA dosing guidance to achieve a busulfan Css of 615 to 1025 ng/mL without TDM has been variable.[15,40-42] Recently, Trame *et al.* created a busulfan population pharmacokinetic model from 94 children receiving oral (N=54) or IV (N=40) busulfan.[15] Their simulations revealed that only 44% of children would achieve a busulfan Css of 615 to 1025 ng/mL when EMA dosing was used without TDM, and that a higher proportion (70-71%) would achieve this therapeutic window with dosing based on BSA or allometric body weight. Our age- and size-dependent model performed similarly to the Trame model (Supplemental Table 6). In addition, compared to FDA dosing, our model can more accurately estimate the initial IV busulfan dose to more rapidly achieve the therapeutic

busulfan Css (Table 3). Our model did appreciably better than FDA dosing for children < 10 years, and achieved a similar percentage within the therapeutic window as the EMA dosing. The generalizability of our model provides a robust tool for prescribers to dose busulfan with minimal concern regarding the original population from which the model was constructed.

To our knowledge, we are the first to describe the maturation of IV busulfan clearance; our data modeling indicates that at 2.5 years of age IV busulfan clearance is essentially (95th percentile) that of adults. Collection of PMA would be useful for implementation of this model for estimating IV busulfan clearance in children < 2.5 years. These covariates can be used for initial IV busulfan dosing and also for TDM. Dose prediction can be based on a prescriber-chosen target exposure. There has been a practice trend towards a Q24h instead of the traditional Q6h dosing frequency.[2] A target exposure expressed using Css is preferable to expression using AUC because Css (i.e., Css=AUC/dosing frequency) incorporates the dosing frequency. This allows the prescriber to choose a single target Css and dosing frequency independently. Subsequent dose personalization can take place using measured busulfan concentrations. The estimated BOV in clearance (11.3%) indicates that 95% of patients can expect to achieve a Css within the 80-125% acceptable therapeutic window [43] with appropriate dose adjustment.

CONCLUSION

In conclusion, we built a novel population pharmacokinetic model, reliant on the largest busulfan database to date, that spans a wide age range (i.e., neonates to adults), accounting for age, body weight, and body composition (i.e., NFM). The model is based on principles that have already been shown to be robust for predictions with other small molecule agents from neonates to adults.[44] Future work should focus on incorporation of this model into a decision support system that includes relevant clinical data in a user-friendly interface to clearly communicate the optimal busulfan dose for HCT recipients. This model can accurately estimate the initial busulfan dose, hopefully improving upon the current initial dosing practices in which only 24.3% of children achieve the patient-specific therapeutic window of busulfan exposure.[3] Furthermore, by including pharmacokinetic sampling, this model can also be used for more efficient TDM by using Bayesian predictions for personalized busulfan dosing, which has been previously used in HCT recipients.[9,13]

ACKNOWLEDGEMENTS

This work was supported by in part by research funding from Otsuka Pharmaceutical of North America (JSM), UW DMTPR (JSM), University of Auckland (NHGH), Children's Oncology Group Chair's grant NIH U10 CA98543 (AG), SDC U10 CA98413 (AG), U10 HD037255 (JSB), and RC1 LM010367-01 (JSB).

The authors wish to thank Ms. Erin Dombrowsky for her invaluable assistance in streamlining data integration through the development of SAS macros.

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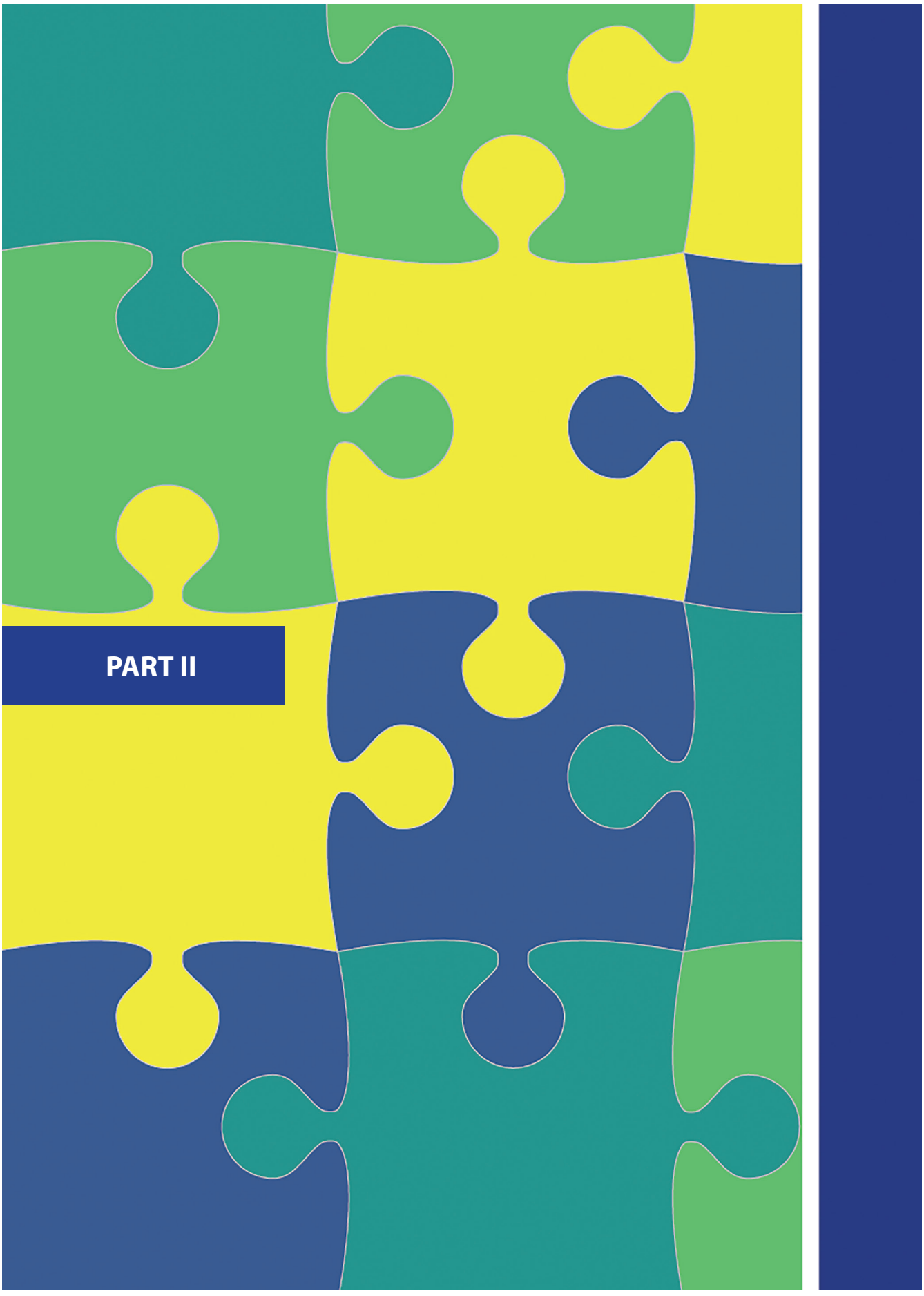
PART II

Strategies for Predicting Busulfan Clearance

CHAPTER 5

Pharmacogenetics of Intravenous and Oral Busulfan in Hematopoietic Cell Transplant Recipients

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J Clin Pharmacol. 2011 Oct;51(10): 1429–1438

ABSTRACT

Kinetics-based dose targeting is often conducted in hematopoietic cell transplant (HCT) patients conditioned with intravenous (IV) or oral busulfan to lower rates of rejection, nonrelapse mortality, and relapse. Using the candidate gene approach, we evaluated whether busulfan clearance was associated with polymorphisms in the genes regulating the predominant metabolizing enzymes involved in busulfan conjugation, specifically glutathione *S*-transferase (GST) isoenzymes A1 *(GSTA1)* and M1 (*GSTM1*). Busulfan clearance was estimated after the morning dose on days 1, 2, and 3; each patient's average clearance was used for analyses. The average ($\pm$ standard deviation) busulfan clearance was 3.2 $\pm$ 0.56 ml/min/kg in the separate population of 95 patients who received oral busulfan and 103 $\pm$ 24 ml/min/m^2 in the 57 patients who received IV busulfan. Oral busulfan clearance was associated with *GSTA1* (p=0.008) but not *GSTM1* (p=0.57) genotypes. However, among the *GSTA1* haplotypes (i.e., **A*A*, **A*B*, **B*B*), there was significant overlap in the observed oral busulfan clearance and similar rates of achieving the target busulfan exposure. Clearance of IV busulfan was not associated with *GSTA1* (p=0.21) or *GSTM1* (p=0.99). These data suggest that personalizing either IV or oral busulfan dosing cannot be simplified on the basis of *GSTA1* or *GSTM1* genotype.

INTRODUCTION

The alkylating agent busulfan (BU) is an integral part of many hematopoietic cell transplantation (HCT) conditioning regimens.[1] Dosing busulfan based on body weight or body surface area (BSA) is associated with considerable interpatient variability in the efficacy and toxicity of busulfan-containing conditioning regimens. This variability in clinical outcomes is due, in part, to interpatient differences in busulfan clearance which result in variable systemic exposure, expressed as area under the plasma concentration-time curve (AUC) or concentration at steady state (C_{ss}=AUC/dosing interval).[2] Pharmacodynamic relationships of busulfan systemic exposure and hepatic veno-occlusive disease (VOD-also referred to as sinusoidal obstruction syndrome), rejection, and disease relapse have been observed in patients receiving BU/cyclophosphamide (CY) conditioning and more recently BU/fludarabine conditioning.[2,3] These observations have led many HCT centers to use therapeutic drug monitoring, which is also termed kinetics-based[4] dose targeting, in patients receiving either intravenous (IV) or oral busulfan.

Kinetics-based dose targeting of busulfan involves the collection of several (usually 4-7) blood samples at known time points after busulfan administration, which are subsequently used to calculate an individual's busulfan clearance. Effective kinetics-based busulfan dose targeting has been shown to lower rates of rejection, nonrelapse mortality, and relapse in select HCT recipients.[5,6] However, the resource intensity of pharmacokinetic sampling has been a barrier to universal acceptance of busulfan dose targeting. Although the recent increase in kinetics-based dose targeting of busulfan shows that this strategy is feasible, more efficient methods to estimate busulfan systemic exposure and clearance (as clearance = dose/AUC) are desirable. In addition, targeting busulfan doses based on genetic polymorphisms may decrease the need for the resource intensive kinetics-based dose targeting that requires quantitation of busulfan concentrations in plasma with subsequent pharmacokinetic modeling.

After IV administration of radiolabeled busulfan, less than 50% of the administered dose is recovered in the urine.[7,8] Approximately one-third (i.e., 32.8$\pm$2.2%) of busulfan is irreversibly bound to plasma proteins, primarily albumin.[9] Only a small fraction (<3%) of a busulfan dose is excreted unchanged in the urine, with neglible amounts in the feces.[10-12] The primary elimination route for busulfan is glutathione conjugation, resulting in formation of gamma -glutamyl-beta -(S-tetrahydrothiophenium)-alanyl-glycine (GS-THT$^+$).[13-15] The initial step in hepatic metabolism of busulfan is conjugation, catalyzed by glutathione S-transferase (GST).[16-18] The enzymatic process is predominantly conjugated by GST isoenzyme A1-1 based on hepatic protein expression of GSTA1.[18] Other GST isoenzymes, GSTM1 and GSTP1, contribute to busulfan conjugation at ~5% and 0.2%, respectively, after accounting for their lower activity for busulfan conjugation and lower hepatic expression relative to GSTA1.[19] Therefore, genetic polymorphisms regulating GSTA1 and GSTM1 hepatic protein expression are likely to be of most importance to busulfan conjugation and thus, clearance.[19-21] Conflicting *in vitro* data have been presented regarding the relationship between hepatic expression of GSTA1 and single nucleotide polymorphisms (SNPs) in the proximal promoter region of the *GSTA1* gene (i.e., *GSTA1*A, *B*).[19,20] The *GSTM1* null genotype is associated with lower hepatic protein expression, and

an increased risk of VOD in β-thalassemia patients receiving BU/CY with antithymocyte globulin (ATG) conditioning.[22]

Thus, we sought to evaluate the association of *GSTA1* haplotype and *GSTM1* genotype with IV or oral busulfan clearance in two separate HCT populations.

MATERIALS AND METHODS

Study populations

This was a retrospective study in two patient cohorts who received HCT conditioning with either IV or oral busulfan. The cohorts were separate and no patients received both IV and oral busulfan. All patients had their busulfan dose personalized to a target busulfan C_{ss} using kinetics-based busulfan dosing. Patients were enrolled in Fred Hutchinson Cancer Research Center (FHCRC) clinical treatment protocols from June 2002 to November 2006 (IV busulfan) and from July 2004 to January 2006 (oral busulfan). Approval of the FHCRC Investigational Review Board was obtained prior to data analysis. Inclusion criteria were as follows: patients receiving their first HCT, receiving a conditioning regimen containing busulfan, and being enrolled in protocols that stipulated busulfan kinetics-based dose targeting. For oral busulfan only, children less than 10 years of age were excluded from this analysis because of previously documented age-dependent clearance.[13] For IV busulfan, 67 patients met the inclusion criteria, but genomic DNA was not available from 10 participants; thus, the final database contained information on 57 patients who received IV busulfan. The criteria were met in 131 patients receiving oral busulfan; however, *GSTA1* and *GSTM1* genotypes could not be obtained for 34 and 2 patients, respectively. Thus, the final database contained 95 patients who received oral busulfan.

Records were examined for demographic data (i.e., age, sex, height, weight, body surface area), and clinical data (i.e., disease, conditioning regimen). The ideal body weight in adults was calculated as follows: for males = 50 kg + (2.3 kg for each inch over 5 feet); for females = 45.5 kg + (2.3 kg for each inch over 5 feet). The adjusted ideal body weight (AIBW = ideal body weight + 0.25 (actual body weight - ideal body weight) was determined.[23] Body surface area (BSA) was calculated using height and AIBW; BSA was calculated by taking the square root of actual weight × height, divided by 60. Standard practice for prophylaxis to busulfan-induced seizures was phenytoin.

Intravenous busulfan dosing and pharmacokinetic sampling

Intravenous busulfan was administered every six hours (Q6hr) to 18 patients and every 24 hours (i.e., daily) to 39 patients. All patients received busulfan for 4 days for a total of 16 doses (if administered Q6hr) or 4 doses (if administered daily). The first busulfan dose and the target busulfan C_{ss} were based upon the FHCRC treatment protocol and could be adjusted by the attending physician. In those patients receiving Q6hr IV busulfan, the mean (± standard deviation) first busulfan dose was 0.92 ± 0.15mg/kg and the cumulative busulfan dose ranged from 11-23 mg/kg. For 22 of the patients receiving daily IV busulfan, the first IV busulfan dose was 3.2 mg/kg for a total IV busulfan dose of 9.7-20 mg/kg. Based upon the average clearance after daily IV busulfan in this cohort, the first IV busulfan dose

was increased to 4 mg/kg in the subsequent 17 patients. These patients received a total dose of 10-19 mg/kg.

A total of 21 pharmacokinetic blood samples were obtained from each patient receiving IV busulfan. Patients receiving IV busulfan Q6hr had pharmacokinetic sampling after the morning dose on days 1 through 3 at the following times relative to the start of the 2 hour infusion: end of infusion, and at 2.25, 2.5, 3, 4, 5, and 6 hours (i.e., prior to subsequent dose). Those patients receiving daily IV busulfan had pharmacokinetic sampling on days 1 through 3 at the following time points relative to the start of the 3 hour infusion: end of infusion, and at 3.25, 4.5, 6, 8, 11 and 24 hours (i.e., prior to subsequent dose).

Oral busulfan dosing and pharmacokinetic sampling

Oral busulfan was administered every six hours (Q6hr) for 4 days for a total of 16 doses. The first busulfan dose and the target busulfan C_{ss} were based upon the FHCRC treatment protocol and could be adjusted by the attending physician. For all patients receiving oral busulfan, the mean first oral busulfan dose was 1 $\pm$ 0.01 mg/kg and a total oral busulfan dose ranging from 9.3-24 mg/kg.

For each patient receiving oral busulfan, a total of 19 pharmacokinetic blood samples were obtained. Pharmacokinetic sampling was conducted after the morning dose on days 1 through 3. Samples were obtained immediately before and 30, 60, 90, 120, 180, 240, 300, and 360 minutes after oral administration on day 1. On days 2 and 3, samples were obtained immediately before and 60, 120, 240, and 360 minutes after oral busulfan administration.

Quantitation and Pharmacokinetic Modeling of Busulfan Samples

Busulfan concentrations were determined by gas chromatography with mass spectrometry detection as previously described.[24] Dynamic range was from 25 to 4500 ng/mL, and the inter-day CV was less than 8%. After quantitation of busulfan samples, the concentration-time data were fit using WinNonlin (version 5.0.1) via noncompartmental (IV or oral) or compartmental (IV only) modeling, determined by visual inspection of the model fit to the individual concentration-time data. The AUC from time 0 to infinity (∞) was calculated after the first dose. The AUC from 0 to the end of the dosing interval (abbreviated tau [τ]) was calculated after the day 2 and day 3 doses in patients receiving busulfan Q6hrs (IV and oral) and the AUC from time 0 to infinity (∞) was calculated after the day 2 and day 3 doses in patients receiving busulfan daily (IV only). The $AUC_{0\ to\ \infty}$ after the first dose equals the $AUC_{0\ to\ \tau}$ at steady state (i.e., days 2 and 3).[25] Clearance was calculated by dividing the dose by the AUC. After calculation of the patient's clearance, the target dose for subsequent doses was calculated linearly to achieve the target C_{ss}, which was chosen by the attending physician. Successful targeting is confirmed by determining the busulfan clearance after the morning dose on days 2 and 3, with further dose adjustments as needed.

Preparation of DNA

Peripheral blood leukocytes were collected from freshly obtained samples and stored at the FHCRC DNA repository (Clinical Research Division). Genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) from peripheral blood leukocytes.

The quality of DNA samples was tested by spectrophotometric analysis at 260 and 280 nm before genotyping.

Determination of GSTA1 and GSTM1 Genotypes
Genotyping was performed at the DNA Sequencing and Gene Analysis Center (Department of Pharmaceutics, University of Washington, Seattle, WA). The four sequence variants of the human *GSTA1* proximal promoter that define the **A* and **B* alleles (-631T or G, -567T, -69C, -52G and –631G, -567G, -69T, -52A, respectively) were determined by direct sequencing of a 780 bp amplicon.[20] The cycling conditions for PCR were as follows: 95˚C for 2 min; 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 2 min; 72˚C for 7 min; hold at 4˚C. The PCR reaction contained 1.25 U PfuTurbo Hotstart DNA polymerase (Stratagene, La Jolla, CA) in 1X reaction buffer with 200 µM dNTPs and 500 nM oligonucleotides with ~50-150 ng genomic DNA template in a total volume of 25 µl. The PCR amplicons were purified on QIAquik PCR purification columns (Qiagen) according to manufacturer's instruction. The primer sequences for PCR (AF7 and AR1) were taken from the original citation[20] and BigDye Terminator V 3.1 (Applied Biosystems, Foster City, CA) sequencing of the purified amplicon was performed using both primer AF7 and a second internal primer (hGSTA1.seq.for 5'CCAGCTATGCTCACAGTAGAG3') to identify the SNPs at –631/-567 and –69/-52, respectively.

GSTM1 gene deletion genotyping was performed using the Applied Biosystems "Taqman® Gene Copy Number Assay" reagents according to the manufacturer's protocol. In brief, DNA (~25 ng) was combined with 5 mL Taqman 2X MasterMix, 1 mL 20X GSTM1 6-FAM-labeled assay (PN4331182) and 1 mL 20X RnaseP VIC-labeled assay (PN4316844) in a total volume of 10 mL. Each sample was run in triplicate in a 96 well PCR plate on a ABI 7900HT (Applied Biosystems) instrument with the following thermal cycling conditions: 95˚C for 10 min and 40 cycles at 95˚C for 15 sec, 60˚C for 1 min. The real-time PCR data were collected using ABI sequence detection system software (SDS V2.1) (Applied Biosystem) and the cycle threshold (C_t) values for GSTM1 and RnaseP triplicates calculated. The C_t values for the RnaseP gene served as a reference when analyzing the samples for the presence or absence of *GSTM1* sequences. Each assay included controls known to contain (or not to contain) the *GSTM1* gene, and patient genotypes were called as either being *GSTM1* positive or *GSTM1* null; no attempt was made to determine zygosity.

Statistical Methods.
An *a priori* power analysis was conducted using our clinical database to assist with kinetics-based dosing of IV or oral busulfan (reported in part in McCune and Holmberg[26]). Eight patients per *GSTA1* genotype group would provide 80% power to observe a 20% difference in IV busulfan clearance. For oral busulfan clearance, six patients per *GSTA1* genotype group would provide 80% power to observe a 20% difference. All statistical analyses were performed using SAS version 9 (SAS, Inc., Cary, NC).

Each participant's average busulfan clearance was calculated from busulfan clearance on days 1, 2 and 3. Log transformation of the average busulfan clearance was performed to normalize the distribution of data before inferential analysis, as specified *a priori*. Categorical patient variables included frequency of administration (Q6hr vs. daily, IV

clearance only), *GSTA1* genotype (categorized as **A*A*, **A*B*, or **B*B*) and *GSTM1* genotype (categorized as null or present). Continuous patient variables included age at the time of HCT, BSA, and AIBW, which was done for the IV busulfan dataset only as this population had greater heterogeneity.

A linear regression analysis was conducted on log average busulfan clearance as the dependent variable to identify which variables were significant predictors of clearance. All predictors with a $p<0.05$ were considered significant.

Genotype frequencies of *GSTA1* and *GSTM1* were determined to be in Hardy-Weinberg equilibrium using χ^2 analysis.

RESULTS

Patient characteristics

Patient pre-transplant demographics and HCT characteristics are described in Table 1.

Table 1. Description of patient population.[a]

Group	IV Busulfan	Oral Busulfan
N	57	95
Age (years)		
Mean ± standard deviation (SD)	38 ± 21	45 ± 12
N < 18 years	13	1
AIBW (kg)		
Mean ± SD	58 ± 25	66 ± 10
N < 12 kg[a]	8[a]	0
Body mass index[c] (kg/m²)		
Mean	27 ± 6.9	66 ± 10
Underweight, n (%)	8 (14)	0
Normal (18.5-24.9) , n (%)	22 (39)	34 (36)
Overweight (25.0-29.9) , n (%)	18 (32)	30 (32)
Obesity I (30.0-34.9) , n (%)	5 (9)	21 (22)
Obesity II (35.0-39.9, n (%))	1 (2)	7 (7)
Extreme Obesity (40.0+), n (%)	3 (5)	3 (3)
Ethnic background, n (%)		
White	46 (81)	76 (80)
Asian	2 (4)	6 (6)
African American	1 (2)	5 (5)
East Indian	1 (2)	3 (3)
Hispanic	1 (2)	2 (2)
Native American	1 (2)	2 (2)
Unknown	5 (9)	1 (1)
Gender, n (%)		
Male	34 (60)	48 (51)
Female	23 (40)	47 (49)

Group	IV Busulfan	Oral Busulfan
Conditioning Regimen, n (%)		
Busulfan + Flu ± ATG	39 (68)	0
Busulfan + CY ± ATG ± etoposide	15 (26)	94 (98)
Busulfan + Flu + CY	2 (4)	1 (1)
Busulfan + melphalan	1 (2)	0
Diagnosis, n (%)		
Myelodysplastic syndrome	17 (30)	27 (28)
Acute myeloid leukemia	13 (23)	29 (31)
Acute myeloid leukemia/myelodysplastic syndrome	0	6 (6)
Myelofibrosis	6 (11)	9 (10)
Chronic myelomonocytic leukemia	5 (9)	2 (2)
Wiskott-Aldrich syndrome	3 (5)	0
Osteopetrosis	3 (5)	0
Chronic myeloid leukemia	2 (4)	16 (17)
Hemophagocytic lymphohistiocytosis	2 (4)	0
Agnogenic myeloid metaplasia	1 (2)	2 (2)
Multiple myeloma	1 (2)	0
Blackfan-Diamond anemia	1 (2)	0
Congenital dyserythropoietic anemia	1 (2)	0
Sickle cell anemia	1 (2)	0
β-Thalassemia	1 (2)	0
Myeloprolierative disorder	0	3 (3%)
Non-Hodgkin lymphoma	0	1 (1%)

AIBW, adjusted ideal body weight; CY, cyclophosphamide; Flu, fludarabine; IV, intravenous; ATG, antithymocyte globulin, [a]within the package insert, children less than 12 kg should receive a higher initial busulfan dose than heavier children and adults; [c]categorized per National Heart Lung and Blood Institute

For patients receiving IV busulfan, the median age was 43 years (range: 0.5-66). Thirteen of the patients (23%) were less than 18 years of age, and eight patients (14%) weighed less than 12 kg, at which weight higher initial IV busulfan doses are necessary per the package insert.[12] Sixty percent (34 of 57) of the patients receiving IV busulfan were male. The majority of patients receiving IV busulfan (41 of 57, 71%) received busulfan plus fludarabine conditioning. Forty-six patients (81%) received ATG as part of the conditioning regimen. Additionally, etoposide and melphalan were given to a limited number of patients (4% and 2%, respectively). No other antineoplastic agents or irradiation were given immediately before or concomitantly with busulfan.

Of those patients receiving oral busulfan, the median age was 48 years (range: 16-63); only one patient (1%) was less than 18 years of age. Fifty percent (48 of 95) of the patients were male. The majority of patients receiving oral busulfan received busulfan plus cyclophosphamide (60 mg/kg/dose, once daily for two days) conditioning. Three patients (3%) received ATG as part of the conditioning regimen. Additionally, fludarabine was given to one patient (1%). No other antineoplastic agents or irradiation were given immediately before or concomitantly with busulfan.

IV busulfan pharmacokinetics

The average (± standard deviation) IV busulfan clearance was 103 ± 23.6 ml/min/m^2, consistent with busulfan clearance in other HCT populations receiving concomitant phenytoin.[27] Busulfan clearance did not differ by age (Figure 1) or by administration frequency (i.e., Q6 hours compared to daily, data not shown). Thus, subsequent statistical analyses were conducted using busulfan clearance standardized by BSA (i.e., ml/min/m^2) in all 57 patients. Of note, IV busulfan clearance standardized to BSA (i.e., ml/min/m^2) is optimal because liver weight expressed relative to BSA (g/m^2) is similar for children and adults.[13,28] Liver weight expressed relative to body weight (g/kg) is higher in young children than in older children and adults.[13,28]

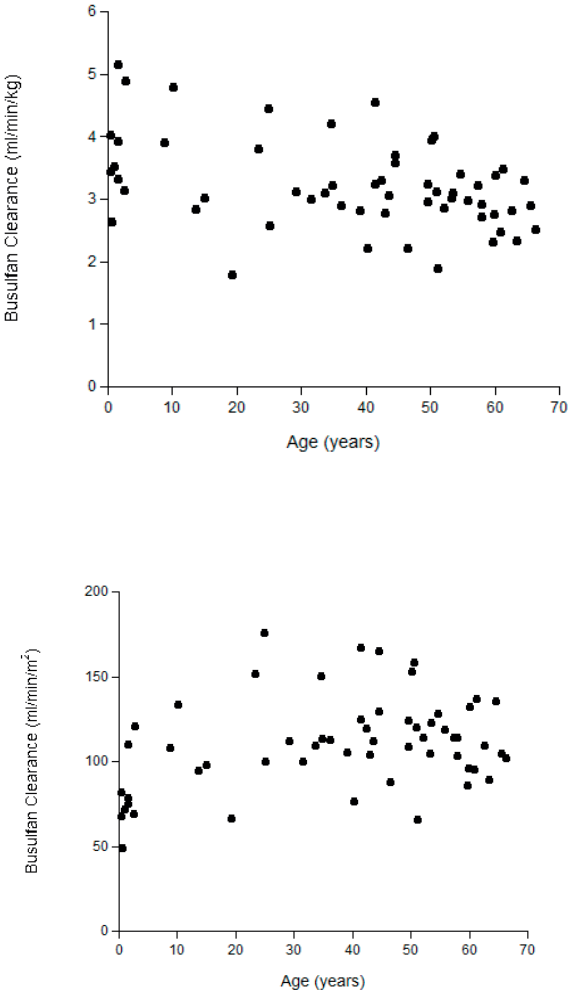

Figure 1. Association of Age with IV Busulfan Clearance Standardized by Body Weight (panel A) or by Body Surface Area (panel B).
Figure 1a (top) and 1b (bottom).

Oral busulfan pharmacokinetics

The average (± standard deviation) busulfan clearance was 3.2 ± 0.56 ml/min/kg (119 ± 20 ml/min/m^2), consistent with busulfan clearance in other HCT populations receiving concomitant phenytoin.[26]

Genotype Frequencies

The frequencies of *GSTA1* and *GSTM1* genotypes observed in the IV and oral busulfan cohort are displayed in Table 2. All genotypes were within the Hardy-Weinberg equilibrium (P>0.05).

Table 2. *GSTA1* and *GSTM1* Frequencies.

Gene	Allele	Reported frequency (%)[20,21]	IV Busulfan Cohort, n (%)	Oral Busulfan Cohort, n (%)
GSTA1	haplotype			
	**A*A*	38	23 (40)	36 (38)
	**A*B*	48	23 (40)	40 (42)
	**B*B*	14	11 (19)	19 (20)
GSTM1	genotype			
	Null (-/-)	47	30 (47)	40 (42)
	Present (+/- or +/+)	53	27 (53)	55 (58)

IV, intravenous

Pharmacogenetic Associations with IV Busulfan Clearance

We analyzed the association between IV busulfan clearance and the genetic variants of *GSTA1* and *GSTM1*. Figure 2 shows the average and the range of IV busulfan clearance by genotype. Neither genotype was statistically associated with busulfan clearance [*GSTA1* (p=0.21, panel A) and *GSTM1* (p=0.99, panel B)].

Pharmacogenetic Associations with Oral Busulfan Clearance

We analyzed the associations between oral busulfan clearance and the *GSTA1* and *GSTM1* genotypes in this study population. Figure 3 shows the average and the range of oral busulfan clearance by genotype, specifically *GSTA1* (p=0.008, panel A) and *GSTM1* (p=0.57, panel B). The *GSTA1* haplotype was statistically significantly associated with oral busulfan clearance, with those patients with germline *GSTA1*A*A* or **A*B* haplotype having a higher oral busulfan clearance than those carrying the *GSTA1*B*B* haplotype. Those patients with the *GSTA1*A*A* haplotype had an oral busulfan clearance that was 0.45 ml/min/kg (16%) higher, on average, than those with *GSTA1*B*B* genotype. Similarly, the oral busulfan clearance of patients with the *GSTA1*A*B* genotype was, on average, 0.41 ml/min/kg (14%) higher than patients with *GSTA1*B*B*. *GSTM1* genotype was not associated with oral busulfan clearance (Figure 3B).

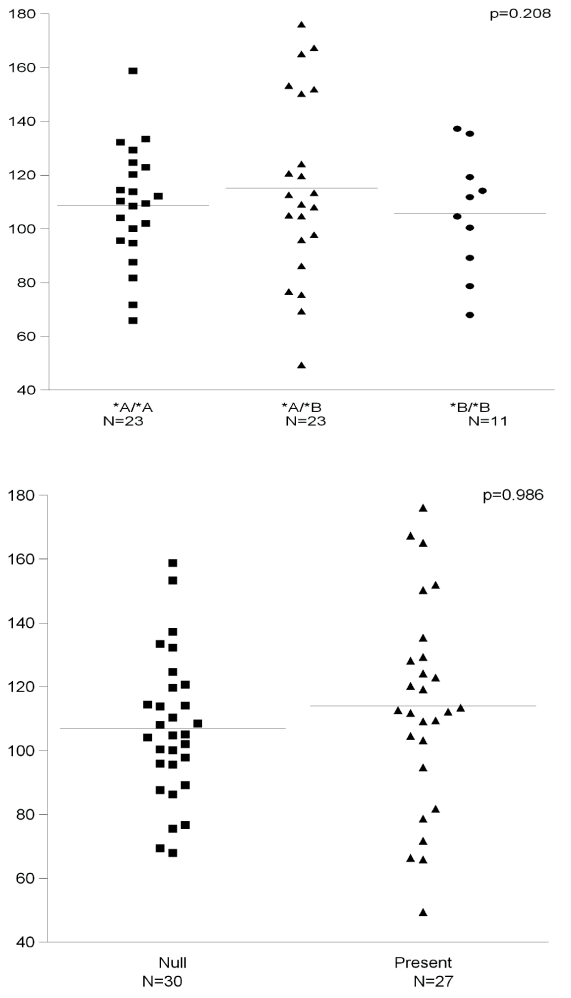

Figure 2. Lack of Association of IV Busulfan Clearance with *GSTA1* (panel 2A (top), p=0.208) and *GSTM1* (panel 2B (bottom), p=0.986). Bars indicate mean values for each category.

We then sought to evaluate the impact of *GSTA1* haplotype upon kinetics-based dosing of oral busulfan to a target busulfan C_{ss}. As shown in Table 3, those patients with the *GSTA1*B*B* haplotype often had their oral busulfan dose decreased from the starting weight-based dose (i.e., dose #1), as evidenced by the mean ratio of dose 5/dose 1 of 0.88. There is considerable interpatient variability in the range of the dose adjustments. The most common busulfan C_{ss} target in the oral busulfan cohort was 800 to 900 ng/ml, which was the target C_{ss} for 76 of 95 patients (80%). Across all three haplotypes, the percentage of patients achieving the target C_{ss} after dose 1 was low (i.e., 20-32%). Specifically, the target C_{ss} was achieved after dose 1 in 32% of the 28 *GSTA1*A*A* carriers, 20% of the 30 *GSTA1*A*B*

carriers and 28% of the 18 *GSTA1*B*B* carriers. The target C_{ss} after all the busulfan doses was achieved among almost all the patients (i.e., 93-94%).

Table 3. Oral busulfan dose adjustments to achieve target busulfan C_{ss} according to *GSTA1* haplotype.

	GSTA1 Haplotype		
	**A/*A*	**A/*B*	**B/*B*
Number of patients (Total = 95)	36	40	19
Dose 5 / Dose 1	1.04 ± 0.15 (0.79 – 1.52)	1.01 ± 0.20 (0.46 – 1.41)	0.88 ± 0.13 (0.60 – 1.16)
Dose 9 / Dose 5	1.02 ± 0.09 (0.88 – 1.24)	1.02 ± 0.09 (0.80 – 1.23)	1.00 ± 0.07 (0.91 – 1.14)

Data are presented as mean ± standard deviation (range). Busulfan dose 1 was 1 mg/kg for all patients; doses 5 and 9 were adjusted to achieve target C_{ss}.

DISCUSSION

We sought to gain a better understanding of the genetic covariates associated with busulfan clearance with the long-range goal of discovering less resource-intensive methods to target both IV and oral busulfan doses in HCT patients. The key findings of this analysis were: 1. Using the candidate gene approach, no significant pharmacogenetic associations were observed between IV busulfan clearance and reported genetic variants of *GSTA1* or *GSTM1* (Figure 2); 2. Although oral busulfan clearance was associated with *GSTA1* haplotype, targeting oral busulfan doses appeared unlikely to be simplified by genetics-based dosing due to the considerable interpatient variability amongst the three *GSTA1* haplotypes (Figure 3A).

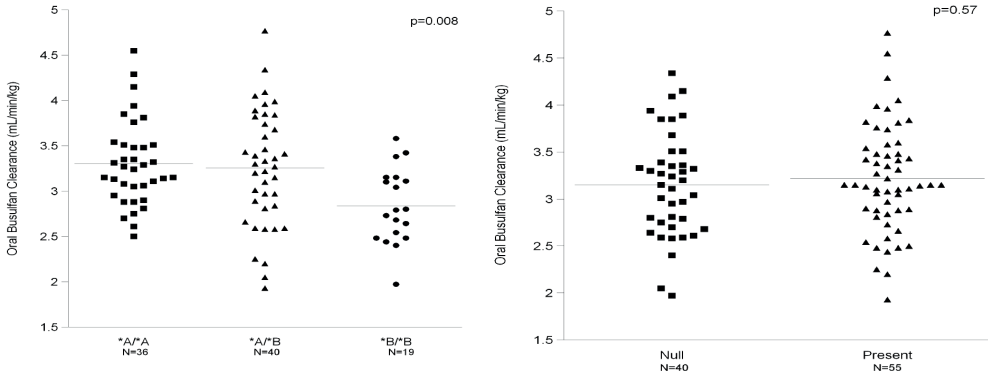

Figure 3. Association of Oral Busulfan Clearance with *GSTA1* (panel 3A (left) P=0.008) but not *GSTM1* (panel 3B (right) p=0.57). Bars indicate mean values for each category.

Use of the established *GSTA1* and *GSTM1* genotypes to predict IV and oral busulfan clearance cannot be recommended at this time. The genotypes evaluated were chosen based on their association with hepatic protein expression.[19-21] Our dataset was adequately powered to reveal a 20% difference in busulfan clearance between *GSTA1* haplotypes. The data regarding the importance of these *GST* polymorphisms to HCT patients receiving busulfan are conflicting. In vitro, *GSTA1*A/*A* is associated with higher hepatic protein expression of this most active GST enzyme but not busulfan conjugation. Hepatic expression of GSTA1 protein is associated with *GSTA1* haplotype, with the *GSTA1*A*A* haplotype having the highest protein expression.[20] However, a separate in vitro study did not find an association between *GSTA1* or *GSTM1* genotype and busulfan conjugation.[19]

The data have been conflicting regarding the association of *GSTA1* and *GSTM1* genotypes with the pharmacokinetics of busulfan. In a small population of twelve Japanese patients receiving oral busulfan, Kusuma et al observed that the apparent oral clearance of busulfan was lower in patients with the *GSTA1*A*B* haplotype than with the *GSTA1*A*A* haplotype.[29] Notably, no patients were homozygotes for the *GSTA1*B*B* variant. With IV busulfan, the data have been conflicting with reports indicating that *GSTA1* is[30] or is not associated[31,32] with IV busulfan clearance. Data with *GSTM1* suggests that this genotype is not associated with IV busulfan clearance,[31] although contradictory data do exist.[32] Not surprisingly, the majority of these studies were from small patient populations (numbers accrued ranging from 12[29] to 77[31]). Similarly, our study was from a single institution; however, we powered our sample size *a priori* to observe a 20% difference in busulfan clearance. Two separate studies in pediatric populations noted that *GSTA1* haplotype was[30] or was not[31] associated with IV busulfan clearance. Our results suggest that *GSTA1* and *GSTM1* are not associated with IV busulfan clearance.

To date, the association between *GST* genotypes and circulating busulfan metabolites – such as the thiophenium ion[13] – in blood has not been evaluated in patients receiving busulfan. However, such studies may elucidate a physiologic rationale, potentially regulation of intestinal GSTA1 expression, for the association of *GSTA1* haplotype with oral (Figure 3A) but not IV (Figure 2A) busulfan clearance.

Consideration should be given to factors which may influence the busulfan clearance phenotype. Concomitant medications, specifically phenytoin, may have altered busulfan clearance and obscured a genotype-phenotype relationship. Phenytoin is commonly used to prevent busulfan-induced seizures, and its effects upon busulfan clearance are difficult to assess since the majority of busulfan pharmacokinetic data has been obtained in patients also receiving phenytoin. However, phenytoin has been reported to increase busulfan clearance[33,34] and may alter busulfan clearance such that an association between *GST* genotype and busulfan clearance phenotype cannot be discerned under these conditions. Unfortunately, no metabolic study data are available that compare busulfan metabolism in patients receiving phenytoin to that in patients not receiving phenytoin. The potential impact of phenytoin upon busulfan metabolism and thus, genotype – phenotype relationships, remains to be evaluated.

The *GST* genotypes have also been associated with clinical outcomes in HCT recipients receiving BU/CY, although a mechanistic rationale for their association remains elusive. Because pharmacokinetics-based busulfan dose targeting was performed in our dataset, we did not evaluate the association of these *GST* genotypes with clinical outcomes. In Korean patients receiving BU/CY conditioning prior to an allogeneic graft, *GSTA1*A*A* is associated with a lower incidence of acute graft versus host disease (GVHD) but not hepatic VOD.[35] The authors hypothesized that the underlying mechanism is that *GSTA1*A*A* metabolizes busulfan and CY more rapidly, thus decreasing tissue injury which has been implicated as a key event in the etiology of GVHD.[1] While busulfan C_{ss} is associated with VOD, it is not consistently associated with acute GVHD.[6,36] Thus, the mechanistic rationale for this observation is more likely to be due to altered metabolism of CY.[6,36] *GSTM1-1* also mediates busulfan conjugation, accounting for ~5% of total GST activity.[18] The *GSTM1* null genotype is associated with increased risk of VOD, lower busulfan C_{ss}, and faster oral busulfan clearance in β-thalassemia patients receiving BU/CY ± ATG regimen.[22,37] These results led to the hypothesis that VOD was caused by liver damage due to a metabolite of busulfan but not busulfan itself, potentially due to depletion of the glutathione pool. Our results suggest that these pharmacogenetic associations between clinical outcomes and *GST* genotypes are multifactorial, since we did not observe an association between *GST* genotypes and IV busulfan clearance.

After IV administration, the busulfan clearance was similar between those receiving busulfan Q6hr and daily, confirming the results of Madden et al.[27] The increasing popularity of daily administration of busulfan makes it imperative that more efficient tools are developed to personalize busulfan doses. Daily administration of busulfan leads to fewer busulfan doses being administered in HCT conditioning (i.e., 4 days of busulfan conditioning, formerly Q6 hour × 16 doses to now Q24 hour × 4 doses).[27,38] For example, with the current practice of weight-based dosing of daily IV busulfan in conjunction with kinetics-based dose adjustments, patients will receive at least one (i.e., 25% of the total busulfan therapy) to three (i.e., 75% of the total busulfan therapy) doses before information on the individual's busulfan clearance is available. In addition, shorter courses (e.g., 2 days) of IV or oral busulfan are being evaluated in reduced-intensity conditioning regimens and prior to infusion of genetically modified cells.[38-41] With these latter regimens, there is inadequate time to target busulfan doses without an on-site laboratory quantitating busulfan concentrations. Current approaches to targeting require extended and intensive blood sampling schedules to characterize an individual's busulfan clearance. Alternative methods to target IV busulfan doses are needed due to the continuing trend for shorter busulfan courses.[27,38-41] We have created a population pharmacokinetic model for daily IV busulfan[42] with the intent of addressing both major busulfan dose targeting hurdles: modeling of variability and mitigation of resource intensity of kinetics-based targeting. Population pharmacokinetic modeling can also identify genetic and non-genetic covariates of busulfan conjugation and elimination. These models, coupled with further in vitro studies, may provide insight regarding why *GST* polymorphisms are associated with hepatic protein expression[20] but not busulfan conjugation *in vitro*.[19] Population pharmacokinetic models also facilitate development of limited blood sampling schedules, which require fewer blood samples to characterize an individual patient's busulfan clearance. Such population pharmacokinetic models and limited sampling schedules

could lead to less resource-intensive methods to target busulfan doses and have been utilized with oral busulfan dosing.[43]

CONCLUSIONS

In summary, IV busulfan clearance is not associated with the genetic variants of the most promising genes related to its metabolism: *GSTA1* and *GSTM1*. Similarly, *GSTM1* genotype was not associated with oral busulfan clearance. Although oral busulfan clearance was associated with *GSTA1* haplotype, the considerable interpatient variability within each haplotype would not provide sufficient confidence in prediction of oral busulfan clearance, and therefore the dose needed to achieve target busulfan exposure. Thus, intensive pharmacokinetic sampling remains the standard for targeting busulfan doses in HCT recipients.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the contributions of the technical staff in the Seattle Cancer Care Alliance Pharmacokinetics Laboratory with busulfan dose targeting; Mr. Eric Mickelson in the FHCRC Hansen Laboratory for DNA isolation; and Ms. Tot Bui Nguyen in the University of Washington the DNA Sequencing and Gene Analysis Center for GST sequencing.

Financial Support: Supported by in part by PDL Biopharma (JSM), UW School of Pharmacy DMTPR Funds (JSM), and the Milo Gibaldi Endowment Fund (RJYH). The clinical hematopoietic cell transplant protocols were supported by HL036444**,** CA 18029. The DNA repository at FHCRC is supported by NIH PO1-AI33484 and R01 HL087690 (Principal Investigator: John Hansen, MD).

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CHAPTER 6

Pharmacometabonomic Prediction of Busulfan Clearance in Hematopoietic Cell Transplant Recipients

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J. Proteome Res. 2016, 15, 2802-2811

ABSTRACT

Intravenous (IV) busulfan doses are often personalized to a concentration at steady state (Css) using the patient's clearance, which is estimated with therapeutic drug monitoring. We sought to identify biomarkers of IV busulfan clearance using a targeted pharmacometabonomics approach. A total of 200 metabolites were quantitated in 106 plasma samples, each obtained before IV busulfan administration in hematopoietic cell transplant (HCT) recipients. Both univariate linear regression with false discovery rate (FDR), and pathway enrichment analyses using the Global test were performed. In the univariate analysis, glycine, *N*-acetylglycine, 2-hydroxyisovaleric acid, creatine, serine, and tyrosine and were statistically significantly associated with IV busulfan clearance at $P<0.05$, with the first three satisfying the FDR of $q<0.1$. Using pathway enrichment analysis, the glycine, serine, and threonine metabolism pathway was statistically significantly associated with IV busulfan clearance at $P<0.05$ and $q<0.1$, and a pathway impact >0.1. Glycine is a component of glutathione, which is conjugated with busulfan via glutathione transferase enzymes. These results demonstrate the potential utility of pharmacometabonomics to inform IV busulfan dosing. Future studies are required to validate these findings.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) offers a curative treatment for a variety of malignant and nonmalignant disorders.[1,2] The alkylating agent busulfan is often part of an HCT conditioning regimen. Recent data has shown improved overall survival in HCT recipients conditioned with intravenous (IV) busulfan compared to total body irradiation (TBI); however, toxicities of the HCT conditioning treatments persist.[1-3] Busulfan has a narrow therapeutic index, and plasma exposure, expressed as concentration at steady state (Css) or area under the plasma concentration-time curve (AUC), forecasts the efficacy of busulfan-containing conditioning regimens.[4,5] Low busulfan Css, caused by rapid clearance, is associated with reduced efficacy, e.g., increased risk of relapse[6] or rejection,[7,8] while high busulfan Css is associated with hepatotoxicity[7,9-12] and non-relapse mortality (NRM).[13] Personalizing busulfan doses to target plasma Css, using a patient-specific busulfan clearance, improves these clinical outcomes.[9,14-17] This process of pharmacokinetic sampling and modeling to personalize busulfan dosing is termed targeted busulfan (^{T}BU), therapeutic drug monitoring or pharmacokinetic (PK)-guided dosing; the latter will be used for the remainder of the manuscript. However, this approach is insufficient as relapse and NRM persist even with PK-guided IV busulfan dosing. Novel biomarkers that can be used to predict IV busulfan clearance before treatment begins, and potentially improve overall survival in HCT recipients, are therefore needed. Identification of such markers could lead to estimating a patient-specific IV busulfan clearance and their personalized dose before IV busulfan administration, reducing the resource intensity of PK-guided dosing of busulfan in HCT conditioning. Furthermore, such analyses could identify novel biomarkers associated with NRM or overall survival.

Substantial insight regarding drug metabolism and response has been gained through the use of metabolomics – the profiling of a broad range of small molecules present in biological fluids.[18-24] For example, recent metabolomics studies have provided insight regarding potential biomarkers for graft versus host disease (GVHD) in allogeneic HCT recipients.[25,26] More specifically, Clayton, et al., introduced the concept of personalized drug treatment using pre-dose metabolite profiling to predict drug response in individual subjects, which the authors termed, "pharmacometabonomics".[22,23] Against this background, we sought to identify biomarkers predictive of IV busulfan clearance using a targeted pharmacometabonomics approach consisting of 200 metabolites in plasma prior to IV busulfan administration in 106 allogeneic HCT recipients.

METHODS

Study population

This was an ancillary retrospective study of 108 subjects who received HCT conditioning with IV busulfan and PK-guided dosing from April 2006 to November 2012 under the aegis of protocols approved by the Fred Hutchinson Cancer Research Center (Fred Hutch) Institutional Review Board. Of the 108 subjects, insufficient sample available for pharmacometabonomics analysis for two subjects, leaving a total of 106 samples for analysis. All subjects were diagnosed with hematologic disorders and had adequate renal

(i.e., serum creatinine < 1.5 mg/dl, and creatinine clearance or radioisotope glomerular filtration rate > 60 ml/min/1.73 m^2) and liver (i.e., total bilirubin < 1.5 mg/dl and alanine aminotransferase < 300 units/l) function. Demographic data were taken from the subjects' medical charts [age, sex, height, total body weight (i.e., actual; TBW), dosing weight (calculated as previously described;[27]), body surface area, and clinical information (disease, conditioning regimen)].

All subjects underwent PK-guided dosing of IV busulfan as part of HCT conditioning. Other conditioning agents included cyclophosphamide (n=70) or fludarabine monophosphate (fludarabine; n=36). Standard practice for prophylaxis of busulfan-induced seizures was phenytoin. Similar antiemetics, antibiotics and antifungals were given per institutional Standard Practice Guidelines. All subjects provided written informed consent before participating in the treatment protocols. The Fred Hutch Institutional Review Board approved both the treatment protocol and the retrospective analysis of samples to identify biomarkers of IV busulfan clearance using pharmacometabonomics.

IV busulfan dosing

Clearance after the first busulfan dose (dose 1), administered in the morning for all subjects, was the primary outcome of interest. The busulfan dose 1 was calculated using TBW if it was less than ideal body weight, or adjusted ideal body weight (AIBW, which equals 0.25 (TBW – ideal weight) + ideal weight) if it was greater than IBW. The IBW in adults was calculated as follows: for males = 50 kg + (2.3 kg for each inch over 5 feet); for females = 45.5 kg + (2.3 kg for each inch over 5 feet). Subsequent IV busulfan doses were personalized to achieve the desired target busulfan Css, chosen for the treatment protocol by the attending physician (i.e., clinician-chosen).

Blood samples (3 ml/sample) were collected in sodium heparin tubes before the morning doses of days 1, 2, and 3 of IV busulfan administration. For those subjects receiving daily IV busulfan, pharmacokinetic samples were drawn at the end of the 3-hour infusion, and at 3.25, 4.5, 6, 8, 11, and 24 hours (i.e., prior to subsequent dose) after the beginning of the infusion.[27] For those subjects receiving IV busulfan every 6 hours (Q6h), PK samples were drawn at the end of the 2-hour infusion, and at 2.25, 2.5, 3, 4, 5, and 6, hours (i.e., prior to subsequent dose) after the beginning of the infusion. All samples were stored on wet-ice or refrigerated, and transported to the Seattle Cancer Care Alliance Busulfan Laboratory, a College of American Pathology-certified laboratory that has focused exclusively on PK-guided dosing of busulfan since 1996. Plasma busulfan concentrations were analyzed by gas chromatography with mass-selective detection as previously described.[27] The dynamic range was from 1.97 to 4.54 ml/min/kg normal fat mass (NFM; see Supporting Methods for further details)[28] and the intraday and interday coefficients of variation were less than 5% and 8%, respectively. After quantitation of busulfan samples, the individual subject's concentration-time data underwent pharmacokinetic modeling using Phoenix WinNonlin (Certara USA, Princeton, NJ) to obtain each individual's busulfan area under the curve (AUC) from time 0 to infinity ($AUC_{0\ to\ \infty}$). After dose 1, the patient-specific clearance and Css were calculated based on the following equations: clearance = dose divided by $AUC_{0-\infty}$ and Css = $AUC_{0-\infty}$ divided by the IV busulfan dosing frequency. After calculation of the patient-specific clearance, the personalized dose was calculated linearly to achieve

the target Css and this personalized dose was administered for subsequent doses. The patient-specific clearance was measured after the morning doses of 2 and 3 of IV busulfan administration as well; these data were not used in this analysis. For the present dataset, the concentration-time data underwent noncompartmental analysis and all clearances are reported based on NFM, the optimal body metric for IV busulfan clearance over a population of pediatric to adult allogeneic HCT recipients.[28]

Targeted pharmacometabonomics sample collection

All samples for pharmacometabonomics analyses were conducted on baseline plasma samples collected prior to IV busulfan administration. Each subject had one sample (i.e., one sample per subject). In 59 subjects, the blood sample was obtained before any conditioning agents were administered (i.e., no conditioning) and blood had been drawn into citrate blood collection tubes (BCTs). In the remaining 47 subjects, samples were drawn after administration of other conditioning agents (n=40 cyclophosphamide/busulfan[27] and n=7 fludarabine/busulfan/thymoglobulin)[29]. For these subjects, the pre-transplant pharmacometabonomics sample had been drawn into an EDTA BCT, refrigerated shortly thereafter at a target temperature of 4°C until transport (within 12 hrs) to the University of Washington/Fred Hutch Pharmacokinetics Laboratory. The sample was subsequently centrifuged and the resultant plasma frozen at -80°C for either cyclophosphamide or fludarabine pharmacokinetic analysis as previously reported.[27,29] The samples underwent two freeze-thaw cycles before the targeted pharmacometabonomics analysis (i.e., the analysis was conducted with the second thaw).

Pharmacometabonomics analysis

Metabolite profiling of plasma was completed at the University of Washington's Northwest Metabolomics Research Center. Targeted pharmacometabonomics analysis was carried out using a liquid chromatography tandem mass spectrometry (LC-MS/MS) platform in both positive and negative ion modes against 200 standard metabolites (see Supporting Information Table S1) from numerous Kyoto Encyclopedia of Genes and Genomes (KEGG)-defined metabolic pathways[30] (e.g., glycolysis, TCA cycle, amino acid metabolism, glutathione, etc.) of potential significance to monitor diet effects, along with 24 internal standards for concentration determinations.[31-33] All plasma samples were prepared in batches of 30 samples. A standard protocol was used[34-37] where 25 µL plasma and 150 µL high performance liquid chromatography (HPLC) grade methanol were combined in an Eppendorf vial and vortexed for 2 min. After 20 min storage at -20 °C the samples were centrifuged at 18,000 g for 10 min. A fixed volume of 150 µL supernatant was collected and placed in a new Eppendorf vial. The protein pellets were mixed with another 300 µL HPLC grade methanol, then vortexed for 10 min and centrifuged for 10 min at 18,000 g. 250 µL was collected and combined with the previous 150 µL sample. Samples were then dried at 30 °C in a SpeedVac for 3 h.

Prior to each LC run, samples were reconstituted with 100 µL 5 mM ammonium acetate in 95% water/5% acetonitrile + 0.5% acetic acid, and filtered through 0.45 µm PVDF filters (Phenomenex, Torrance, CA) prior to analysis on an AB Sciex QTrap 5500 LC-MS/MS system (AB Sciex, Toronto, ON, Canada).[35-37] The LC system was composed of two Agilent 1260 binary pumps, an Agilent 1260 auto-sampler and Agilent 1290 column compartment

containing a column-switching valve (Agilent Technologies, Santa Clara, CA). Each sample was injected twice, 10 µL for analysis using negative ionization mode and 2 µL for analysis using positive ionization mode. Both chromatographic separations were performed in reverse phase (RP) on Thermo Accucore PFP columns (150 x 2.1 mm, 2.6 µm particle size, Thermo Fisher Scientific Inc., Waltham, MA). The flow rate was 0.250 mL/min, auto-sampler temperature was kept at 4 °C, and the column compartment was set at 40 °C. The mobile phase was composed of Solvents A (5 mM ammonium acetate in water + 0.5% acetic acid + 0.5% acetonitrile) and B (acetonitrile + 0.5% acetic acid + 0.5% water). After chromatographic separation, MS ionization and data acquisition was performed using AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Toronto, ON, Canada) with electrospray ionization (ESI) source. The collision gas was 99.99% pure nitrogen. The data gathered through the multiple reaction monitoring were integrated using MultiQuant 2.1 software (AB Sciex, Toronto, ON, Canada).[33] A pooled QC sample was run for every 10 biological samples to assess instrument performance. The intra-assay average CV was 7.8% across all samples.

Statistical analysis

Of the 200 metabolites measured, 118 had detectable signal in all samples and were retained for analysis. The majority of metabolites were skewed to higher values and were therefore log-transformed using the natural logarithm to approximate a normal distribution. A univariate linear regression model was used to assess marginal associations of each metabolite individually on IV busulfan clearance (continuous) after dose 1. Because two different BCTs were used, the effect of BCT type was assessed. Although the results were similar with and without adjustment (data not shown), BCT type was included in the univariate analyses. In our previous evaluation of 1,610 HCT recipients (n=904 male and n=689 female), used to inform the present analysis, we found no effect of gender or weight on IV busulfan clearance, the endpoint of interest.[28] However, to ensure that gender or weight did not confound our results, both were tested and found not to affect significance of the metabolites evaluated with the exception of a single metabolite. Homovanilate became significant with adjustment for gender (data not shown), but this would be expected by chance due to the high number of individual tests. Therefore, results without adjustment for gender and weight are presented. To determine the percentage of variance explained in our model, R^2 was calculated including all significant metabolites in a single regression model. Benjamini-Hochberg methods were used to control for false discovery rate (FDR).[38] Individual metabolites were considered for both $P<0.05$ and $q<0.1$.

To consider metabolites that coordinately predict IV busulfan clearance, pathway analyses using all metabolites were carried out using MetaboAnalyst 3.0 (see Supporting Information Table S2),[39,40] integrating pathway enrichment analysis and pathway topology analysis for visualization. Within the pathway analysis module, metabolites were auto-scaled (mean-centered and divided by the standard deviation of each variable), and IV busulfan clearance was evaluated as a continuous outcome. Four metabolites from our panel, aminoisobutyric acid, cystamine, inositol, and *N*-acetylneuraminate, were not present in the MetaboAnalyst compound library [derived from KEGG,[30] Small Molecule Pathway Database[41] (SMPDB)], and Human Metabolome Database[42] (HMDB)], and thus were not included in the pathway analyses. The Global test,[43] which evaluates changes

among groups of metabolites, was used for statistical significance of pathway enrichment analysis, with FDR of $q<0.1$ for multiple comparisons. While 56 pathways contained at least one metabolite from our panel, only 26 pathways contained 4 or more metabolites, sufficient for meaningful pathway analysis (see Supporting Information Table S2). Betweeness centrality (shortest path between nodes), based on metabolite centrality in a given metabolic network, was used to calculate metabolite importance.[44] Pathway impact was calculated as the sum of the importance measures of the pathway-specific metabolites, normalized by the sum of the importance measures of all metabolites in each pathway.[45]

A post-hoc univariate receiver operating characteristic (ROC) curve was performed on the most significant metabolite, glycine, to evaluate performance as a predictive biomarker using the ROCR package in R.[46] For this ROC analysis we defined two groups of subjects based on their IV busulfan clearance being above or below the median. The area under the ROC (AUROC) curve and 95% confidence interval (95% CI) are also reported.

RESULTS

Patient characteristics

Pre-transplant characteristics and diagnoses of the 106 HCT subjects are given in Table 1. Mean age was 50.4 y (range 22-66), and BMI was 20.0 ± 2.2 kg/m^2. Slightly more subjects were male (60%). All samples were collected prior to IV busulfan administration; however, 47 subjects (44%) began other conditioning regimens prior to sample collection. Mean IV busulfan clearance after dose 1 was 3.2 ± 0.5 ml/min/kg of dosing weight and 3.3 ± 0.6 ml/min/kg NFM (Figure 1), which is in agreement with previous studies.[10]

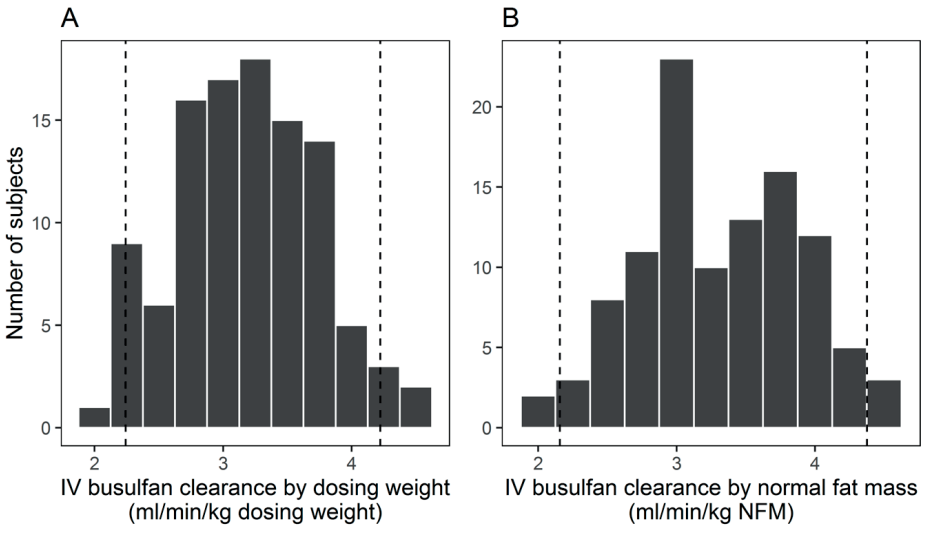

Figure 1. IV busulfan clearance of the population, shown by dosing weight (A) and normal fat mass (NFM; B). Dashed vertical lines border 95% of the observed values.

Table 1. Demographic and clinical data for the HCT study population (N=106)

Parameter	N[a]
Age (y)	50.4 (21.6-65.8)
Male sex	64 (60%)
Dosing weight (kg)[b]	69.4 ± 11.1
Body mass index [BMI; weight (kg)/height (m)^2]	20.0 ± 2.2
Normal fat mass (NFM; kg)	67.6 ± 13.9
HCT Conditioning[c]	
Cyclophosphamide/Busulfan	67 (63%)
Busulfan/Cyclophosphamide	3 (3%)
Fludarabine/Busulfan	27 (25%)
Fludarabine/Busulfan/Thymoglobulin	9 (8%)
Busulfan dosing frequency	
Every 6 hours	11 (10%)
Every 24 hours	95 (90%)
IV busulfan clearance (ml/min/kg NFM)	3.33 ± 0.59
Diagnosis	
Aplastic anemia	1 (1%)
Acute lymphoblastic leukemia	1 (1%)
Acute myeloid leukemia	45 (42%)
Chronic myeloid leukemia (CML)	4 (4%)
Chronic myelomonocytic leukemia	2 (2%)
Myelodysplastic syndrome (MDS)	21 (20%)
MDS/CML	1 (1%)
Myelofibrosis	26 (25%)
Myeloproliferative disease	5 (5%)
Blood collection tube	
Citrate	59 (56%)
EDTA	47 (44%)
Drugs present in sample	
None	59 (56%)
Cyclophosphamide	40 (38%)
Fludarabine	7 (7%)

[a] Data presented as: number (%) or mean ± standard deviation; percentages may not add up to 100 due to rounding
[b] Total body weight was used for busulfan dosing if total body weight was less than ideal body weight, whereas adjusted ideal body weight was used if total body weight was greater than ideal body weight.
[c] Listed in administration order; all subjects received PK-guided dosing of busulfan, in which the IV busulfan dose was personalized based on clearance

Pharmacometabonomics

In the univariate analysis, six metabolites were statistically significantly associated with IV busulfan clearance at $P<0.05$: glycine, *N*-acetylglycine, creatine, and serine, were positively associated with clearance, and tyrosine and 2-hydroxyisovaleric acid, were negatively associated. Three of these metabolites satisfied the FDR of $q<0.1$: glycine, *N*-acetylglycine and 2-hydroxyisovaleric acid (Table 2). Inclusion of all six nominally significant metabolites in a single regression model explained ~16% of the variability ($R^2=0.16$).

Table 2. Endogenous metabolites significantly associated with IV busulfan clearance by univariate analysis.

Metabolite	Function[a]	Direction[b]	P-value	q-value[c]
Glycine	Amino acid; involved in biosynthesis of proteins, including glutathione, involved the metabolism of busulfan, as well as purines, heme, bile salts, and creatine; inhibitory neurotrasmitter	+	0.0006	0.08
N-Acetylglycine	Acetylated glycine; important for synthesis, stability and localization of proteins	+	0.002	0.09
2-Hydroxyisovaleric Acid	Fatty acid; derived from metabolism of valine, leucine and isoleucine; may originate from ketogenesis	-	0.002	0.09
Creatine	Involved in energy production, primarily in muscle; produced from glycine and arginine	+	0.006	0.23
Serine	Amino acid; involved in production of purines and pyrimidines; inhibitory neurotransmitter	+	0.03	0.80
Tyrosine	Amino acid; biosynthesis of proteins; essential component of neurotransmitters; receptor kinases involved in immune signaling	-	0.03	0.80

[a] Information pertaining to function is derived from Kyoto Encyclopedia of Genes and Genomes, Human Metabolome Database or PubChem unless otherwise noted
[b] Indicates whether the metabolite was positively or negatively associated with IV busulfan clearance
[c] False discovery rate (Benjamini-Hochberg)

In the pathway enrichment analysis, considering all metabolites together, the only pathway meeting both a statistical significance level of $P<0.05$ and $q<0.1$ and any measureable pathway impact factor (e.g., <0.1) was the glycine, serine, and threonine metabolism pathway, which was significantly associated with IV busulfan clearance ($P=0.002$, FDR=0.028, impact=0.53; Figures 2, 3 and Table 3). Other pathways that were statistically significant at $P<0.05$ and $q<0.1$ were thiamine, porphyrin, cyanoamino acid, methane, and glutathione metabolism, but all had pathway low impact values (<0.1). The post-hoc univariate AUROC curve for glycine was 0.66 (95% CI: 0.55, 0.75, Figure 4).

Table 3. Top pathways, significance and impact from pathway enrichment analyses, sorted by increasing *P*-values.

Pathway Name	Total Metabolites[a]	Matched Metabolites[b]	P-value	-log(P)[c]	q-value[d]	Impact[e]
Thiamine metabolism	24	2	0.0009	6.97	0.028	0.0
Porphyrin and chlorophyll metabolism	104	2	0.002	6.49	0.028	0.0
Cyanoamino acid metabolism	16	4	0.002	6.34	0.028	0.0
Glycine, serine and threonine metabolism	48	11	0.002	6.18	0.028	0.53
Glutathione metabolism	38	5	0.003	5.97	0.028	0.0
Methane metabolism	34	4	0.003	5.79	0.028	0.02
Sphingolipid metabolism	25	1	0.01	4.55	0.07	0.0
Sulfur metabolism	18	1	0.01	4.55	0.07	0.0

Pathway Name	Total Metabolites[a]	Matched Metabolites[b]	P-value	-log(P)[c]	q-value[d]	Impact[e]
Nitrogen metabolism	39	8	0.02	4.11	0.10	0.01
Glycerolipid metabolism	32	1	0.03	3.45	0.17	0.02
Primary bile acid biosynthesis	47	4	0.03	3.42	0.17	0.03

[a] Total number of metabolites in the pathway
[b] Number of matched metabolites, explained in Statistical Analysis section
[c] -log(*P*) is the negative natural log of the *P* value for each pathway shown in Figure 2
[d] False Discovery Rate (Benjamini-Hochberg)
[e] Impact is the pathway impact value on IV busulfan clearance calculated from pathway topology analysis

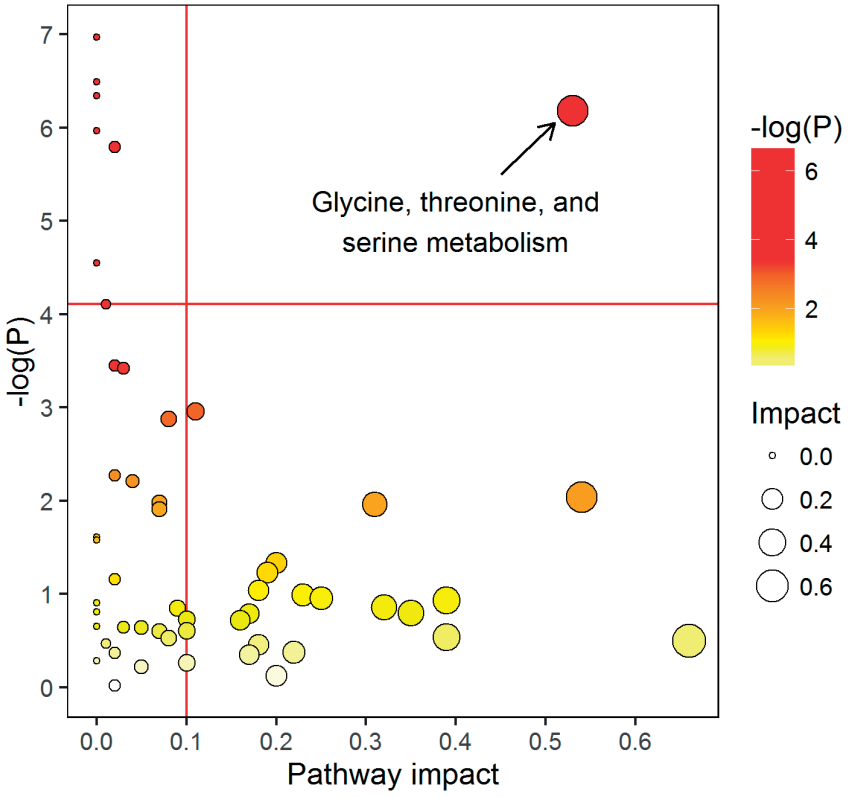

Figure 2. Overview of pathway enrichment analysis. All dots represent matched pathways from topology pathway analysis. Pathways are colored according to their significance values from pathway enrichment analysis, with gradations from yellow, having the least significance, to red having the highest significance (exact *P* values are given in Tables 3 and S2). Pathways above the horizontal red line correspond to $q<0.1$. Pathway impact is indicated on the x-axis. Pathways to the right of the vertical red line on the x-axis have an impact score >0.1. Glycine, threonine and serine metabolism was the only significant pathway with $q<0.1$ and a pathway impact value >0.1. Other pathways that were significant but had a negligible pathway impact (e.g., <1: thiamin, porphyrin, cyanoamino acid, glutathione, methane and sphingolipid metabolism; all values are given in Table 3) all included glycine.

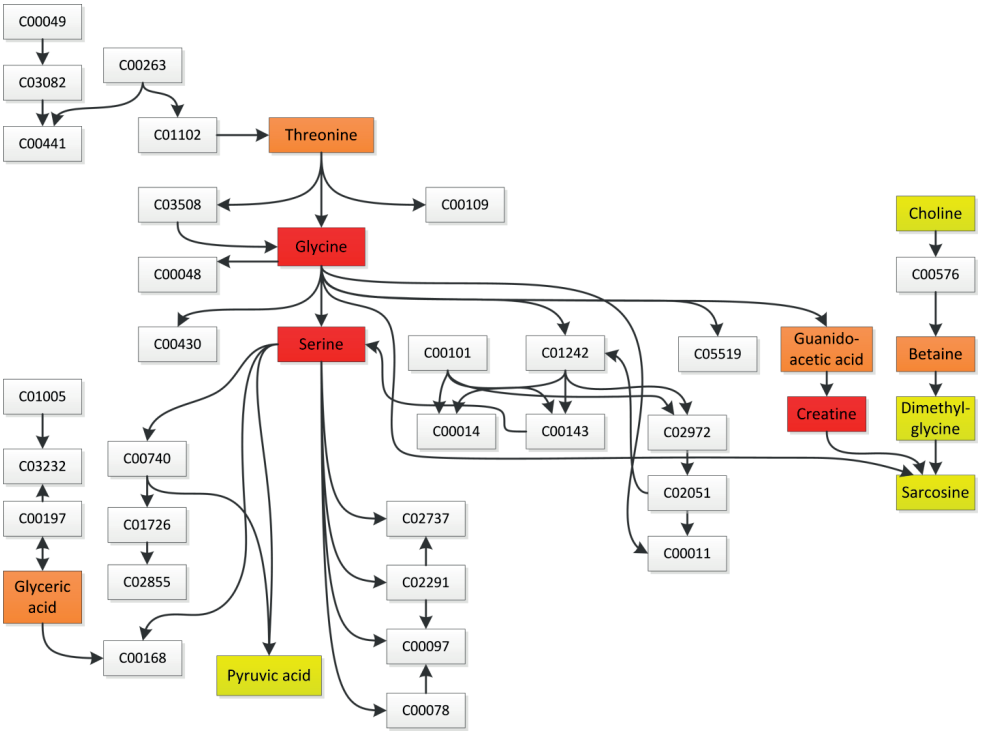

Figure 3. Glycine, Serine, and Threonine Metabolism pathway. Named metabolites were included in the pharmacometabonomics panel; boxed metabolites shown by letter and number combination show metabolites in the pathway but not included in the metabolite panel. Metabolites are colored according to their significance values from pathway enrichment analysis, with yellow having the least significance (*P*=0.2-0.8), orange having moderate significance (*P*=0.03-0.12), and red having the highest significance (*P*=0.01-0.0006). C00049: aspartic acid; C03082: aspartyl-4-phosphate; C00441: aspartate-semialdehyde; C00197: 3-phospho-D-glycerate; C00168: hydroxypyruvic acid; C03508: 2-amino-3-oxobutanoic acid; C03232: phosphohydroxypyruvic acid; C00740: D-serine; C01726: lombricine; C02855: N-phospho-D-lombricine; C00048: glycoxylic acid; C00263: homoserine; C01102: O-phosphohomoserine; C00430: 5-aminolevulinic acid; C02737: phosphatidylserine; C00014: ammonia; C00078: tryptophan; C00109: 2-ketobutyric acid; C00101: tetrahydrofolic acid; C00143: 5,10-methylene-THF; C02291: cystathionine; C00097: cysteine; S-aminomethyldihydrolipoylprotein; C02972: dihydrolipoylprotein; C02051: lipoylprotein; C00011: carbon dioxide; C05519: allothreonine; C00576: betaine aldehyde; C01242: S-aminomethylidihydrolipoyl-protein; Nine metabolites are omitted from the figure for ease of presentation: C03283: 2,4-diaminobutanoate; C06442: N-gamma-acetyldiaminobuturate; C06231: ectoine; C01005: phosphoserine; C16432: 5-hydroxyectoine; C01888: aminoacetone; C00546: pyruvaldehyde; C03194: 1-aminopropan-2-ol; C05235: hydroxyacetone.

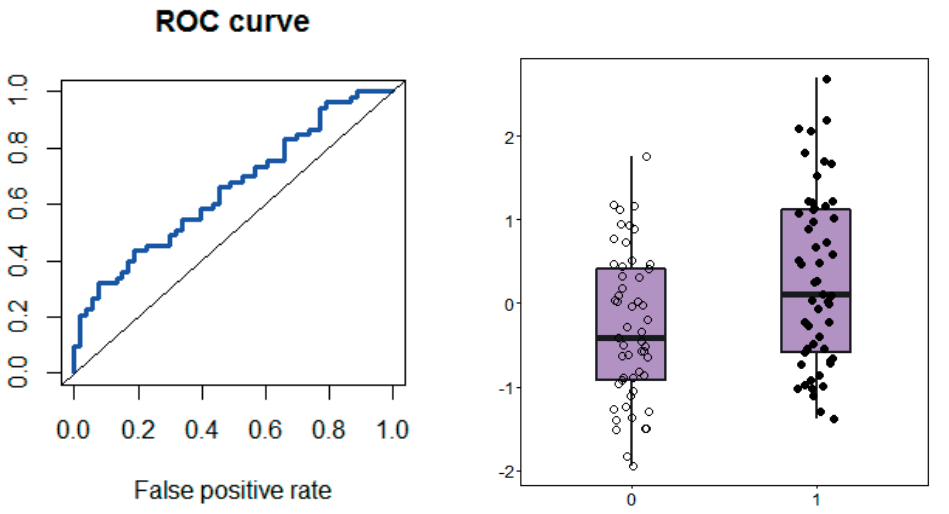

Figure 4. Evaluation of glycine as a predictor for IV busulfan clearance. Area Under the Receiver Operator Characteristic (AUROC) curve for the most promising metabolite, glycine = 0.66 (95% CI: 0.55, 0.75). Box plots represent mean (interquartile range) of low busulfan clearance (0; below the median) and high busulfan clearance (1; above the median).

DISCUSSION

The key findings of this analysis are: 1) glycine, *N*-acetylglycine, creatine, serine, tyrosine and 2-hydroxyisovaleric acid plasma concentrations were associated with IV busulfan clearance, with the AUROC curve for glycine alone being 0.66; and 2) of the 26 pathways with sufficient metabolites for analysis, the glycine, serine, and threonine metabolism pathway was most highly associated with IV busulfan clearance. In this analysis, we took a first step towards identifying endogenous plasma metabolites associated with IV busulfan clearance with the long-range goal of personalizing IV busulfan doses using biomarkers identified via pharmacometabonomics.

Busulfan is a widely-used alternative to TBI in preparation for HCT.[47] A bi-functional anti-neoplastic alkylating agent, busulfan hydrolyzes in aqueous solutions to release methanesulfonate moieties. The resulting reactive carbonium ions alkylate DNA, destroying existing blood cells and remaining cancer cells. While overall survival after HCT is improved with busulfan conditioning, increased efficacy and reduced toxicity of busulfan-based conditioning is needed. It is well known that busulfan has a narrow therapeutic index, with many HCT centers obtaining busulfan pharmacokinetic data in allogeneic HCT recipients.

The patient-specific IV busulfan clearance, not busulfan Css, is the relevant endpoint because the goal with PK-guided dosing is to obtain the patient-specific IV busulfan clearance to be used for dose personalization to achieve the clinician-chosen target

busulfan Css. Currently, weight-based dosing of the initial busulfan dose achieves the clinician-chosen target busulfan Css in 22.6% of patients.[48] After the initial busulfan dose, serial pharmacokinetic samples are obtained to determine the plasma exposure and the patient-specific IV busulfan clearance (as AUC = dose/clearance). That patient-specific clearance is then used with the clinician-chosen target Css (as Css=AUC/dosing frequency) to personalize the subsequent IV busulfan doses with the intent of achieving the clinican-chosen target Css. This process is referred to as PK-guided dosing or therapeutic drug monitoring (TDM). Personalizing IV busulfan doses using PK-guided dosing results in over 85% of patients achieving the busulfan clinician-chosen target Css at the end of 4-days of busulfan therapy.[48] Thus, busulfan Css was not the endpoint of interest because it is confounded by the personalized IV busulfan dose adjustments– made using the patient-specific IV busulfan clearance– to achieve the clinician-chosen target busulfan Css. PK-guided dosing improves clinical outcomes, but it cannot be conducted with the shorter (i.e., <4 day) busulfan courses included in reduced intensity conditioning regimens. Even with the traditional four days of busulfan conditioning, PK-guided dosing is time sensitive and resource-intensive. It is anticipated that 'omics techniques can improve –or ideally replace– PK-guided busulfan dosing to decrease its resource intensity.

Numerous small studies have evaluated the association of busulfan pharmacokinetics with the constitutional pharmacogenomics of genes regulating the enzymes involved in busulfan disposition.[49] The major elimination pathway of busulfan is through conjugation with glutathione to form an unstable *S*-glutathione sulfonium conjugate γ-glutamyl-β -(S-tetrahydrothiophenium)-alanyl-glycine ($GS^{+}THT$).[50] This reaction is mainly catalyzed by glutathione transferase (GST) isoenzymes A1-1, with GSTM1-1 and GSTP1-1 having minor roles.[51,52] While highly polymorphic, variants in *GSTA1* and *GSTM1* are not associated with IV busulfan clearance[53-55] possibly due to redundancy in function across GST enzymes.[56] Therefore, other methods or biomarkers for personalization are needed.

Metabolomics is a commonly used approach for biomarker discovery.[57-59] While there are no other studies evaluating IV busulfan clearance in HCT subjects, a few studies have examined the metabolome to identify subsequent clinical outcomes among allogeneic HCT recipients. Reikvam, et al.,[26] used metabolite profiling of 766 analytes to evaluate whether pre-transplant metabolic status in 75 HCT subjects was associated with GVHD. Altered pre-transplant levels of several immunoregulatory metabolites, including BCAA and tyrosine derivatives, were found among subjects who later developed GVHD. The authors hypothesized that these metabolites may be involved in the development of GVHD. Another study evaluated 40 thiol/redox metabolites associated with early stages of GVHD between syngeneic and allogeneic HCT recipients. Reduced glutathione was significantly decreased while oxidized glutathione was increased among allogeneic compared to syngeneic recipients as well as non-transplant controls, indicating early shifts in oxidative stress.[25] Further, an accumulation of cysteine, cystathione and cysteinylglycine was associated with early GVHD among the allogeneic HCT subjects.[25] These studies, as well as our own, highlight the opportunity pharmacometabonomics could offer to improve clinical outcomes in HCT recipients.

In the present study, we analyzed 200 metabolites representing over 25 pathways. Six metabolites measured pre-administration were associated with subsequent IV busulfan clearance. Glycine, *N*-acetylglycine and 2-hydroxyisovaleric acid remained significant with FDR <0.1. In addition, glycine, serine, and threonine metabolism was significant in pathway enrichment analyses. Other pathways were statistically significant, but were driven mainly by glycine, and contained few metabolites from our panel, such that pathway impact values were negligible (e.g., < 0.02). Glycine is a non-essential amino acid that can be endogenously synthesized from serine, threonine or choline. In addition to roles in the production of purines, bile acids, creatine and heme, glycine is a component of glutathione— which is involved in the metabolism of busulfan.[60]

It is tempting to speculate that more substrate for glutathione production may be driving the association between glycine and IV busulfan clearance. In fact, glutathione was one of the pathways significantly associated with increased IV busulfan clearance. However, our panel contained only five of the 38 metabolites in the pathway: glycine, pyroglutamic acid, ornithine, glutamic acid and cadaverine, with glycine being the only metabolite to reach statistical significance individually. Further, the other four metabolites were either inversely associated with IV busulfan clearance or only slightly positive. Other components of glutathione consist of the amino acids cysteine and glutamate (see Supporting Information Figure S1). While cysteine was not included in our panel due to difficulty in measuring it by mass spectrometry, glutamate was inversely associated with IV busulfan clearance. This would suggest that increased substrate for the production may not explain the relation between glycine and increased IV busulfan clearance. However, whereas we had 11 of the 48 metabolites represented in the glycine, serine and threonine pathway, only five metabolites were included in the glutathione pathway, which may have been insufficient for a complete evaluation. Nonetheless, we cannot rule out another mode of action contributing to increased IV busulfan clearance, e.g., an altered amino acid pool pre-administration, as all metabolites were amino acids or their derivatives. In addition to amino acids in the glycine, serine, and threonine pathway (glycine, serine and creatine), tyrosine and 2-hydroxyisovaleric acid were negatively associated with IV busulfan clearance. Tyrosine is a non-essential amino acid which can be synthesized from phenylalanine, while 2-hydroxyisovaleric acid is a fatty acid derivative of leucine, a branched chain amino acid (BCAA). Although a decrease in 2-hydroxyisovaleric acid was observed, no associations were found in other BCAA, including isoleucine and valine, or their metabolites. These observations may also reflect dietary or other exposures at the time of measurement.

Strengths of this work include the large population of over 100 HCT subjects, a well characterized IV busulfan pharmacokinetic database, and the targeted panel providing high accuracy of metabolite identification and relative abundances. As with all studies, there were some limitations worth noting. Importantly, the current dataset had insufficient coverage of the glutathione pathway involved in busulfan metabolism. While 13 metabolites in the glutathione pathway were measured, only five had detectable signal in our plasma samples. Thus, greater sensitivity of the eight that did not have detectable signals (i.e., putrescine, spermidine, spermine, cysteine, cysteinylglycine, glutathione, oxidized glutathione, and ascorbic acid) is needed. Having more information

about this pathway pre-administration would have provided a more complete picture. Future studies should further focus the targeted analysis on this pathway. In addition not all 118 metabolites had matches in MetaboAnalyst. Four metabolites (i.e., aminoisobutyric acid, cystamine, inositol, N-acetylneuraminate) were therefore not included in pathway enrichment analyses, although none of these metabolites were significant on their own in univariate analyses. Another potential limitation is the two types of BCTs used for sample collection. However, we evaluated associations both with and without adjustment of BCT type and found no differences among the significant metabolites; thus, it is unlikely that this factor had any effect on the results. An analysis of the targeted metabolome with clinical outcomes is also needed. Finally, these finding should be replicated in an independent cohort.

CONCLUSIONS

In conclusion, this work demonstrates that glycine, and potentially other metabolites in the glycine, serine, and threonine metabolism pathway predict IV busulfan clearance in HCT subjects. Further studies, including greater interrogation of the glutathione pathway, are needed to validate these results which may have the prospect of personalizing IV busulfan dosing and potentially improve clinical outcomes.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health/National Cancer Institute grants: CA182963, CA18029, and 5P30CA015704. We are also grateful to the physicians, nurses, physician assistants, nurse practitioners, pharmacists, and support staff caring for our patients, and to the patients who participated in this study.

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CHAPTER 7

Prediction of Intravenous Busulfan Clearance by Endogenous Plasma Biomarkers Using Global Pharmacometabolomics

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Metabolomics (2016) 12:161

ABSTRACT

Introduction: High-dose busulfan (busulfan) is an integral part of the majority of hematopoietic cell transplantation conditioning regimens. Intravenous (IV) busulfan doses are personalized using pharmacokinetics (PK)-based dosing where the patient's IV busulfan clearance is calculated after the first dose and is used to personalize subsequent doses to a target plasma exposure. PK-guided dosing has improved patient outcomes and is clinically accepted but highly resource-intensive.

Objective: We sought to discover endogenous plasma biomarkers predictive of IV busulfan clearance using a global pharmacometabolomics-based approach.

Methods: Using LC-QTOF, we analyzed 59 (discovery) and 88 (validation) plasma samples obtained before IV busulfan administration.

Results: In the discovery dataset, we evaluated the association of the relative abundance of 1885 ions with IV busulfan clearance and found 21 ions that were associated with IV busulfan clearance tertiles ($r^2 \geq 0.3$). Identified compounds were deoxycholic acid and/or chenodeoxycholic acid, and linoleic acid. We used these 21 ions to develop a parsimonious seven-ion linear predictive model that accurately predicted IV busulfan clearance in 93% (discovery) and 78% (validation) of samples.

Conclusion: IV busulfan clearance was significantly correlated with the relative abundance of 21 ions, seven of which were included in a predictive model that accurately predicted IV busulfan clearance in the majority of the validation samples. These results reinforce the potential of pharmacometabolomics as a critical tool in personalized medicine, with the potential to improve the personalized dosing of drugs with a narrow therapeutic index such as busulfan.

INTRODUCTION

The goal of an allogeneic hematopoietic cell transplant (HCT) is to cure the patient – termed the host or recipient – of their underlying disease by replacing their hematopoietic cells with cells from a healthy donor [1]. The transplantation of donor cells that are not genetically identical (i.e., allogeneic) can result in bi-directional immunologic reactions (i.e., host-versus-graft and graft-versus-host) [1]. In HCT, grafting of cells from one individual to another provokes immunologic reactions involved in engraftment of the donor cells, graft-versus-host disease, control of a malignancy (termed graft versus tumor), the development of tolerance, and immune reconstitution [1]. High-dose busulfan (busulfan) plays a key role in the majority of HCT conditioning regimens that do not include total body irradiation. Improving the efficacy and reducing the toxicity of intravenous (IV) busulfan is critical to avoid the devastating effects of conditioning regimens using total body irradiation [2]. Furthermore, recent data has shown improved overall survival in HCT recipients conditioned with IV busulfan as compared to total body irradiation [3].

Busulfan has a narrow therapeutic index, and busulfan plasma exposure – typically expressed as area under the plasma concentration-time curve (AUC) – is a predictive biomarker that forecasts the likely response to IV busulfan-containing conditioning regimens[1,4]. An individual's IV busulfan dose and clearance estimate their plasma exposure, which can be expressed as AUC or concentration at steady state ($C_{ss,}$ defined as AUC divided by the dosing interval) [4]. Busulfan clearance, which is a measure of how rapidly busulfan is eliminated from the body, has moderate between-subject variability (coefficient of variation of 20.5% after IV administration) [4]. Low IV busulfan AUC, caused by rapid IV busulfan clearance, is associated with an increased risk of rejection or relapse, while high IV busulfan AUC is associated with an increased risk of hepatotoxicity and non-relapse mortality._Personalizing IV busulfan doses to a target plasma AUC – termed pharmacokinetics (PK)-based dosing or targeted busulfan (^{T}BU)– improves each of these clinical outcomes [4]. Because of IV busulfan's narrow therapeutic index, PK-guided IV busulfan dosing is the standard of care [5-7]. Body weight is used to estimate IV busulfan dose 1. After dose 1 administration, six to seven serial PK samples [8] are collected and transported to the analytical facility for quantification of the plasma busulfan concentrations. The patient's busulfan clearance is estimated using non-compartmental analysis of the busulfan concentration-time data. The busulfan clearance and target AUC for that patient are used to personalize the IV busulfan dose. Unfortunately, the current dosing method of using body weight for dose 1 IV busulfan dose rarely achieves the target AUC. Specifically, using body weight to determine dose 1 of IV busulfan results in only 24% of children [9] and 23% of adults [8] achieving their personalized target IV busulfan AUC.

Despite the acceptance of AUC as a predictive biomarker of the patient's response to busulfan-containing HCT regimens, identifying novel biomarkers is desirable because of the rapidly evolving trend of shorter IV busulfan courses [10] and because relapse and non-relapse mortality continue to be problematic even with PK-guided IV busulfan dosing [5,7]. McCune et al. recently developed a population pharmacokinetic model for IV busulfan from a large cohort of HCT recipients (N=1610, 92% pediatric) [11]. Age and normal fat mass (NFM) were identified as patient-specific characteristics that were associated with

IV busulfan clearance [11]. Additionally, to date, predictors of clinical outcomes for the other common components of the HCT conditioning regimens, such as cyclophosphamide or fludarabine pharmacokinetics, have not been found [6,12]. Therefore, we propose using global pharmacometabolomics profiling to identify potential biomarkers of IV busulfan clearance. Following statistical analyses, candidate ions can be selected for metabolite identification and the analysis of metabolic pathways [13].

In recent years, the use of pre-dose metabolite profiling to predict drug response has been evaluated for other fields[14,15]. With the current focus on precision medicine, metabolomics is emerging as a tool to predict both pharmacokinetic and pharmacodynamic outcomes.[16-19] Targeted and global metabolomics have been used to identify altered metabolic pathways, thereby providing insight into the underlying causes of variability and degree of response to drug treatment. . Using the metabolomics analyses of pre-dose samples, investigators have been able to predict the efficacy and the potential for toxicity for various classes of drugs.

Thus, we sought to determine whether endogenous pharmacometabolomics-based biomarkers obtained before IV busulfan administration can predict IV busulfan clearance in a HCT patient population.

METHODS

Study population

Between April 2006 to November 2012, HCT recipients aged 21.5 to 65.8 years underwent PK-guided dosing of IV busulfan (Table 1) under the auspices of their HCT treatment protocol.

Table 1. Description of subject population[a]

	Discovery dataset	Validation dataset	Entire population[b]
Number	59	88	108
Blood collection tube (BCT)[c]			
Citrate	59 (100%)	0	59 (40.1%)
EDTA	0	88 (100%)	88 (59.9%)
Drugs present in pharmacometabolomics sample			
None	59 (100%)	31 (35.2%)	[c]
Cyclophosphamide	0	50 (56.8%)	[c]
Fludarabine	0	7 (8.0%)	[c]
Age, in years	48.7 ± 12.4 (21.6 – 64.0)	50.8 ± 11.2 (21.6 – 65.8)	50.4 ± 11.2 (21.6 – 65.8)
Dosing weight (DWT, kg)[d]	70.4 ± 11.6	69.0 ± 11.0	69.4 ± 11.2
Normal fat mass (NFM, kg)	68.8 ± 14.9	67.3 ± 13.6	67.5 ± 13.9
Male sex	35 (59.3%)	54 (61.4%)	65 (60.2%)
HCT Conditioning[e]			
Cyclophosphamide/Busulfan	27 (45.8%)	62 (70.5%)	69 (63.9%)
Busulfan/Cyclophosphamide	3 (5.1%)	2 (2.3%)	3 (2.8%)

	Discovery dataset	Validation dataset	Entire population[b]
Fludarabine/Busulfan	27 (45.8%)	17 (19.3%)	27 (25%)
Fludarabine/Busulfan/Thymoglobulin	2 (3.4%)	7 (8%)	9 (8.3%)
Busulfan dosing frequency			
Every 6 hour	11 (18.6%)	4 (4.5%)	11 (10.2%)
Every 24 hour	48 (81.4%)	84 (95.5%)	97 (89.8%)
IV busulfan clearance (ml/min/kg NFM)	3.17 ± 0.53	3.26 ± 0.56	3.20 ± 0.55
Diagnosis			
Aplastic anemia	1 (1.7%)	1 (1.1%)	1 (0.9%)
Acute lymphoblastic leukemia	1 (1.7%)	1 (1.1%)	1 (0.9%)
Acute myeloid leukemia	30 (50.8%)	35 (39.8%)	46 (42.6%)
Chronic myeloid leukemia (CML)	2 (3.4%)	4 (4.5%)	4 (3.7%)
Chronic myelomonocytic leukemia	2 (3.4%)	1 (1.1%)	2 (1.9%)
Myelodysplastic syndrome (MDS)	11 (18.6%)	17 (19.3%)	21 (19.4%)
MDS/CML	1 (1.7%)	1 (1.1%)	1 (0.9%)
Myelofibrosis	9 (15.3%)	24 (27.3%)	27 (25%)
Myeloproliferative disease	2 (3.4%)	4 (4.5%)	5 (4.6%)

[a]Data presented as : number (%) or mean ± standard deviation (range) ; percentages may not total 100 because of rounding. [b]Number of samples (discovery and validation datasets) and number of subjects (entire population). [c]Of the 108 subjects, 39 subjects had samples collected on two separate occasions, once in citrate BCT (i.e., part of the discovery dataset) and once in EDTA BCT (i.e., part of the validation dataset). See "Evaluation of blood collection tubes (BCT)" section in Methods. [d]Dosing weight described in "IV busulfan pharmacokinetics". [e]listed in administration order; all patients received PK-guided dosed IV busulfan, as described in "IV busulfan pharmacokinetics" section.

The samples were quantitated at the College of American Pathologists (CAP)-certified Busulfan Pharmacokinetics Laboratory. Information on the subjects' age, sex, height, total (i.e., actual) body weight (TBW), dosing weight (calculated as previously described[7] and HCT conditioning regimen were available. Total body weight was used for dosing if the total body weight was less than the ideal body weight, whereas adjusted ideal body weight was used if the total body weight was greater than the ideal body weight). Subjects received antiemetics, antibiotics, and antifungals per Fred Hutch Standard Practice Guidelines, minimizing potential confounders. All subjects received phenytoin to prevent IV busulfan-induced seizures.

All subjects gave written informed consent before participating in the treatment protocols. The Fred Hutch Institutional Review Board approved the treatment protocol as well as this retrospective analysis of samples to identify biomarkers of IV busulfan clearance using pharmacometabolomics.

IV busulfan pharmacokinetics

The dose 1 IV busulfan clearance was the primary outcome. The pharmacokinetic sampling schema and quantitation of plasma IV busulfan concentrations were previously described[6,7]. In brief, for each patient, the IV busulfan concentration-time profile after dose 1 underwent noncompartmental analysis to estimate IV busulfan clearance using Phoenix WinNonlin (Certara USA, Princeton, NJ)[9]. The IV busulfan clearances were expressed relative to NFM, which accounts for body size differences in children and adults

so that one population pharmacokinetic model could be used for all HCT recipients. NFM was calculated as previously described (Online Resource - Methods) [11]. Briefly, all PK parameters, including clearance, were scaled for body size using allometric theory and the predicted free fat mass (FFM), which was calculated from height and weight [20-23]. The FFM was subsequently used with additional body metrics and IV busulfan-specific PK parameters to estimate NFM [24]. The range of IV busulfan clearances in these patients was similar to other adult HCT populations (Online Resource Supplemental Figure 1) [11]. The dataset was divided into three IV busulfan clearance tertiles (slow, moderate or rapid) (Figure 1).

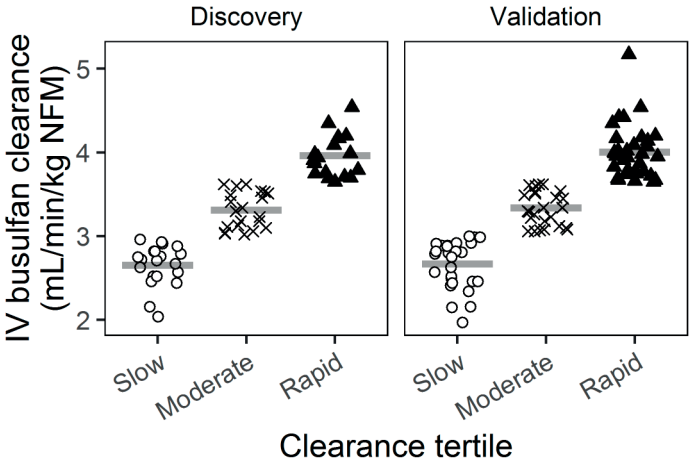

Figure 1. IV busulfan clearance is shown in tertiles for the discovery and validation datasets. Open circles – slow clearance tertile, "X" symbols – moderate clearance tertile, black triangles – rapid clearance tertile. Horizontal lines represent the mean clearance for that group.

Metabolomic sample collection

Metabolomic profiling was conducted on plasma samples obtained before IV busulfan administration (N=147, Table 1) from 108 subjects. Plasma samples were collected in citrate or EDTA blood collection tubes (BCT) before administration of any HCT conditioning or following administration of either cyclophosphamide or fludarabine, which were administered as part of HCT conditioning.

Metabolomic sample extraction and processing

Samples for global profiling were prepared as described previously [25]. In brief, 200 µL of plasma were combined with 800 µL of ice-cold acetonitrile containing deuterated internal standards to monitor for shifts in retention time. Samples were vortexed for 30 seconds and centrifuged at 20,000 RCF at 4°C for 10 min. The supernatant was transferred to glass tubes and evaporated under nitrogen gas at room temperature. Samples were reconstituted by adding 25 µL of methanol and 25 µL of 0.4% acetic acid in water, vortexed, and transferred to vials for analysis.

Liquid chromatography-quadrupole time-of-flight (LC-QTOF) analysis
Global metabolomics analyses of samples were performed using an Agilent (Santa Clara, CA) 1200 LC coupled to an Agilent 6520 QTOF mass spectrometer. Samples (2 µL injection) were separated chromatographically using a 3.5 µm, 2.1 x 30 mm Agilent Zorbax SB-C8 guard column and a 1.8 µm, 2.1 x 50 mm Agilent Zorbax SB-Aq analytical column heated to 60°C. The flow rate was 0.6 mL/min with A: 0.2% acetic acid in water and B: 0.2% acetic acid in methanol (2% to 98% B in 13 min, 98% B until 19 min followed by re-equilibration for 5 min). The total run time was 24 min per sample. The MS source was maintained at 350°C with a capillary voltage of 3500 V, and the desolvation gas flow was 12 L/min. Scans were obtained between *m/z* 100 and 1000 at an acquisition rate of 3 spectra/sec. Data were collected using electrospray ionization (ESI) in positive and negative modes.

Data processing, filtering and normalization
The data analysis and metabolite identification are summarized in Online Resource Supplemental Figure 2. Raw data files were exported to mzData format, and the R package xcms was used to analyze raw mass spectral data [26-28]. Feature detection was performed using the xcms "centWave" algorithm, and retention-time correction was performed using the function "peakgroups" with smoothing via a loess function. The resulting list of ion features was exported for filtering, normalization, and statistical analysis. All analyses were performed using the statistical programming language R [29].

Ions were deemed uninformative and excluded when observed in fewer than 25% of all samples. A pseudo-count of one was added to every ion abundance value to allow for log-transformation, and each sample was scaled by its total ion abundance. Scaled abundances were multiplied by $1x10^6$ for ease of presentation and then log-transformed for subsequent analyses. To remove redundant isotopic peaks, only the major peak was retained among ions that exhibited the following: (1) correlation among chromatograms greater than 0.9; (2) matching retention times; and (3) mass differences of multiples of 1.0087 Da. Finally, to ensure reproducibility, low-signal ions (mean scaled abundance below zero) were removed. The statistical analysis was based on the resulting 1885 total ions (1286 positive and 599 negative).

Selection of the discovery and validation datasets
An initial evaluation of the data using principal component analysis explored whether the global metabolome differed by the characteristics of sex, HCT conditioning regimen, diagnosis or BCT. There were no observed differences with any of these four characteristics. For statistical analyses, the samples were split into separate discovery and validation sets in a manner that maintained the greatest degree of homogeneity within each dataset while maintaining the objective evaluation of associations between ion abundance and busulfan clearance. The discovery dataset (n=59 samples) was comprised of samples collected in citrate BCT with no prior HCT conditioning administered to the subject. Samples in the validation dataset (n=88) were collected in EDTA but had greater heterogeneity with regards to conditioning, having had no conditioning (n=31), cyclophosphamide (n=50) [7] or fludarabine (n=7) [6] administered before sample collection.

Statistical analysis

Rather than fitting a separate univariate model for each of the 1885 ions, we allowed for correlations among ion abundances by first applying a multivariate regression model. For this, we fit a penalized least squares (ridge) regression model[30] with all ion abundances using IV busulfan clearance as the outcome. This was implemented using the "refund" R package [31] in which the penalty tuning parameter is chosen automatically via a restricted maximum likelihood approach (Randolph et al.[32]). From this model, ions were selected by keeping only those whose regression coefficient had a 95% confidence interval not containing zero. Among the resulting 167 ions, this list was further reduced by retaining ions whose abundances exhibited an r^2 of at least 0.3 in a (marginal) univariate linear model of association with IV busulfan clearance *and* a monotonic increase or decrease across subjects ordered by tertiles of IV busulfan clearance. The latter criterion served to focus on ions that would be of most predictive of IV busulfan clearance. The possible identities of these 21 ions, which are referred to as "selected" ions, were determined as described below.

In parallel, we constructed a statistical predictive model by starting with the selected ions and applying backward and forward stepwise variable-selection. The goodness of fit was assessed using the Akaike information criterion (AIC). The final linear model contained seven ions, which are referred to as "predictive" ions.

Validation dataset

To verify the ability of the seven-ion predictive model to estimate IV busulfan clearance, this model was applied to the validation dataset, and IV busulfan clearance was calculated for each subject. A predicted IV busulfan clearance was considered to be accurate for each subject if the value was within 80 to 125% of the observed IV busulfan clearance. This 80 to 125% range is used by the FDA to ascertain bioequivalence [33] and is a reasonable metric by which to determine prediction accuracy. The model validation was completed by applying the predictive model, created using the discovery dataset, to the samples in the validation dataset.

Evaluation of blood collection tubes (BCT)

Of the 108 subjects, 39 subjects had samples collected on two separate occasions, once in citrate BCT (i.e., part of the discovery dataset) and once in EDTA BCT (i.e., part of the validation dataset). These samples allowed for a comprehensive evaluation of the within-subject variability and the effect of the BCT upon the 21 selected ions. To ascertain whether the BCT affected the ion abundance in the predictive model, the correlation was determined for each ion abundance measured in the citrate versus EDTA BCT for the 39 subjects.

Ion identification

Identification of the 21 selected ions was carried out following established methods [34] and by searching major metabolomics databases using the accurate mass (within 15 ppm) and MS/MS fragmentation spectra when available. Databases queried included METLIN (http://metlin.scripps.edu/), Massbank (http:// massbank.imm.ac.cn/MassBank) and HMDB (Version 3.0 http://hmdb.ca/) using exact molecular weights. Commercially available standards were purchased from Sigma-Aldrich (St. Louis, MO) to confirm the identity of ions with a putative identification.

RESULTS

Patient characteristics

Patient pre-transplant demographics and HCT characteristics are described in Table 1. The mean age was 50.4 years (range: 21.6 to 65.8) with a slightly higher percentage of males (60.5%). The mean (± standard deviation) of the IV busulfan clearance was 3.17 ± 0.53 ml/min/kg NFM for the discovery dataset and 3.26 ± 0.56 ml/min/kg NFM for the validation dataset. The IV busulfan clearance by dosing weight and NFM are shown in Online Resources Supplemental Figures 2A and 2B.

Pharmacometabolomics

The goal of these analyses was to determine whether endogenous pharmacometabolomics-based biomarkers obtained before IV busulfan administration could be used to predict IV busulfan clearance. After aligning, filtering and normalizing the data, a total of 1885 ions were included in the analyses (Online Resource Supplemental Figure 2). The data were split into discovery and validation datasets based on the BCT. The 21 selected ions were detected within a two-minute window using our LC-QTOF conditions (range: 10.5 to 12.5 min). Of the selected ions, 11 ions were detected in negative ESI mode, and 10 ions were detected in positive ESI mode (Table 2). The ion with the strongest correlation with IV busulfan clearance (r^2 = 0.57) was *m/z* = 626.353 with a retention time of 11.0 min and was detected in negative mode. Twelve of these selected ions were positively correlated with IV busulfan clearance, whereas the other nine ions had abundances that decreased as clearance increased (Figure 2).

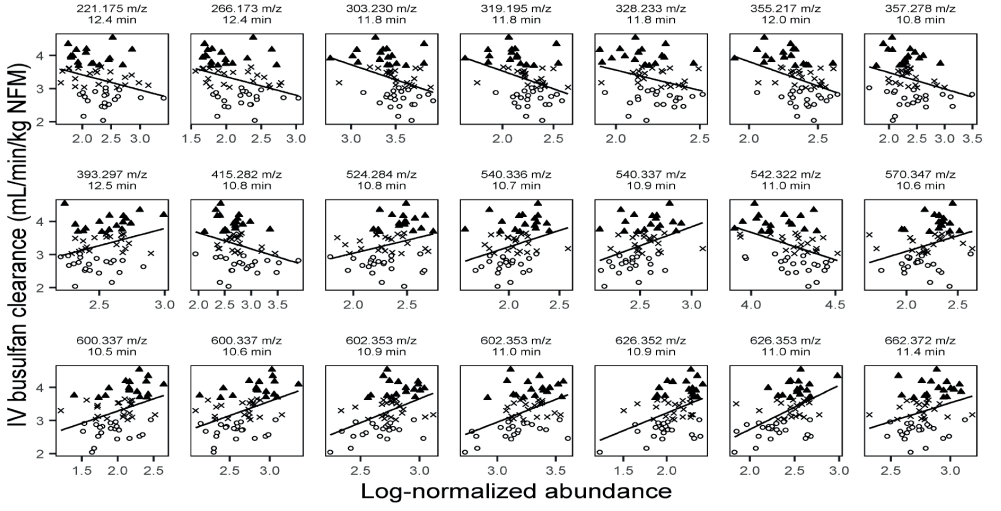

Figure 2. Linear regressions of discovery dataset ion abundance with IV busulfan clearance of the selected 21 ions with r^2 = 0.3 and monotonic change by IV busulfan clearance tertile. Open circles – slow clearance tertile, "X" symbols – moderate clearance tertile, black triangles – rapid clearance tertile. Each ion is identified by the mass-to-charge ratio (m/z) and retention time (RT in min).

Table 2. Selected ions (n=21) and the predictive ion subset (n=7) from discovery dataset based on IV busulfan clearance.

m/z	Retention Time (min)	Ionization Mode	Ion abundance by IV busulfan clearance tertile (mean ± s.d.)			Weighted r^2	ANOVA p value	Number Data base Matches	Primary Database Classification/ Metabolite Identified[b]
			Slow Clearance	Moderate Clearance	Rapid Clearance				
221.175	12.4	+	2.41 ± 0.34	2.27 ± 0.45	2.16 ± 0.34	0.33	0.1	27	Isoprenoid
266.173	12.4	+	2.25 ± 0.31	2.13 ± 0.40	1.99 ± 0.33	0.48	0.09	7	Unknown
303.230[a]	**11.8**	**+**	**3.55 ± 0.18**	**3.52 ± 0.22**	**3.36 ± 0.27**	**0.39**	**0.02**	**278**	**Linoleic acid**
319.195	11.8	+	2.29 ± 0.16	2.27 ± 0.19	2.12 ± 0.23	0.31	0.02	17	Linoleic acid
328.233	11.8	+	2.25 ± 0.14	2.24 ± 0.14	2.15 ± 0.14	0.38	0.05	8	Fatty Acyl
355.217	12.0	+	2.40 ± 0.13	2.39 ± 0.14	2.26 ± 0.17	0.38	0.007	12	Isoprenoid
357.278	10.8	+	2.47 ± 0.44	2.37 ± 0.26	2.27 ± 0.28	0.36	0.2	46	Chenodeoxy, cholic acid or Deoxycholic acid
393.297[a]	**12.5**	**+**	**2.45 ± 0.16**	**2.54 ± 0.16**	**2.57 ± 0.20**	**0.40**	**0.1**	**168**	**Bile Acid**
415.282[a]	**10.8**	**+**	**2.85 ± 0.45**	**2.73 ± 0.27**	**2.62 ± 0.29**	**0.34**	**0.1**	**121**	**Chenodeoxycholic acid or Deoxycholic acid**
524.284	10.8	-	2.26 ± 0.22	2.29 ± 0.23	2.45 ± 0.16	0.33	0.01	9	Glycerophospholipid
540.336	10.7	-	1.97 ± 0.16	2.06 ± 0.18	2.13 ± 0.22	0.31	0.05	2	Steroid Conjugate
540.337	10.9	-	2.41 ± 0.17	2.55 ± 0.19	2.58 ± 0.22	0.38	0.02	2	Steroid Conjugate
542.322[a]	**11.0**	**+**	**4.28 ± 0.14**	**4.27 ± 0.14**	**4.14 ± 0.14**	**0.31**	**0.01**	**9**	**Glycerophosphocholine**
570.347	10.6	-	2.14 ± 0.19	2.21 ± 0.24	2.30 ± 0.15	0.40	0.05	11	Glycerophospholipid
600.337[a]	**10.5**	**-**	**1.91 ± 0.26**	**1.98 ± 0.29**	**2.16 ± 0.31**	**0.38**	**0.03**	**20**	**Glycerophospholipid**
600.337[a]	**10.6**	**-**	**2.55 ± 0.25**	**2.63 ± 0.27**	**2.83 ± 0.31**	**0.34**	**0.01**	**21**	**Glycerophospholipid**
602.353	10.9	-	2.65 ± 0.19	2.75 ± 0.17	2.83 ± 0.15	0.31	0.007	7	Unknown
602.353	11.0	-	3.14 ± 0.23	3.27 ± 0.13	3.30 ± 0.18	0.32	0.03	7	Unknown
626.352	10.9	-	1.96 ± 0.27	2.06 ± 0.21	2.18 ± 0.18	0.34	0.01	4	Glycerophospholipid
626.353[a]	**11.0**	**-**	**2.26 ± 0.25**	**2.45 ± 0.20**	**2.54 ± 0.17**	**0.57**	**0.0006**	**4**	**Glycerophospholipid**
662.372	11.4	-	2.73 ± 0.16	2.75 ± 0.17	2.87 ± 0.16	0.33	0.02	1	Fatty Acyl

[a]The seven ions included in the predictive model are presented in bold text. These ions had a ridge regression coefficient whose 95% confidence interval did not contain zero, had $r^2 \geq 0.3$, and had a monotonic increase or decrease in abundance across busulfan clearance tertiles. [b]Database classification was determined from the most common classification in database hits.

A predictive model for IV busulfan clearance

A linear predictive model was subsequently built from these 21 selected ions. As described in the Statistical Analysis section, we used stepwise variable selection to construct a linear model based on seven ions as predictors of IV busulfan clearance (Table 3). The prediction of clearance was defined as accurate when the predicted IV busulfan clearance fell within 80 to 125% of the observed IV busulfan clearance.[33] Of the 59 samples in the discovery dataset, IV busulfan clearance was accurately predicted for 55 samples (93.2%). For the 88 samples in the validation dataset, which were not used in constructing the predictive model, IV busulfan clearance was accurately predicted for 69 samples (78.4%). Figure 3 shows the observed versus predicted IV busulfan clearance for both the discovery and validation datasets.

Table 3. Model coefficients for the seven-ion predictive model.

Ion	Retention Time (min)	♌ Estimate ± Standard Error	P-value
[Intercept]		3.15 ± 2.36	0.2
600.337	10.5	-2.14 ± 1.11	0.06
600.337	10.6	2.27 ± 1.13	0.05
626.353	11.0	0.80 ± 0.31	0.01
303.230 (Linoleic acid)	11.8	-0.53 ± 0.27	0.06
393.297	12.5	1.10 ± 0.33	0.002
415.282 (Chenodeoxycholic acid or deoxycholic acid)	10.8	-0.26 ± 0.16	0.1
542.522	11.0	-0.89 ± 0.41	0.03

The predictive model is of form $E(y)=\beta_0 + \beta_1 x_1 + \ldots \beta_7 x_7$ where y is IV busulfan clearance and x_j is the abundance of the j^{th} ion. The coefficients, β_j, were estimated using the discovery data. These values of β_j were then fixed in this equation while the x_j values obtained from the validation dataset were used to obtain the predicted values, y*, of the observed y.

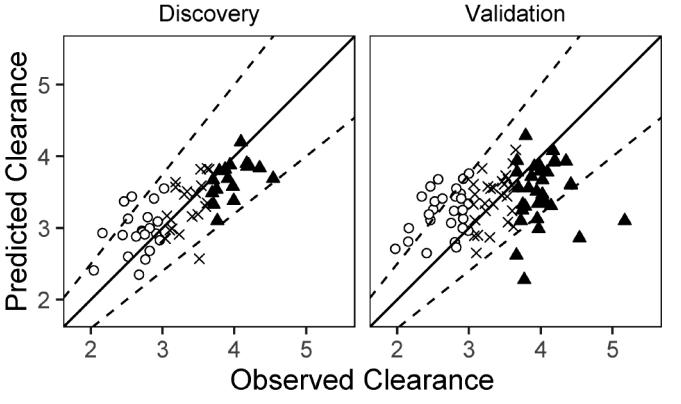

Figure 3. The performance of the seven-ion predictive model to estimate IV busulfan clearance in the discovery and validation datasets. The middle solid line represents the line of unity, and the outer dotted lines represent 80 and 125% of the observed IV busulfan clearance. Observed clearance is IV busulfan clearance (ml/min/kg NFM) estimated using noncompartmental analysis. Predicted clearance is IV busulfan clearance (ml/min/kg of NFM) estimated using the seven-ion predictive model. Open circles – slow clearance tertile, "X" symbols – moderate clearance tertile, black triangles – rapid clearance tertile.

Evaluation of BCT

For the seven predictive ions, the ion abundances were similar when plasma was collected in citrate or EDTA BCTs. A strong positive correlation (r^2 from 0.50 to 0.85) was found for six of the seven ions, suggesting that the BCT did not greatly affect ion abundance (Online Resource Supplemental Figures 3). The predicted IV busulfan clearances from the citrate BCT samples were comparable to the predicted IV busulfan clearances from the EDTA BCT samples, suggesting that the BCT did not substantially affect the results of the predictive model (Online Resources, Supplemental Figures 3 and 4).

Ion identification

Four of the 21 selected ions were potentially identified. For the remaining 16 ions, the hypothesized classes as determined by METLIN is listed in Table 2. The authentic standards for both chenodeoxycholic acid and deoxycholic acid, which differ only in the position of a hydroxyl group and thus have identical molecular weights, eluted at the same time (~10.8 min) and with peaks at the same *m/z* as the ions with *m/z* of 357.279 and 415.282. These ions represent the loss of two water molecules (*m/z* of 357.279; i.e., [M+H - 2 H_2O]+) and the sodium adduct (*m/z* of 415.282; i.e., [M+Na]+) of chenodeoxycholic acid and/or deoxycholic acid (monoisotopic MW = 392.293 Da). When selected samples were analyzed at a collision energy of 20 V, the fragmentation pattern of the 357.279 *m/z* ion was similar to that of authentic standards of both chenodeoxycholic acid and deoxycholic acid. We were not able to fragment the 415.282 *m/z* ion. Chenodeoxycholic acid and deoxycholic acid could not be resolved chromatographically using the stated LC-QTOF conditions.

The ions with *m/z* of 303.230 and 319.193 eluting at 11.8 minutes are consistent with the sodium (i.e., [M+Na]+) and potassium (i.e., [M+K]+) adducts of linoleic acid. The authentic standard for linoleic acid gave rise to peaks with *m/z* of 281.247 ([M+H]+), 303.230 ([M+Na]+), and 319.193 ([M+K]+) that co-eluted within 0.1 min of the peaks seen in clinical plasma samples. The putative [M+H]+ peak was also observed in clinical samples, but a slightly overlapping peak at the same *m/z* likely interfered with its accurate integration, and that ion was not significantly correlated with busulfan clearance. The hypothesized sodium and potassium adducts of linoleic acid were unable to be fragmented successfully, precluding confirmation of its identity based on fragmentation pattern.

DISCUSSION

These analyses provide encouraging results demonstrating the potential of pharmacometabolomics-based markers for informing IV busulfan dosing. Using a semi-quantitative, untargeted LC-MS platform to measure predose endogenous metabolite ions in plasma in citrate BCT, we built a statistical model from a small number of ions that provides a modest but reproducible capability to predict IV busulfan clearance in a separate dataset of subjects whose plasma samples were collected in a different BCT (i.e., EDTA).

Busulfan is a bi-functional alkylating agent that rapidly (i.e., within 2 minutes) damages DNA in a dose-dependent manner *ex vivo* [35]. Busulfan plasma exposure has proven to be a predictive marker for outcomes, and PK-guided busulfan dosing has been shown to lower rates of rejection, nonrelapse mortality, and relapse in select HCT recipients [36,37]. Although the recent increase in PK-guided dosing of busulfan shows that this strategy is feasible, the resource intensity of pharmacokinetic sampling has been a barrier. PK-guided dosing is resource-intensive, involving the collection of multiple blood samples after IV busulfan administration, quantitation of plasma busulfan concentrations, and subsequent pharmacokinetic analysis to calculate patient-specific busulfan clearance (as clearance = dose/AUC) for personalizing future doses to the target AUC. More efficient methods to estimate IV busulfan systemic exposure and clearance are desirable because shorter (i.e., 2-day) busulfan regimens are increasingly popular. The combination of estimating busulfan doses to achieve target busulfan AUC based on a population pharmacokinetic model [11] and pre-dose pharmacometabolomics may decrease the need for resource-intensive PK-guided dosing.

After IV administration of radiolabeled busulfan, less than 50% of the administered dose is recovered in the urine [38,39]. Approximately one-third (i.e., 32.8 ± 2.2%) of busulfan is irreversibly bound to plasma proteins, primarily albumin [40]. Only a small fraction (<3%) of a busulfan dose is excreted unchanged in the urine, with negligible amounts in the feces [41,42] The primary route of elimination for busulfan is by hepatic glutathione conjugation, catalyzed by glutathione transferases (GSTs) [43-45]. Of the various classes of GSTs, busulfan is predominantly conjugated by GSTA1-1 (alpha class), with GSTM1-1 (mu class) and GSTP1-1 (pi class) participating to a lesser degree [45]. The conjugated metabolite, γ-glutamyl-β-(*S*-tetrahydrothiophenium)-alanyl-glycine (THT^+) [46,47], is subsequently metabolized by various cytochrome P450 enzymes, specifically CYP1A1, 2B6, 2C8, 2C9, and 2C19 [48].

The associations of IV busulfan clearance with various genetic polymorphisms, usually those regulating GSTA1-1 and GSTM1-1 hepatic protein expression, have been examined. Polymorphisms in *GSTA1* and *GSTM1* are not consistently associated with IV busulfan clearance, making constitutional genetics-based dosing infeasible [49,50]. In HCT recipients, plasma alpha GST activity was only moderately correlated with hepatic GST expression (r^2=0.567) [51] as was blood GSH with IV busulfan clearance (r^2=0.45) [52]. Thus, lymphocyte mRNA or protein activity will not sufficiently reflect hepatic mRNA or protein expression of IV busulfan-metabolizing enzymes, making RNA or protein techniques obtained from a blood sample infeasible biomarkers for IV busulfan PK. Alternative techniques, such

as pharmacometabolomics, can be used to identify biomarkers to replace PK-guided IV busulfan dosing, with the eventual goal of improving overall survival after IV busulfan-containing HCT conditioning regimens. The work herein is encouraging proof of principle that IV busulfan clearance can be predicted by endogenous pharmacometabolomics.

Of the 21 selected ions associated with IV busulfan clearance tertiles, deoxycholic acid and/or chenodeoxycholic acid as well as linoleic acid were identified. We could not find any published literature evaluating the effect of these bile acids upon busulfan metabolism, though bile acids have been shown to inhibit human GST activity *in vitro*.[53] Singh et al. showed that human GSTs are inhibited by bile acids, including chenodeoxycholic acid, with varying potency based on the GST isozyme studied.[53] Conjugation by GST is the first step in busulfan metabolism and inhibition of this step could lead to a decrease in busulfan clearance. In our study, while we did observe that individuals with slow clearance showed elevated levels of linoleic acid and deoxycholic acid and/or chenodeoxycholic acid when compared to those with rapid clearance, further work needs to be done to confirm this association.

A different bile acid – ursodeoxycholic acid – is administered to HCT recipients to lower the risk of hepatotoxicity. In HCT recipients, there were no statistically significant differences in cyclosporine pharmacokinetics between ursodeoxycholic acid and placebo but its effect upon other HCT medications – including busulfan – have not been examined. Chenodeoxycholic acid increases hepatic mRNA expression of CYP2B6, 2C8, 2C19 and 3A4 in a human hepatocyte model [54], and induces CAR, FXR, and PXR in primary hepatocytes and Caco-2 cells [55]. CAR induces expression of CYP1A1, and CAR and PXR both induce expression of CYP2B6, 2C9, and 2C19 [56]. These CYPs are involved in the metabolism of the busulfan metabolite THT^+ [48]. Also, GSH conjugates of chenodeoxycholic acid have been found in the bile of human infants (although not adults) [57], which may be relevant since the primary route of busulfan metabolism is via conjugation of GSH by GSTs. If GST-mediated glutathionylation of busulfan is lower in some patients than in others, then GST-mediated glutathionylation of bile acids could also be low in these patients, leading to relatively higher concentrations of the bile acids chenodeoxycholic acid and/or deoxycholic acid (i.e., the ions with *m/z* of 357.278 and 415.282 with retention time of 10.8 minutes) – and lower concentrations of the products of that reaction, GSH-bile-acid conjugates (not measured). In this analysis, chenodeoxycholic acid or deoxycholic acid were negatively correlated with IV busulfan clearance. The putative mechanism of deoxycholic acid and chenodeoxycholic acid association with IV busulfan clearance could be via their influence upon THT^+ metabolism or could reflect GST activity, but *in vitro* studies are needed to test and to clarify these hypotheses.

There are some limitations to this work. IV busulfan plasma exposure was not the primary endpoint because it is determined by the administered IV busulfan dose [9], the administration interval, and the patient-specific IV busulfan clearance. The purpose of PK-guided dosing is to estimate the patient-specific IV busulfan clearance, which was the primary endpoint. Given the substantive number of ions identified detected with global pharmacometabolomics approaches, we focused upon a discrete and well-defined endpoint (i.e., busulfan clearance) over clinical outcomes because of the heterogeneity

of the patient population (Table 1). Typical of an HCT analysis, there was considerable heterogeneity in the additional components of the HCT conditioning regimens, in the underlying diagnoses of the patient population, and in the type of allogeneic graft, all of which can affect clinical outcomes [1]. The samples were collected in two different BCT, which fortunately did not appear to influence the ions of interest. We assigned samples to the discovery dataset because of their homogeneity (i.e., no HCT conditioning medications in the pharmacometabolomics samples). Despite the difference in HCT conditioning in the discovery and validation datasets, IV busulfan clearance was adequately estimated by the predictive model in the validation datasets (i.e., 78.4% of the validation samples were within 80% to 125% of the observed IV busulfan clearance values). More precise estimation of IV busulfan clearance is desirable to allow more accurate personalization of IV busulfan doses to achieve the desired busulfan plasma exposure. Finally, as in other pharmacometabolomics studies, identification of ions of interest is a hurdle. We identified deoxycholic acid and/or chenodeoxycholic acid and linoleic acid as part of the 21 selected ions, but only one ion as part of the seven predictive ions. An analysis of deoxycholic acid, chenodeoxycholic acid, and linoleic acid concentrations, quantitated using targeted assays, and subsequent correlation with IV busulfan clearance should be conducted. Finally, these results should be confirmed in a prospective study to determine how precisely the predictive model is able to estimate IV busulfan clearance and what other information can be incorporated (e.g., population-based PK model) [11] to improve the estimation of the IV busulfan dose for HCT patients.

CONCLUSIONS

We evaluated the association of the relative abundance of 1885 ions with IV busulfan clearance and found 21 ions that were significantly correlated with IV busulfan clearance. The seven-ion predictive model accurately predicted IV busulfan clearance in the majority of the validation samples. These results reinforce the potential of pharmacometabolomics as a critical tool in personalized medicine, with the potential to improve the personalized dosing of drugs with a narrow therapeutic index such as busulfan.

ACKNOWLEDGEMENTS

The authors thank the study subjects, their caregivers, and the patient care staff for their support of this study. This work was supported by research funding from the National Institute of Health (CA182963, ES07033, CA18029, and CA15704)

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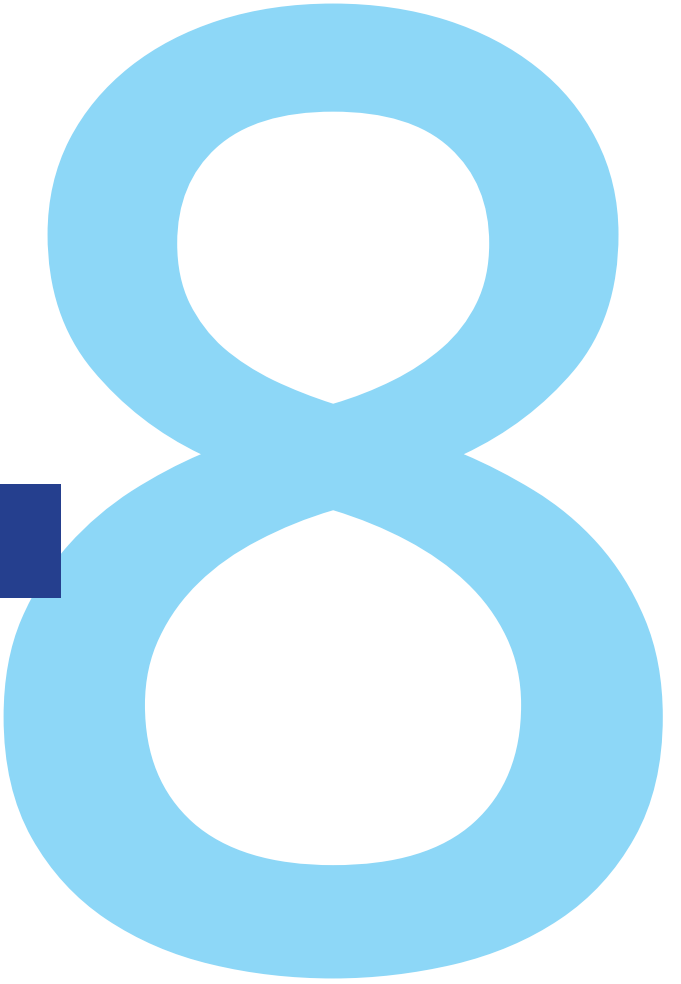
Supplemental Materials: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5562150/

CHAPTER 8



Prediction of Busulfan Clearance by Predose Plasma Metabolomic Profiling

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Submitted for publication

ABSTRACT

Intravenous (IV) busulfan doses are often personalized to a target plasma exposure (targeted busulfan) using an individual's busulfan clearance. Previously, we found that glycine metabolism was associated with busulfan clearance in a targeted panel of endogenous metabolomic compounds (EMCs). With the ultimate goal of finding a panel of plasma EMCs that predict busulfan clearance, in the present study we extend our findings using a global pharmacometabonomics approach in a cohort of 132 hematopoietic cell transplant (HCT) patients receiving busulfan before allogeneic HCT. A total of 841 EMCs were quantitated in 228 longitudinal blood samples before IV busulfan administration. We performed both univariate linear regression and pathway analyses using global testing. All analyses were controlled for false discovery. For the pre-busulfan and 2-week pre-busulfan time points, we found 37 and 27 EMCs, respectively, significantly associated with busulfan clearance. Using pathway enrichment analysis, 18 pathways were statistically significantly associated with IV busulfan clearance. Lysine degradation was the top pathway, followed by Steroid biosynthesis, which aligned with several androgen metabolites significantly associated with IV busulfan clearance in the univariate analysis. These results suggest pharmacometabonomics can improve or replace pharmacokinetics to personalize busulfan doses.

INTRODUCTION

Many allogeneic hematopoietic cell transplantation (HCT) patients receive high-dose busulfan (busulfan) as part of their HCT conditioning regimen.[1,2] Busulfan has a narrow therapeutic index. Busulfan exposure, expressed as area under the plasma concentration-time curve (AUC)[3], is associated with the efficacy and toxicity of many busulfan-based conditioning regimens.[4] The plasma AUC is determined by the administered dose and the patient's clearance (i.e., $AUC = \frac{dose}{clearance}$). Low busulfan AUC, caused by underdosing or rapid clearance (or both), is associated with reduced efficacy (e.g., increased risk of relapse or rejection). Conversely, high busulfan AUC, caused by overdosing or a slow clearance, is associated with an increased risk of liver toxicity and non-relapse mortality (NRM).[4]

These clinical outcomes are improved by pharmacokinetic (PK)-guided busulfan dosing, in which busulfan doses are personalized to clinician-chosen target plasma AUC using the individual patient's (i.e., patient-specific) busulfan clearance.[4] Ideally, the target AUC is achieved with the initial busulfan dose. The current clinical practice of using various age- and weight-based dosing schemes for the initial busulfan dose achieves the clinician-chosen target busulfan AUC in only 22.6% of patients.[5] PK-guided busulfan dosing is often successful: personalizing IV busulfan doses using PK-guided dosing results in over 85% of patients achieving the clinician-chosen target busulfan AUC at the end of 4-days of busulfan therapy.[5] Although PK-guided busulfan dosing is feasible and improves clinical outcomes, the current process (Table 1) is resource- and labor-intensive and time-sensitive, in part because of the short (up to four days) administration schedule of busulfan. In addition, PK-guided dosing is rarely feasible with the shorter (i.e., <4 day) busulfan courses included in reduced intensity or gene therapy conditioning regimens.

Table 1. Steps involved in personalizing busulfan doses: PK-guided busulfan dosing of the initial (first) therapeutic busulfan dose to achieve the target AUC

Steps	Current	Validated alternative	Undergoing investigation
1. Chose and then administer initial busulfan dose	Body weight, age	Population pharmacokinetic-model guided[4,45,46]	Before busulfan administration: Test dose[47] Pharmacogenomics[48] Pharmacometabonomics[9,10]
2. Pharmacokinetic blood sampling	Extensive sampling	Limited sampling schedules[45,46]	Sampling in saliva[49]
3. Quantitation of busulfan concentrations	LC-MS	None available	
4. Pharmacokinetic modeling of concentration-time data to estimate patient's AUC	Noncompartmental or compartmental analysis of one patient's data	PopPK-guided dosing with a posterior Bayesian prediction[46]	
5. Estimation of one patient's busulfan clearance (CL)	*CL = dose/AUC*	No alternative needed (fundamental principal of pharmacokinetics)	
5. Determine one patient's personalized dose to achieve their target AUC	*Dose = Target AUC X clearance*		

It is anticipated that metabolomics could improve –or ideally replace– PK-guided busulfan dosing to decrease its resource intensity. In the context of drug response, there are two categories of metabolomic study design. The first is pharmacometabonomics, which is "the prediction of the outcome (e.g., efficacy or toxicity) of a drug intervention in an individual, based on a mathematical model of 'preintervention' EMCs.[6] The second is pharmacometabolomics (PMx), which is defined as an "enhanced understanding of mechanisms for drug effect and increased ability to predict individual variation in drug response phenotypes, based on using both baseline EMCs profiles prior to treatment and also effects of drug treatment over time ('longitudinal' metabolomic profiles)" .[7,8] Here, we sought to validate our previous global[9] or targeted[10] pharmacometabonomic analyses of endogenous metabolomic compounds (EMCs) associated with IV busulfan clearance and extend our findings using a larger set of identified EMCs. In our previous global pharmacometabonomic analysis, we found that tertiles of increasing IV busulfan clearance were associated with 21 ions ($R^2 \geq 0.3$). Identified EMCs included the bile acids deoxycholic acid and/or chenodeoxycholic acid, and linoleic acid. Bile acids have been shown to inhibit glutathione-*S*-transferases *in vitro*. Using targeted pharmacometabonomic analysis, we found that the EMCs glycine, N-acetylglycine, 2-hydroxyisovaleric acid, creatine, serine, and tyrosine were significantly associated with IV busulfan clearance, and that pathway analyses were consistent, revealing that the glycine, serine, threonine pathway was also associated with IV busulfan clearance. Glycine is conjugated to busulfan during detoxification, indicating the biological plausibility of our findings in predicting busulfan clearance. Against this background, we sought to validate that these EMCs were

associated with IV busulfan clearance and expand the repertoire of EMCs using a global pharmacometabonomics approach with an evaluation of 783 identified EMCs in plasma prior to IV busulfan administration in 132 allogeneic HCT recipients.

METHODS

Study population

This was an ancillary prospective study of 132 participants who received HCT conditioning with IV busulfan from December 2014 to November 2018 under the aegis of a protocol approved by the Fred Hutchinson Cancer Research Center (Fred Hutch) Institutional Review Board (clinicaltrials.gov protocol number NCT02291965). The inclusion criterion was: scheduled to receive intravenous busulfan (any dose, any number of doses, any dosing frequency) as part of their HCT conditioning. The exclusion criteria were: inability to read English; female patients who were pregnant or breastfeeding; life expectancy severely limited by diseases other than malignancy. All participants provided written informed consent prior to study procedures. The conditioning regimen and post-graft immunosuppression were not affected by participation in this study. Standard practice for prophylaxis of busulfan-induced seizures was phenytoin (adults) or levetiracetam (children). Antiemetics, antibiotics, and antifungals were given per Institutional Standard Practice Guidelines. Demographic data were taken from the participants' medical charts [age, sex, height, total body weight (i.e., actual; ABW), body surface area, and clinical information (disease, conditioning regimen)].

Busulfan dosing

Busulfan clearance was the primary outcome of interest. The patient-specific IV busulfan clearance, not the busulfan AUC, is the relevant endpoint because the goal with PK-guided dosing is to obtain the patient-specific IV busulfan clearance to be used for dose personalization to achieve the clinician-chosen target busulfan AUC.

The initial busulfan dose, chosen by the treatment protocol or the participant's treating physician, was based on actual body weight (ABW) or body surface area. The busulfan dose 1 was calculated using ABW if it was less than ideal body weight or adjusted ideal body weight (AIBW, which equals 0.25 (ABW – ideal weight) + ideal weight) if it was greater than IBW. The IBW in adults was calculated as follows: for males = 50 kg + (2.3 kg for each inch over 5 feet); for females = 45.5 kg + (2.3 kg for each inch over 5 feet). In total, 125 (94.7%) participants underwent PK-guided dosing of IV busulfan, also called targeted busulfan (^{T}BU). In those receiving ^{T}BU, subsequent IV busulfan doses were personalized to achieve the desired target busulfan AUC, chosen for the treatment protocol by the attending physician (i.e., clinician-chosen). In the seven participants conditioned with the CNS regimen (busulfan/cyclophosphamide/thiotepa), busulfan doses were dosed based on body weight and not personalized to a target AUC.

Busulfan pharmacokinetic sampling and quantitation

Because of the circadian variability of busulfan pharmacokinetics,[11] all busulfan pharmacokinetic sampling occurred in the morning. Blood samples (3 ml/sample) were

collected in sodium heparin tubes before the morning doses of days 1, 2, 3, and 4 of IV busulfan administration. For those participants receiving IV busulfan every 24 hours (Q24h), pharmacokinetic samples were drawn at the end of the 3-hour (hr) infusion and at 3.25, 4.5, 6, 8, and 24 hours (i.e., prior to the subsequent dose) after the beginning of the infusion.[12] For patients receiving IV busulfan every 12 hours (Q12h), PK samples were drawn at the end of the 2-hour infusion, 2.25; 4, 5, 6, and 8 hours. For those participants receiving IV busulfan every 6 hours (Q6h), PK samples were drawn at the end of the 2-hour infusion and at 2.25, 2.5, 3, 4, 5, and 6 hours (i.e., prior to subsequent dose) after the beginning of the infusion. All samples were stored on wet ice or refrigerated. For those participants receiving PK-guided busulfan dosing, their samples were transported to the Seattle Cancer Care Alliance Busulfan Laboratory, a College of American Pathology-certified laboratory that has focused exclusively on PK-guided dosing of busulfan since 1996. Plasma busulfan concentrations were analyzed by gas chromatography with mass-selective detection as previously described.[12] The remaining (i.e., after the doses without subsequent PK-guided busulfan dosing) busulfan pharmacokinetic samples were analyzed using reverse-phase high-performance liquid chromatography with mass spectrometry detection (LC-MS), using a method modified from Snyder et al.[13] For each sample, 25 µL of plasma were combined with the internal standard (80 ng of d8-busulfan). This sample then underwent protein precipitation with 200 µL methanol, followed by vortexing and centrifugation. The supernatant was transferred to a 96-well plate, and 1 mL was injected on an Agilent 1100 series high-performance liquid chromatography coupled to an Agilent G1946D mass spectrometer operating in positive electrospray ionization (ESI) mode. Separation was achieved on an Agilent Zorbax column (RX-C8, 2.1 mm x 150 mm, 5 micron) using methanol and 10mM ammonium formate, pH 3.5 (67.5%) as an isocratic mobile phase. Ions monitored were 264 (busulfan) and 272 (d8-busulfan) *m/z*. The correlation coefficient was used to evaluate the linearity of the calibration curves and was >0.99 in all experiments. The limit of quantitation was 50 ng/mL (coefficient of variation (CV)=5.7%; accuracy 107.3%) and signal to noise (S/N) ratio of 7.5. The limit of detection was 25 ng/mL, with a S/N ratio of 3.8. Both laboratory values were within the defined specifications for comparable results (i.e., within 15% of quality control samples) participated in interlaboratory proficiency exercises of busulfan quantitation.

Busulfan population pharmacokinetic modeling

This validation cohort had busulfan clearance over the entire duration of busulfan treatment as the endpoint of interest, as compared to dose 1 busulfan clearance in our retrospective cohort. This change was made for a few reasons: 1. The busulfan clearance did not consistently change over time in this validation cohort; 2. An increasing number of HCT programs do PK-guided busulfan dosing after multiple doses, so the clearance over the entire duration of treatment is of increasing importance.[4]

NONMEM . Supplemental methods 1 contains the NONMEM code. Supplemental **Figure 1** and **2** show the Goodness of Fit and Simulated vs. Actual concentration-time data, respectively. Estimation of busulfan AUCs was accomplished using maximum a posteriori probability (MAP) Bayesian estimation of pharmacokinetic parameters, incorporating a blend of individualized pharmacokinetic data and a population parameter prior. The MAP Bayesian method used individual patient busulfan concentration-time data, together

with a pharmacokinetic model and mean parameter values plus their variance (derived from a prior population pharmacokinetic analysis of 1610 HCT recipients).[14] All reported clearances are based on normal fat mass (NFM), the optimal body metric for IV busulfan clearance over a population of pediatric to adult allogeneic HCT recipients.[14] The use of NFM to characterize IV busulfan clearance was based on the findings of McCune et al., who reported a population pharmacokinetic model based on normal NFM and maturation based on post-menstrual age built from 12,380 busulfan concentration-time points obtained after IV busulfan administration in 1,610 HCT recipients, aged 0.1 – 66 years.[14] All clearance (CL,Q) and volume (V1,V2) parameters were scaled for body size and composition using allometric theory and predicted fat free mass (FFM).[15-17] The population prior parameters were not changed during this study. The estimated busulfan clearance was calculated by dividing the Bayesian AUC estimate by the busulfan dose. **Supplemental Methods** contains the NONMEM code. **Supplemental Figure 1 and 2** show the Goodness of Fit and Simulated vs. Actual concentration-time data, respectively.

Pharmacometabonomics sample collection

Longitudinal blood samples (3 ml/sample) were scheduled to be collected in sodium heparin tubes before IV busulfan dosing: up to two weeks prior to the first conditioning dose (2-week pre-busulfan sample), and immediately before administration of IV busulfan dose 1 (pre-busulfan sample).

Because we sought to identify EMCs predictive of IV busulfan clearance, samples were obtained before busulfan administration. The 2-week pre-busulfan sample collection time was the earliest feasible time within the final HCT workup (i.e., time period, typically up to 2-weeks, in which patients undergo final assessment to determine whether they can receive an HCT). If a predictive association was found and pharmacometabonomics-guided IV busulfan dosing was subsequently used clinically, this sample would allow for more time for pharmacometabonomic quantitation and data interpretation. The immediately pre-busulfan sample was the latest feasible time before busulfan administration. If a predictive association was found and pharmacometabonomics-guided IV busulfan dosing was subsequently used clinically, this sample necessitates a rapid quantitation and data interpretation. A total of 228 pharmacometabonomic samples were obtained: 96 participants had 2-week pre-busulfan samples and 132 participants had pre-busulfan samples.

The pharmacometabonomic samples were immediately refrigerated at 4°C, stored for up to 4 hours from the time of collection, centrifuged to plasma, and immediately stored at -80°C. The samples underwent at most one freeze-thaw cycle before pharmacometabonomic analysis (i.e., the analysis was conducted after the first or the second thaw).

Global pharmacometabonomics analysis

EMCs profiling of plasma was completed by Metabolon (Durham, North Carolina, USA), as we have previously described.[18] The samples were shipped on dry ice to Metabolon's facility and stored at -80°C upon receipt. Samples were divided into 5 aliquots; one was held in reserve while each of the others was analyzed by one of four different mass spectrometry methods. Raw data were extracted, peak-identified, and QC processed; then Metabolon's

proprietary software was used to confirm the consistency of peak identification across the various samples. EMCs were identified by comparison of the processed data to Metabolon's library entries of purified standards or recurrent unknown entities. Library matches for each EMC were checked for each sample and corrected if necessary. Using the criteria established by the Chemical Analysis Working Group as part of the Metabolomics Standards Initiative,[19] most EMCs met the level 1 standards for identification with the remainder meeting the level 2 standards per the same criteria. ANCOVA contrasts were performed to examine differences in EMC levels between the samples at each time point. See **Supplemental Methods** for more details about each EMC quantitation and quality control.

Statistical analysis

All data transformations and analyses were carried out using Stata (v17, College Station, TX). A total of 841 EMCs were reliably measured and retained for analysis. The majority of EMCs were skewed to higher values and were therefore centered log-transformed to approximate a normal distribution. A univariate linear regression model was used to assess marginal associations of each EMC individually on IV busulfan clearance (continuous) over the whole duration of busulfan treatment. In our previous evaluation of 1,610 HCT recipients (n=904 male and n=689 female), used to inform the present analysis, we found no effect of sex or weight on IV busulfan clearance, the endpoint of interest.[14] However, to ensure that sex or weight did not confound our results, sex was included in all univariate models and normal fat mass was used to normalize the busulfan clearance. To determine the percent of variance explained in our model, R^2 was calculated including all significant EMCs in a single regression model. Benjamini-Hochberg methods were used to control for false discovery rate (FDR).[20] Individual EMCs were considered significant at FDR<0.05.

To consider EMC that coordinately predict IV busulfan clearance, pathway analyses inputting all EMCs were carried out using MetaboAnalyst 5.0[21,22] integrating pathway enrichment analysis and pathway topology analysis for visualization. Within the pathway analysis module, EMCs were auto-scaled (mean-centered and divided by the standard deviation of each variable), and IV busulfan clearance was evaluated as a continuous outcome. EMCs were matched to those supported in the MetaboAnalyst compound library [derived from KEGG,[23] Small Molecule Pathway Database[24] (SMPDB), and Human Metabolome Database[25] (HMDB)], which included EMCs in 49 metabolic pathways with sufficient coverage for meaningful pathway analysis. The Global test [26], which evaluates changes among groups of EMCs, was used for statistical significance of pathway enrichment analysis, with FDR<0.1 for multiple comparisons. Betweeness centrality (shortest path between nodes), based on EMC centrality in a given metabolic network, was used to calculate EMC importance.[27] Pathway impact was calculated as the sum of the importance measures of the pathway-specific EMCs, normalized by the sum of the importance measures of all EMCs in each pathway.[28]

RESULTS

Patient characteristics

Pre-transplant characteristics and diagnoses of 132 HCT participants are given in Table 2. Mean age was 46.4 y (range 1.7-70). Slightly more participants were male (65%).

Table 2. Demographic and clinical data for the HCT study population (N=132)

Parameter	N[a]
Age (y)	46.4 (1.7-70)
Male sex	84 (65%)
Adjusted ideal body weight (kg)[b]	82.6 ± 28.7
Normal fat mass (NFM; kg)	68.8 ± 21.6
HCT Conditioning[c]	
Cyclophosphamide/Busulfan±others	64 (48%)
Busulfan/Cyclophosphamide±others	35 (27%)
Busulfan/Fludarabine±others	19 (14%)
CNS regimen[d]	7 (5%)
Fludarabine/Cyclophosphamide/Busulfan	5 (4%)
Busulfan/Melphalan	2 (2%)
Busulfan dosing frequency	
Every 6 hours	17 (13%)
Every 12 hours	1 (1%)
Every 24 hours	114 (86%)
Diagnosis	
Myelodysplastic syndrome (MDS)	71 (54%)
Acute myeloid leukemia	32 (24%)
Chronic myeloid leukemia (CML)	11 (8%)
Non-Hodgkins lymphoma	8 (6%)
Non-malignant diseases	7 (5%)
Neuroblastoma	3 (2%)

[a] Data presented as: number (%) or mean ± standard deviation; percentages may not add up to 100 due to rounding
[b] Total body weight was used for busulfan dosing if total body weight was less than ideal body weight, whereas adjusted ideal body weight was used if total body weight was greater than ideal body weight.
[c] Listed in administration order; Except for those participants treated with the CNS regimen, all received PK-guided dosing of busulfan, in which the IV busulfan dose was personalized based on clearance

Pharmacometabonomics

All 132 participants had a metabolomics sample at the pre-busulfan time point and 96 participants also had a 2-week pre-busulfan sample.

In the univariate analysis, 37 EMCs at the pre-busulfan time point were associated with IV busulfan clearance at FDR<0.05 (**Table 3**). For the 2-week pre-busulfan time point, 27 EMCs were associated with IV busulfan clearance at FDR<0.05 (**Table 4**). Inclusion of these 24 EMCs in a single regression model explained 77% of the variability (R^2=0.77). Several

androgen steroid conjugates (11 and 12 of 17 for pre-busulfan and 2-week pre-busulfan, respectively) were statistically significant at both time points. Of note, all significant associations with busulfan clearance were positive with the exception of 3 EMCs at the pre-busulfan time point: cortisol, cortisone, and methyl-4-hydroxybenzoate sulfate.

In the pathway enrichment analysis, considering all EMCs together, 18 pathways were significant at FDR<0.05 for the pre-busulfan time point (**Table 5**). For the 2-week pre-busulfan time point, there was one significant pathway at FDR<0.05 (**Table 6**). Lysine degradation was the most significant pathway across both time points. This pathway contained 7 EMCs, including lysine, 4-trimethylamonio-butanoate, aminoadipate, N6,N6,N6, trimethyl-L-lysine, N6,N6, dimethyl-lysine, and pipecolate, all of which were positively associated with busulfan clearance, and hydroxy-lysine, which was inversely associated.

Table 3. Association of IV busulfan clearance with endogenous metabolomic compounds (EMC) in the pre-busulfan time point (FDR<0.05).

EMC[a]	Beta Coefficient[b]	P-value[b]	FDR[c]	Evaluated previously
5alpha-androstan-3beta,17beta-diol disulfate	0.18	3.60E-11	3.03E-08	
mannonate	0.06	6.60E-10	5.52E-07	
androstenediol (3beta,17beta) disulfate (2)	0.11	1.10E-09	8.98E-07	
N,N,N-trimethyl-alanylproline betaine (TMAP)	0.08	1.40E-08	1.16E-05	
androstenediol (3beta,17beta) disulfate (1)	0.13	2.60E-08	2.16E-05	
androstenediol (3beta,17beta) monosulfate (2)	0.11	9.50E-08	0.00008	
5alpha-androstan-3beta,17alpha-diol disulfate	0.12	2.10E-07	0.0002	
androsterone glucuronide	0.12	2.40E-07	0.0002	
urate	0.03	5.90E-07	0.0005	Navarro, 2016[10] (N/S)
epiandrosterone sulfate	0.15	8.20E-07	0.0007	
N-acetylcarnosine	0.06	1.30E-06	0.001	
hydroxy-N6,N6,N6-trimethyllysine	0.05	1.60E-06	0.001	
androsterone sulfate	0.16	1.70E-06	0.001	
3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	0.13	2.10E-06	0.002	
androstenediol (3alpha, 17alpha) monosulfate (3)	0.12	2.20E-06	0.002	
5alpha-androstan-3alpha,17beta-diol monosulfate (2)	0.09	2.80E-06	0.002	
picolinoylglycine	0.07	4.20E-06	0.003	
creatinine	0.03	4.30E-06	0.004	Navarro, 2016[10] (P=0.006)
N-acetylalanine	0.03	4.90E-06	0.004	
palmitoyl-arachidonoyl-glycerol (16:0/20:4)	0.10	4.90E-06	0.004	
cortisol	-0.13	9.70E-06	0.008	
N6,N6,N6-trimethyllysine	0.04	0.00001	0.01	
cortisone	-0.12	0.00001	0.01	
imidazole lactate	0.04	0.00002	0.01	
methyl-4-hydroxybenzoate sulfate	-0.20	0.00002	0.01	

EMC[a]	Beta Coefficient[b]	P-value[b]	FDR[c]	Evaluated previously
deoxycarnitine	0.03	0.00002	0.01	Navarro, 2016[10] (N/S)
arachidonoylcarnitine (C20:4)	0.06	0.00002	0.02	
1-methylhistidine	0.05	0.00003	0.03	
pyroglutamine	0.07	0.00004	0.03	Navarro, 2016[10] (N/S)
5alpha-androstan-3alpha,17beta-diol disulfate	0.08	0.00004	0.03	
palmitoyl-arachidonoyl-glycerol (16:0/20:4)	0.08	0.00004	0.03	
lysine	0.02	0.00004	0.03	Navarro, 2016[10] (N/S)
gamma-glutamylisoleucine	0.04	0.00004	0.03	
argininate	0.09	0.00005	0.04	
1-myristoyl-2-palmitoyl-GPC (14:0/16:0)	0.05	0.00005	0.04	
1-carboxyethylleucine	0.06	0.00005	0.04	
xanthosine	0.07	0.00006	0.047	

[a] Kyoto Encyclopedia of Genes and Genomes, Human Metabolome Database or PubChem; metabolites with (#) represent isomers of the same compound.
[b] Beta coefficient and P value from linear regression models evaluating the association between log transformed EMCs and IV busulfan clearance
[c] False discovery rate (Benjamini-Hochberg)

Table 4. Association of IV busulfan clearance with endogenous metabolomic compounds (EMC) in the 2-week pre-busulfan time point (FDR<0.05).

EMC	Beta Coefficient[b]	P-value[b]	FDR[c]	Evaluated previously
androstenediol (3alpha, 17alpha) monosulfate (2)	0.12	4.00E-10	3.37E-07	
androsterone glucuronide	0.16	2.40E-09	2.04E-06	
5alpha-androstan-3beta,17beta-diol disulfate	0.20	6.80E-09	5.72E-06	
creatinine	0.04	4.70E-08	3.95E-05	Navarro, 2016[10] (P=0.006)
mannonate	0.08	1.50E-07	0.0001	
N-acetylcarnosine	0.08	2.20E-07	0.0002	
epiandrosterone sulfate	0.17	6.90E-07	0.0006	
N,N,N-trimethyl-alanylproline betaine (TMAP)	0.05	8.00E-07	0.0007	
androstenediol (3beta,17beta) disulfate (2)	0.11	8.40E-07	0.0007	
1-methylurate	0.16	8.70E-07	0.0007	
dehydroepiandrosterone sulfate (DHEA-S)	0.14	1.10E-06	0.0009	
androsterone sulfate	0.19	1.40E-06	0.001	
androstenediol (3beta,17beta) monosulfate (1)	0.15	1.60E-06	0.001	
androstenediol (3beta,17beta) disulfate (1)	0.14	1.80E-06	0.001	
5alpha-androstan-3alpha,17beta-diol monosulfate (2)	0.11	2.00E-06	0.002	
hydroxyasparagine	0.04	8.20E-06	0.007	
imidazole lactate	0.05	8.50E-06	0.007	
androstenediol (3alpha, 17alpha) monosulfate (3)	0.13	0.00002	0.01	
urate	0.03	0.00003	0.02	Navarro, 2016[10] (N/S)
5-acetylamino-6-amino-3-methyluracil	0.18	0.00003	0.02	
5alpha-pregnan-3beta,20beta-diol monosulfate (1)	0.10	0.00003	0.03	

EMC	Beta Coefficient[b]	P-value[b]	FDR[c]	Evaluated previously
pregnenediol sulfate (C21H34O5S)	0.09	0.00005	0.04	
androstenediol (3beta,17beta) monosulfate	0.11	0.00006	0.048	
N-(2-furoyl)glycine	0.20	0.00006	0.048	
2,3-dihydroxy-5-methylthio-4-pentenoate (DMTPA)	0.03	0.00006	0.048	
etiocholanolone glucuronide	0.11	0.00007	0.049	
3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	0.15	0.00007	0.049	

[a] Kyoto Encyclopedia of Genes and Genomes, Human Metabolome Database or PubChem; metabolites with (#) represent isomers of the same compound.
[b] Beta coefficient and P value from linear regression models evaluating the association between log transformed EMCs and IV busulfan clearance
[c] False discovery rate (Benjamini-Hochberg)

Table 5. Top pathways, significance and impact from pathway enrichment analyses at the pre-busulfan time point, sorted by increasing P-values.

Pathway Name	Total[a]	Matched[b]	P-value	FDR[d]	Impact[e]
Lysine degradation	25	7	4.49E-07	2.92E-05	0.16
Steroid hormone biosynthesis	85	7	0.0002	0.003	0.05
Purine metabolism	65	11	0.0003	0.005	0.04
Glycine, serine and threonine metabolism	33	12	0.002	0.03	0.72
Pyrimidine metabolism	39	11	0.003	0.03	0.35
Aminoacyl-tRNA biosynthesis	48	20	0003	0.03	0.17
Cysteine and methionine metabolism	33	7	0.003	0.03	0.26
Arginine and proline metabolism	38	11	0.004	0.03	0.40
D-Arginine and D-ornithine metabolism	4	2	0.005	0.03	0
Alanine, aspartate and glutamate metabolism	28	14	0.006	0.03	0.72
Glutathione metabolism	28	6	0.007	0.03	0.18
Pantothenate and CoA biosynthesis	19	9	0.007	0.03	0.08
Fatty acid degradation	39	2	0.008	0.04	0
Glyoxylate and dicarboxylate metabolism	32	9	0.01	0.04	0.26
Valine, leucine and isoleucine biosynthesis	40	7	0.01	0.045	0.04
Linoleic acid metabolism	5	2	0.01	0.045	1
Inositol phosphate metabolism	30	2	0.01	0.045	0.13
Fatty acid biosynthesis	47	3	0.01	0.045	0.01

[a] Total number of EMCs in the stated pathway
[b] Number of matched EMCs, explained in Statistical Analysis section
[c] -log(*P*) is the negative natural log of the *P* value for each pathway
[d] False Discovery Rate (Benjamini-Hochberg)
[e] Impact is the pathway impact value on IV busulfan clearance calculated from pathway topology analysis

Table 6. Top pathways, significance and impact from pathway enrichment analyses at the 2-week pre-busulfan sample time point, sorted by increasing *P*-values.

Pathway Name	Total[a]	Matched[b]	P-value[c]	FDR[d]	Impact[e]
Lysine degradation	25	7	0.0003	0.02	0.16

[a] Total number of EMCs in the pathway
[b] Number of matched EMCs, explained in Statistical Analysis section
[c] -log(*P*) is the negative natural log of the *P* value for each pathway
[d] False Discovery Rate (Benjamini-Hochberg)
[e] Impact is the pathway impact value on IV busulfan clearance calculated from pathway topology analysis

DISCUSSION

We evaluated if IV busulfan clearance could be predicted using pharmacometabonomic samples, building upon our previous analyses.[9,10] The key findings reported in this manuscript are: 1) Validating our previous findings, the Glycine, Serine, and Threonine pathway was significant in the retrospective[10] and the pre-busulfan sample of this prospective cohort (Table 5). 2) We identified novel pathways associated with IV busulfan clearance. The lysine degradation pathway was most highly associated with IV busulfan clearance for both the 2-week pre-busulfan and the immediately pre-busulfan samples. IV busulfan clearance was associated with several androgen steroid conjugates in the univariate analyses, which aligns the Steroid biosynthesis pathway being significant in the pathway analyses

Busulfan has a narrow therapeutic window, with many HCT recipients receiving PK-guided dosing of busulfan to achieve their target busulfan AUC.[3] Although PK-guided dosing improves clinical outcomes,[4] it is optimal to achieve the target AUC with the first dose – i.e., the "right-dose-first-time" paradigm.[29] Various methods to predict pre-emptively predict IV busulfan clearance have been evaluated (Table 1). One method is to estimate busulfan clearance after administration of a pre-HCT 'test dose', which is a single small dose of busulfan ranging from 0.25 to 0.8 mg/kg. While the use of a test dose has been able to minimize subsequent dose adjustments during the actual conditioning, the test dose strategy does not predict clearance well enough to replace PK-directed busulfan dosing.[4] An additional method to predict an individual patient's IV busulfan clearance is use pre-emptive pharmacogenomics of the genes regulating the glutathione transferase (GST) enzymes involved in busulfan metabolism.[4] GST isoenzymes A1-1 mainly catalyzes this reaction; GSTM1-1 and GSTP1-1 have minor roles.[30,31] However, *GSTA1* and *GSTM1* polymorphisms are not consistently associated with IV busulfan clearance,[4] potentially due to redundancy in function across GST enzymes.[32] The third method is to predict IV busulfan clearance using an EMC panel from a pharmacometabonomic sample obtained pre-busulfan.

The results from this pharmacometabonomic analysis provide novel hypotheses regarding pathways influencing IV busulfan clearance. We designed this independent cohort of prospectively collected samples to replicate our findings from a retrospective cohort analyzed with global[9] or targeted[33] metabolomics assays. In the pre-busulfan sample, 37 EMCs remained significant with FDR <0.05. In addition, numerous (n=19) pathways were

associated with IV busulfan clearance of varying impact. Our working hypothesis for the association of these pathways on IV busulfan clearance is presented in Figure 1.

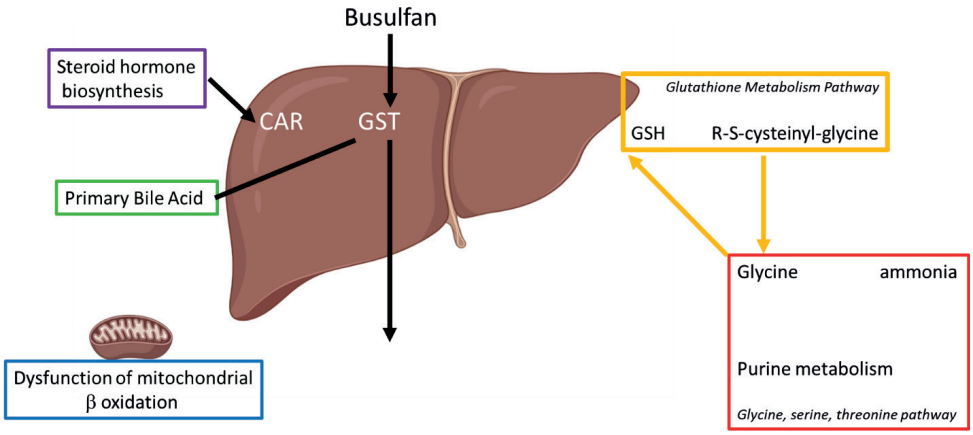

Figure 1. Working hypothesis for the pathways associated with IV busulfan clearance. Our working hypothesis is that glutathione metabolism (yellow box) continues to be central to IV busulfan clearance. We confirmed our prior findings of the the association of the glycine, serine, threonine pathway (red box) and bile acids (green box) with IV busulfan clearance. The impact of mitochondrial dysfunction (blue box) and reactive oxygen species was also found.

Our previous panel included 5 EMCs in the glutathione pathway, i.e., glycine, pyroglutamic acid, ornithine, glutamic acid and cadaverine.[10] However, our current panel contained only six of the 28 EMCs in the pathway: glycine*, glutamate, cysteine, Cys-gly, ornithine*, spermidine. This may have been insufficient for a complete evaluation. Higher EMCs concentrations were associated with faster IV busulfan clearance for all the EMCs except glycine where lower glycine concentrations were associated with a faster IV busulfan clearance. It is tempting to speculate that more substrate for glutathione production may be driving the association between glycine and IV busulfan clearance. In fact, glutathione was one of the pathways significantly associated with increased IV busulfan clearance. We had previously found that deoxycholic or chenodeoxycholic acid[9] were associated with IV busulfan clearance, with the putative mechanism being inhibition of GST activity by bile acids.[34] However, IV busulfan was not associated with bile acids in the univariate or pathway analysis. Notably, linoleic acid was significant in our retrospective analysis[9] and linoleic acid metabolism pathway was significant in this prospective cohort (Table 5).

Our data also supported the importance of mitochondrial dysregulation. Kim et al reported that urinary phenylacetylglutamine and two acylcarnitines were associated with busulfan AUC in 130 pediatric HCT patients receiving PK-directed busulfan.[35] They suggested the mitochondrial dysregulation influenced busulfan clearance; our findings are suggestive of this paradigm. In this prospective cohort, plasma acetylcarnitine, phenylacetylcarnitine, and phenylacetylglutamine were not statistically associated with IV busulfan clearance. Specifically, these EMCs had lower p-values (ranging from 0.021 to 0.129) but did not pass

the FDR (0.993). The pathway enrichment analyses revealed that lysine degradation had the lowest p-value (7.27×10^{-7}) in both the 2-week pre-busulfan and pre-busulfan sample. Lysine degradation occurs through two pathways: 1. via formation of saccharopine, which is a pathway confined to the mitochondria; 2. via the pipecolic acid pathway, which is pathway not yet fully elucidated and known enzymes are localized in the mitochondria, cytosol and peroxisome. The lysine degradation pathway does include two sodium ion-dependent, low affinity carnitine (SLC) transporter (i.e., SLC25A21 and SLC25A29), which do not overlap with the putative SLC transporters possibly involved in busulfan transport (i.e., SLC7A8 (rs7141505) and SLC22A4 (rs1050152).[36] The lysine degradation pathway has no clear association with glutathione,[37] but it does result in glycine formation. Glycine is a non-essential amino acid that can be endogenously synthesized from serine, threonine or choline. Glycine has a role in the production of purines, bile acids, creatine, heme, and glutathione. However, glycine was not associated with IV busulfan clearance (p=0.649). However, consistent with our retrospective analysis, the glycine, serine, threonine pathway was associated with IV busulfan clearance (p=0.006, FDR=0.032) with an impact = 0.51

We found that the steroid biosynthesis and retinoid pathway were associated with IV busulfan clearance. The GSTs, specifically GSTA1-1 and GSTA3-3, catalyze the conversion of androst-5-ene-3,17-dione to androst-4-ene-3.17-dione,[38] the latter of which is part of the steroid hormone biosynthesis pathway. GSTA1-1 mainly catalyzes the metabolism of busulfan.[30,31] Furthermore, the GSTs are regulated by the nuclear receptors Constitutive Androstane Receptor (CAR) and (RXR).[39] EMCs within the steroid biosynthesis pathway are agonists for CAR, while RXR is stimulated by EMCs within the retinoid pathway. We hypothesize that variation in the EMCs within these two pathways modulate the activity of these nuclear receptors, subsequently affecting GST activity and thus IV busulfan clearance. It should be noted that the majority of these participants received the anti-epileptic medication (AEM) phenytoin to prevent busulfan-induced seizures. Human CAR mediates induction of CYP2B6 gene expression by phenytoin,[40] however it is not apparent if phenytoin can induce hepatic GST and IV busulfan clearance as we have previously reviewed.[4]

In recent years, the use of pre-dose EMC [6,41] profiling to predict drug response in patients has been evaluated. There is an increasing number of pharmacometabonomic studies (summarized in Supplemental Table 1 of McCune et al. [18]) examining the association of EMCs with subsequent clinical outcomes among allogeneic HCT recipients. We recently reported that relapse was associated with the cysteine/methionine pathway and the glycine, serine, and threonine metabolism pathway.[18] The latter can be explained by the fact that glutathione S-transferases conjugate both busulfan and glutathione, which contains glycine as a component. The d-arginine and d-ornithine metabolism pathway and arginine and proline metabolism pathway were most associated with acute GVHD.

Strengths of this work include the prospectively collected cohort of over 100 HCT patients with a well-characterized IV busulfan pharmacokinetic dataset, and the global panel providing broad coverage of several pathways of interest. However, this study has limitations worth noting. Importantly, this prospective cohort had insufficient coverage of the glutathione pathway involved in busulfan metabolism. While 28 EMCs in the

glutathione pathway were measured, only six had detectable signal in our plasma samples. Sufficient sensitivity was attained in three (i.e., spermidine, cysteine, cysteinylglycine) EMCs not detected in our retrospective cohort. None of these EMCs were associated with busulfan clearance (p-values ranging from 0.084 to 0.836 with each having a FDR of 0.993). However, five EMCs within the glutathione pathway (i.e., putrescine, spermine, glutathione, oxidized glutathione, and ascorbic acid) were not available in this prospective cohort. Another potential limitation is the different type of blood collection tube (BCT) was used for sample collection than those used for our retrospective cohort.[9,10] Because our long-range goal is to predict clinical outcomes[18] and pharmacokinetics[9,10] after IV busulfan administration, we collected blood samples before, during and after busulfan administration. We used sodium heparin (green top) BCT tubes, which are used to collect blood for quantitating busulfan concentrations for the clinically-indicated PK-guided dosing for this study. We chose this BCT to avoid an error with using the wrong BCT for clinical care of PK-guided busulfan dosing. However, it did introduce a limitation in that our previous studies used with EDTA or citrate BCT, which did not influence the EMC results.[9,10] An additional limitation is the lack of urinary data. Before study initiation, we did consider urine collection during all four days of busulfan administration but deemed complete collection was unlikely because of the substantive urinary output in those participants receiving high-dose cyclophosphamide and, thus, aggressive hydration to minimize the risk of hemorrhagic cystitis. However, collection of urinary EMC data in future studies may provide mechanistic insight. The lysine degradation pathway had the lowest p-value but small impact on IV busulfan clearance (Table 3 and 4). In renal brush border vesicles, the transport of L-lysine was inhibited by basic amino acids (e.g., L-arginine and L-ornithine) but was not significantly altered by the neutral amino acid L-tyrosine.[42] However, the impact of urinary elimination upon IV busulfan clearance is minimal. Only a small fraction (<3%) of a busulfan dose is excreted unchanged in the urine, with negligible amounts in the feces.[43,44]

CONCLUSIONS

In this analysis, we sought to replicate and expand our understanding of the association of plasma EMCs with busulfan clearance, with the long-range goal of personalizing IV busulfan doses using biomarkers identified via pharmacometabonomics. The plasma EMCs predict the majority of the interindividual variability in IV busulfan clearance. This suggests that pharmacometabonomics could sufficiently estimate IV busulfan clearance to personalize the first dose to achieve the target AUC with the first busulfan dose. We also discovered novel pathways associated with IV busulfan clearance. Further studies, including greater interrogation of the lysine degradation, steroid hormone biosynthesis, and glutathione pathway, are needed to validate these results which may have the prospect of personalizing IV busulfan dosing and potentially improving clinical outcomes.

ACKNOWLEDGMENTS

This publication was supported by the National Institutes of Health under the Award Numbers: R01CA182963, R01GM129863, P01CA18029, 5P30CA015704, and UL1 TR002319. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We are grateful to the patients who participated in this study. We are also grateful to the health care providers caring for the participants. The authors wish to thank the research staff (Sheri Ballard, Meagan Bemer, Genecelle Delossantos, Michael Donahue, and Jeffrey Stevens) involved in sample acquisition, sample transport, and data management.

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SUPPLEMENTAL MATERIALS

Supplemental Methods 1: NONMEM code
Supplemental Methods 2: Endogenous metabolomic compounds quantitation
Supplemental Figure 1: Goodness of fit plot of busulfan concentration-time data
Supplemental Figure 2: Predicted and actual concentration-time data

SUPPLEMENTAL METHODS 1. NONMEM CODE

```
;----------------------------------------------
$INPUT      ID TIME AMT RATE OCC DOSENUM DOSEFREQ EVID DV LAB BLQ CMT
            SEX PMAW AWT AIBW IDEALWT HEIGHT FFM2 NFM_CL NFM_Vd BSA
            COND FLUCOND
;----------------------------------------------
$DATA      9117nonmem3allv6.csv IGNORE=@ IGNORE(CMT.GT.1);
;----------------------------------------------
$SUBROUTINE ADVAN3 TRANS4 ; Use CL,V1,Q,V2 parameterization
;----------------------------------------------
$PK

; IF (NEWIND.LE.1) LN2=LOG(2) Nick has this and I have no idea why

;Group parameters from PMID 16176118
  IF (SEX==0) THEN ; female
    WHSMAX=37.99
    WHS50=35.98
    FSEXCL=THETA(11)
    FSEXV1=THETA(12)
  ELSE ; male
    WHSMAX=42.92
    WHS50=30.93
    FSEXCL=1
    FSEXV1=1
  ENDIF

HTM=HEIGHT/100 ; cm -> m

FFM=WHSMAX*HTM**2*AWT/(WHS50*HTM**2 + AWT)
```

```
  FATKG=AWT-FFM
  NFMCL=FFM+THETA(5)*FATKG
  NFMV1=FFM+THETA(6)*FATKG

FSZCL=(NFMCL/70)**THETA(13)
FSZV1=(NFMV1/70)**THETA(14)

FMATCL=1/(1+(PMAW/THETA(7))**(-THETA(8)))

IF (TIME<6) THEN
  FTIMCL=1
ELSE
  IF (TIME<36) THEN
    FTIMCL=THETA(15)
  ELSE
    IF (TIME<60) THEN
      FTIMCL=THETA(16)
    ELSE
          FTIMCL=THETA(16) ; FN_CL
        ENDIF
      ENDIF
    ENDIF

GRPCL=THETA(1)*FSZCL*FMATCL*FSEXCL*FTIMCL
GRPV1=THETA(2)*FSZV1*FSEXV1
GRPQ=THETA(3)*FSZCL
GRPV2=THETA(4)*FSZV1*FSEXV1

IF (OCC==1) THEN
  BOVCL=ETA(5)
  BOVV1=ETA(6)
  BOVQ=ETA(7)
  BOVV2=ETA(8)
ENDIF
```

```
IF (OCC==2) THEN
  BOVCL=ETA(9)
  BOVV1=ETA(10)
  BOVQ=ETA(11)
  BOVV2=ETA(12)
ENDIF
IF (OCC==3) THEN
  BOVCL=ETA(13)
  BOVV1=ETA(14)
  BOVQ=ETA(15)
  BOVV2=ETA(16)
ENDIF

;Individual parameters
CL=GRPCL*EXP(ETA(1)+BOVCL)
V=GRPV1*EXP(ETA(2)+BOVV1)
Q=GRPQ*EXP(ETA(3)+BOVQ)
V2=GRPV2*EXP(ETA(4)+BOVV2)
AUC=AMT/CL
K10=CL/V
Halflife=0.693/K10

V1=V
S1=V1
;----------------------------------------------
$ERROR
IPRED = A(1)/V1
PROP=IPRED*THETA(10)
ADD=THETA(9)
SD=SQRT(PROP*PROP + ADD*ADD) ;*EXP(PPV_RUV)
Y=IPRED + SD*ERR(1)
ETARUV=ETA(17)

;----------------------------------------------
```

```
$THETA  11.4 ; 1 POP_CL L/h/70kg
 13.9 ; 2 POP_V1 L/70kg
 135.2 ; 3 POP_Q L/h/70kg
 29.9 ; 4 POP_V2 L/70kg
 0.509 ; 5 FFAT_CL
 0.203 ; 6 FFAT_V
 45.7 ; 7 TM50_CL
 2.3 ; 8 HILL_CL
 0.106 ; 9 RUV_ADD micromole/L
 0.0387 ; 10 RUV_PROP
 1 ; 11 FFEM_CL
 1.07 ; 12 FFEM_V
 0.75 ; 13 PWR_CL
 1 FIX ; 14 PWR_V
 0.932 ; 15 F1_CL
 0.919 ; 16 F2_CL
 ;
;-----------------------------------------------
$OMEGA  BLOCK(4)
 0.0459  ;     BSV_CL
 0.0172 0.152  ;     BSV_V
 0.0681 0.097 0.814  ;     BSV_Q
 0.0115 -0.00924 0.069 0.00932  ;     BSV_V2
$OMEGA  BLOCK(4)
 0.0138  ;    BOV_CL1
 -0.00918 0.069  ;    BOV_V11
 0.0566 -0.0608 0.324  ;     BOV_Q1
 0.0161 -0.0477 0.0996 0.0454  ;    BOV_V21
$OMEGA  BLOCK(4) SAME
;; BOV_CL2
;; BOV_V12
;; BOV_Q2
;; BOV_V22
$OMEGA  BLOCK(4) SAME
```

```
;; BOV_CL3
;; BOV_V13
;; BOV_Q3
;; BOV_V23
$OMEGA  BLOCK(1) FIX
 0.114  ;    PPV_RUV
;----------------------------------------------------
$SIGMA  1.  FIX  ;      EPS1
;----------------------------------------------------
$ESTIMATION METHOD=CONDITIONAL,INTERACTION MAXEVALS=0 POSTHOC NSIG=3
          SIGL=9 NOABORT PRINT=0 ;
```

SUPPLEMENTAL METHODS 2

EMC profiling of plasma was completed by Metabolon (Durham, South Carolina, USA). The samples were shipped on dry ice to Metabolon's facility and stored at -80°C upon receipt.

Global metabolomics analysis was carried out via 4 different mass spectrometry methods: two separate reverse phase (RP)/ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) methods with positive ion mode electrospray ionization (ESI), one RP/UPLC-MS/MS method with negative ion mode ESI, and one HILIC/UPLC-MS/MS method with negative ion mode ESI. Samples were divided into four aliquots, one for each method and one for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of EMCs were spiked into every analyzed sample to allow instrument performance monitoring and aid chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all EMCs (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

Sample preparation

Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse EMCs, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced

with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below.

Bioinformatics: The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

LIMS

The purpose of the Metabolon LIMS system was to enable fully auditable laboratory automation through a secure, easy to use, and highly specialized system. The scope of the Metabolon LIMS system encompasses sample accessioning, sample preparation and instrumental analysis and reporting and advanced data analysis. All of the subsequent software systems are grounded in the LIMS data structures. It has been modified to leverage and interface with the in-house information extraction and data visualization systems, as well as third party instrumentation and data analysis software.

Data Extraction and Compound Identification

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. These systems are built on a web-service platform utilizing Microsoft's .NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (m/z), and chromatographic data

(including MS/MS spectral data) on all molecules present in the library. Furthermore, EMC identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate EMCs. More than 3300 commercially available purified standard compounds have been acquired and registered into LIMS for analysis on all platforms for determination of their analytical characteristics. Additional mass spectral entries have been created for structurally unnamed EMCs, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

Curation

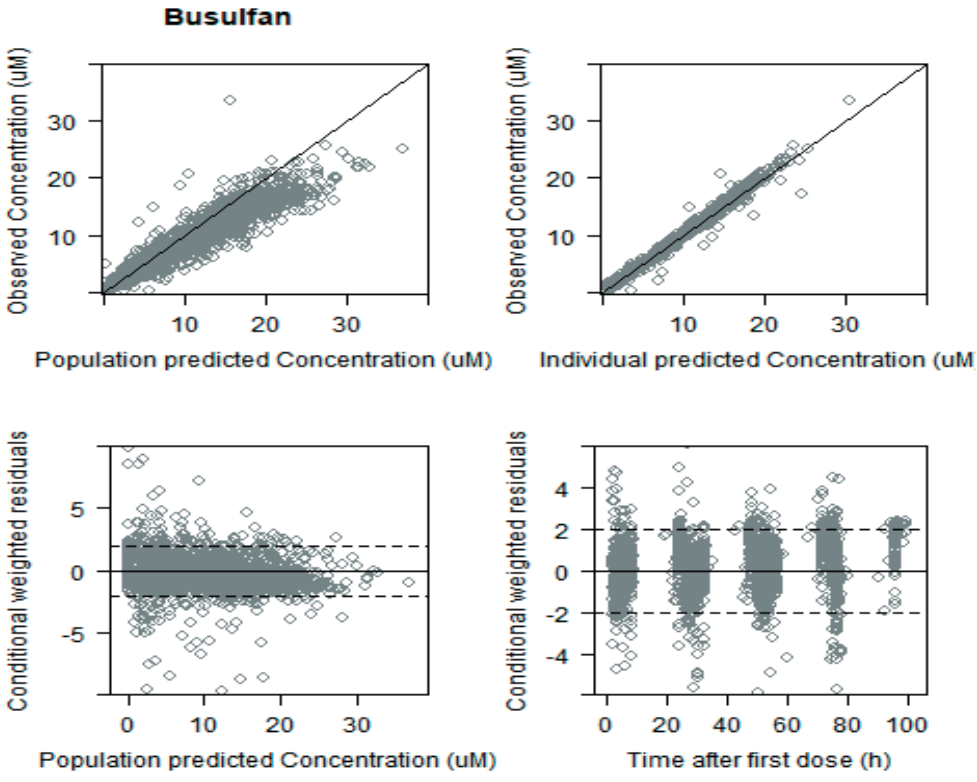
A variety of curation procedures were carried out to ensure that a high quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, mis-assignments, and background noise. Metabolon data analysts use proprietary visualization and interpretation software to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected if necessary.

EMC Quantification and Data Normalization

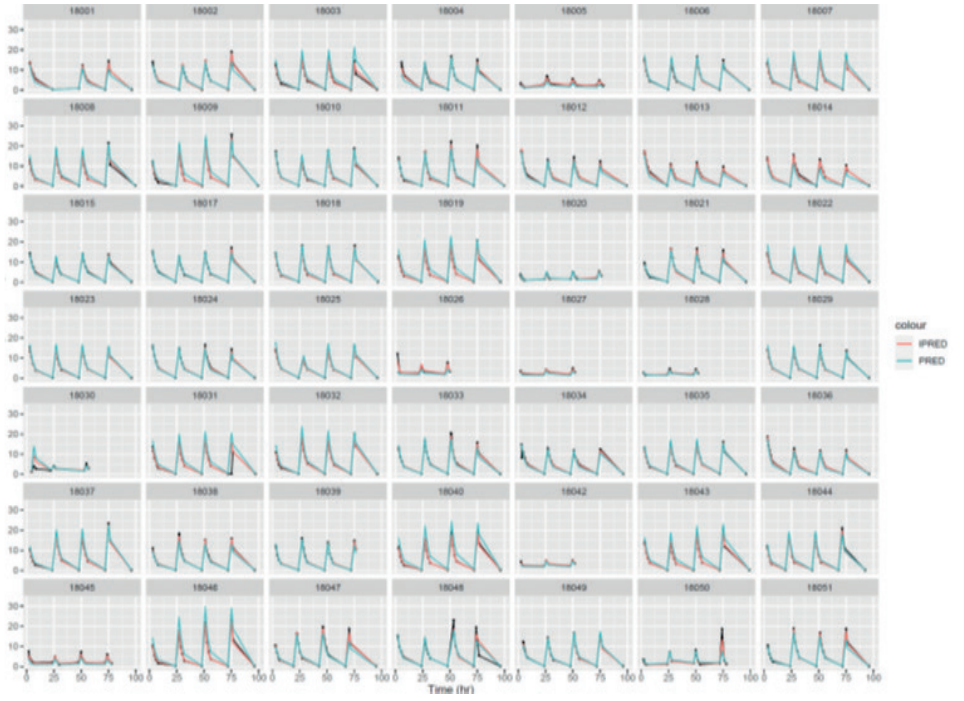
Peaks were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences.

Peaks were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately (block correction). In certain instances, EMC data may have been normalized to an additional factor (e.g., cell counts, total protein as determined by Bradford assay, osmolality, etc.) to account for differences in EMC levels due to differences in the amount of material present in each sample.

A pooled QC sample was run for every 10 biological samples to assess instrument performance. The intra-assay average CV was 7.8% across all samples.



Supplemental Figure 1. Goodness of fit plots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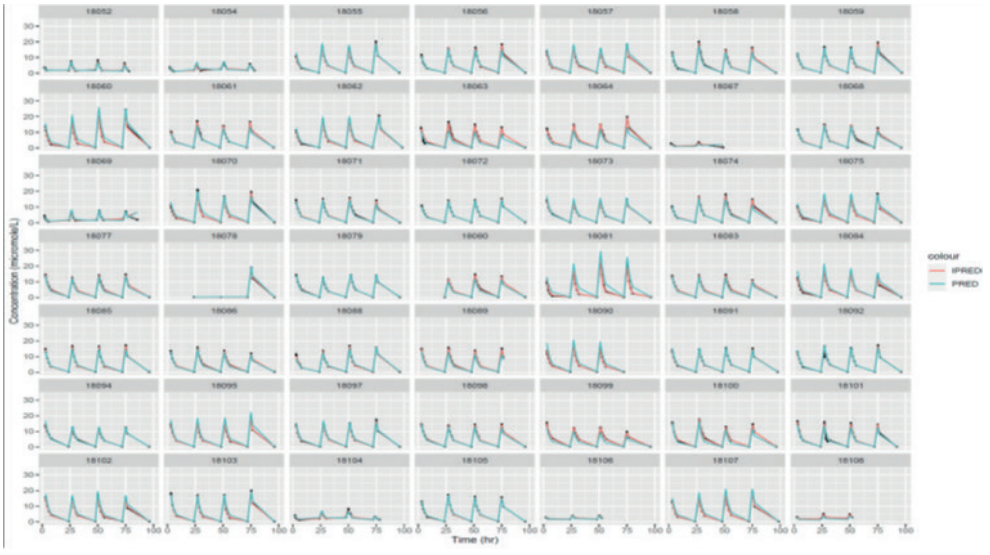
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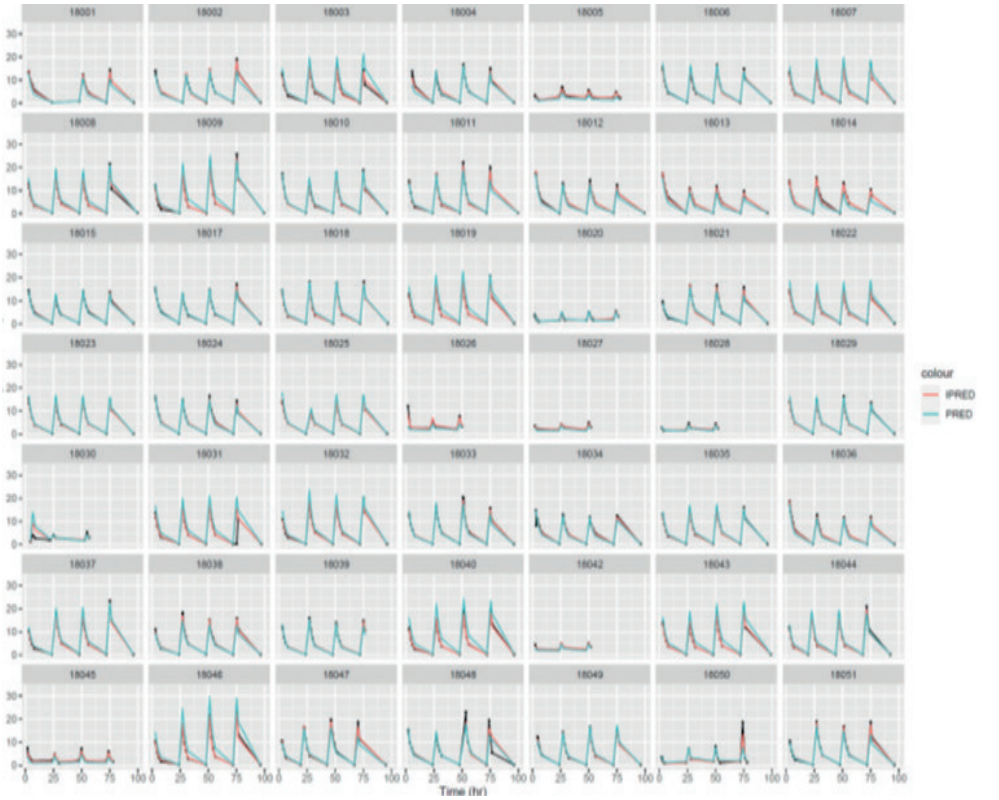
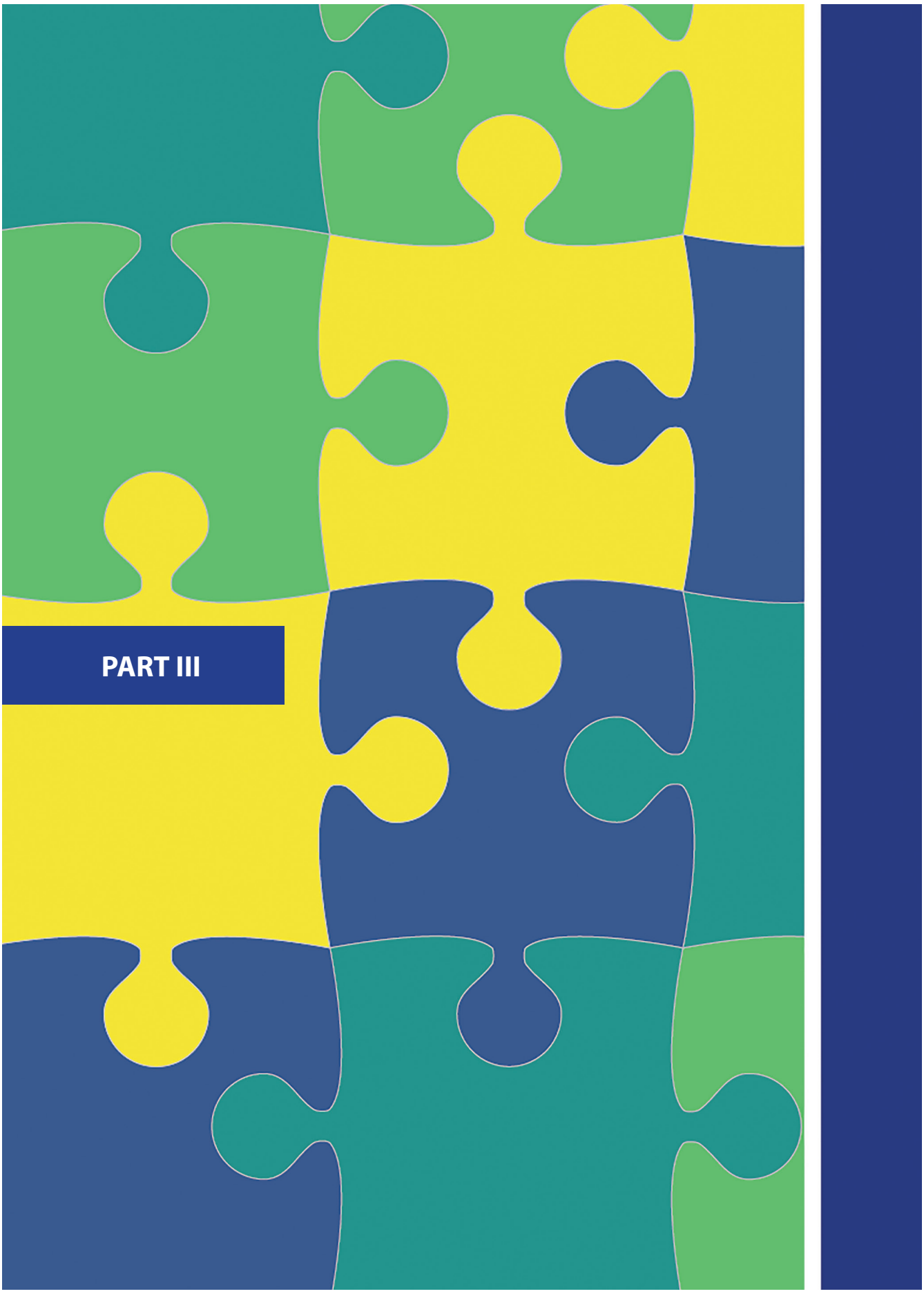
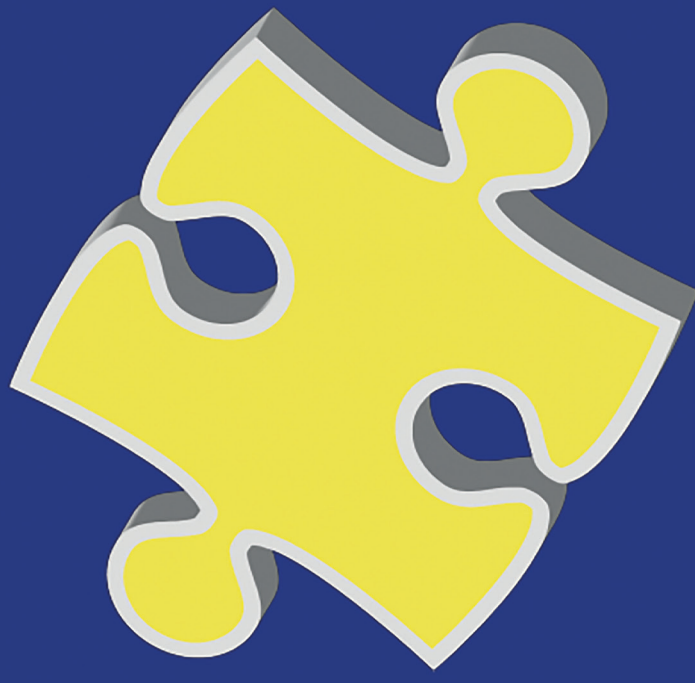


Supplemental Figure 2. Predicted vs. observed busulfan concentration-time profiles


PART III

Identifying New Biomarkers for Busulfan-based Conditioning



CHAPTER 9

Prediction of Acute Graft Versus Host Disease and Relapse by Endogenous Metabolomic Compounds in Patients Receiving Personalized Busulfan-based Conditioning

Jeannine S. McCune[*], Jožefa McKiernan, Erik van Maarseveen[a], Alwin D. R. Huitema, Timothy W. Randolph, H. Joachim Deeg, Ryotaro Nakamura, K. Scott Baker

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J. Proteome Res. 2021, 20, 684-694

ABSTRACT

Busulfan-based conditioning is the most commonly used high-dose conditioning regimen for allogeneic hematopoietic cell transplant (HCT). The alkylating agent busulfan has a narrow therapeutic index, with busulfan doses personalized to a target plasma exposure (targeted busulfan). Using a global pharmacometabonomics approach, we sought to identify novel biomarkers of relapse or acute graft versus host disease (GVHD) in a cohort of 84 patients receiving targeted busulfan before allogeneic HCT. A total of 763 endogenous metabolomic compounds (EMCs) were quantitated in 230 longitudinal blood samples before, during, and shortly after intravenous busulfan administration. We performed both univariate linear regression and pathway enrichment analyses using global testing. The cysteine/methionine pathway and the glycine, serine, and threonine metabolism pathway were most associated with relapse. The latter be explained by the fact that glutathione-S-transferases conjugate both busulfan and glutathione, which contains glycine as a component. The D-arginine and D-ornithine metabolism pathway and arginine and proline metabolism pathway were most associated with acute GVHD. None of these associations were significant after correcting for false discovery rate (FDR) with a strict cutoff of FDR-adjusted $P < 0.1$. Although larger studies are needed to substantiate these findings, the results show that EMCs may be used as predictive biomarkers in HCT patients.

Abstract

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative procedure, with its most frequent indication being hematologic malignancies.[1] In allogeneic transplantation, grafting of hematopoietic stem cells from one individual to another provokes immunologic reactions involved in engraftment of the donor cells, graft-versus-host disease (GVHD), control of a malignancy, the development of tolerance, and immune reconstitution.[2] These immunologic reactions are influenced by the conditioning regimen (also termed preparative regimen), the type and source of the donor graft, and the post-transplant immunosuppressive regimen, all of which are essential components of the HCT procedure. In HCT recipients, overall survival is improved in those conditioned with intravenous (IV) busulfan compared to total body irradiation (TBI); however, potential severe toxicity of busulfan-based conditioning persists.[3-5]

Busulfan-based conditioning is the most commonly used high-dose conditioning regimen for allogeneic HCT. Busulfan is hepatically metabolized through glutathione (GSH) conjugation by glutathione S-transferase (GST) enzymes; this process depletes hepatocyte GSH stores in murine hepatocytes in vitro.[6] Dysregulation of GSH and accumulation of cysteine, cystathione, and cysteinylglycine are associated with GVHD in experimental murine models of HCT (Supplemental Table 1, Table S1).[7]

Table 1. Participant characteristics

Characteristic	N[a]
N	84
Age (y)	53.0 (1.7-66.2)
Male sex	52 (62%)
HLA match	
Matched donor (HLA-identical)	76 (90%)
Mismatched donor	8 (10%)
Donor type	
Unrelated	57 (68%)
Related – matched sibling	26 (31%)
Related – father	1 (1%)
HCT Conditioning[b]	
CY/^{T}BU	51 (61%)
^{T}BU/CY	20 (24%)
FLU/^{T}BU	10 (12%)
FLU/CY/^{T}BU	2 (2%)
^{T}BU/CY/TBI	1(1%)
^{T}BU dosing frequency	
Every 24 h	77 (92%)
Every 6 h	7 (8%)

Characteristic	N[a]
Diagnosis	
Myelodysplastic syndrome	52 (62%)
Acute myeloid leukemia	20 (24%)
Chronic myeloid leukemia	10 (12%)
Other[c]	2 (2%)

[a] Data presented as: number (%) or median (range); percentages may not add up to 100 due to rounding. [b] Listed in administration order; all participants received targeted busulfan (^{T}BU), in which the IV busulfan dose was personalized based on the patient's busulfan clearance to a target plasma exposure; [c] Other diagnoses include; chronic leukemia NOS n=1; and eosinophilic leukemia n=1. Abbreviations. ^{T}BU: Targeted busulfan; CY: cyclophosphamide; FLU: fludarabine; TBI: total body irradiation

Compared to syngeneic HCT mice and non-transplant control mice, allogeneic HCT mice had significant decreases in reduced glutathione and increases in oxidized glutathione, indicating early shifts in oxidative stress.[7] Thus, we hypothesized that plasma endogenous metabolomic compounds (EMCs; i.e., not downstream metabolites of busulfan) are associated with GVHD in patients receiving busulfan-based conditioning regimens prior to allogeneic HCT.

Relapse is reduced by personalized dosing of busulfan to a target area under the plasma concentration-time curve (AUC), termed targeted (^{T}BU) or pharmacokinetic (PK)-directed dosing.[8] However, neither targeted busulfan dosing nor the introduction of IV targeted busulfan have sufficiently reduced relapse. On the basis of preclinical data that GSH is important to busulfan toxicity, we recently conducted a clinical trial that reversed the order of administration – specifically giving cyclophosphamide (CY) first followed by IV targeted busulfan.[9] Hepatic toxicity is low with both the traditional administration order of targeted busulfan followed by CY regimen and the newer regimen of giving CY first followed by IV targeted busulfan (i.e., CY/^{T}BU) regimens. However, for patients with acute myeloid leukemia or myelodysplastic syndrome, the incidence of relapse was higher with CY/^{T}BU compared to the traditional administration sequence of ^{T}BU followed by CY. This raised the hypothesis that GSH dysregulation may also influence relapse.

To test this hypothesis, we sought to identify biomarkers predictive of the efficacy and toxicity (i.e., acute GVHD) of targeted busulfan conditioning regimens in patients diagnosed with a hematologic malignancy. We applied a global pharmacometabonomics approach investigating 763 EMCs in plasma samples obtained longitudinally over the course of IV targeted busulfan administration in 84 allogeneic HCT recipients.

METHODS

Study population

Between December 2014 and November 2018, 84 patients participated in this prospective ancillary biomarker study. This study was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board (December 2014 to November 2018; clinicaltrials.gov protocol number NCT02291965) and the City of Hope Institutional Review Board (March

2017 to November 2018). All participants provided written informed consent prior to study procedures. The conditioning regimen and postgraft immunosuppression were not affected by participation in this study.

As part of HCT conditioning, all 84 participants underwent pharmacokinetic-guided dosing of IV busulfan, also called targeted busulfan (^{T}BU), which personalized each patient's busulfan dose to achieve the desired target busulfan exposure. The first busulfan dose was based on body weight or body surface area. Subsequent doses were personalized using the individual participant's busulfan clearance as previously described;[8] the target busulfan AUC was chosen by the treating physician. Because of the circadian variability of busulfan pharmacokinetics,[10] all busulfan pharmacokinetic sampling occurred in the morning. Antiemetics, antibiotics, and antifungals were given per Institutional Standard Practice Guidelines.

Clinical outcomes

The assessment of clinical outcomes was described previously.[11] Acute GVHD and chronic GVHD were graded according to established criteria.[12-14] We defined disease relapse or disease progression as disease recurrence following complete remission or progression of persistent disease. The primary end points were relapse and acute GVHD (grade 0-1 vs. 2-4). Each endpoint was treated as a binomial outcome and analyzed separately.

Global pharmacometabonomics sample collection

Longitudinal blood samples (3 ml/sample) were scheduled to be collected in sodium heparin tubes at three time points during IV busulfan dosing (Table S2): up to two weeks prior to the first conditioning dose (2-week prebusulfan sample), immediately before administration of IV busulfan dose 1 (prebusulfan sample), and with the last busulfan pharmacokinetic sample after the last morning busulfan dose (last busulfan PK sample).

Because we sought to identify biomarkers predictive of efficacy and toxicity, samples were obtained before busulfan administration. The 2-week prebusulfan sample collection time was the earliest feasible time within the final HCT workup (i.e., time period (typically up to 2-weeks) in which the patient undergoes final assessment if they can receive an HCT). If a predictive association were found and metabolomics-guided HCT were subsequently used clinically, this sample would allow for more time for metabolomics quantitation and data interpretation. The immediately prebusulfan sample was the latest feasible time before busulfan administration. If a predictive association were found and metabolomics-guided HCT were subsequently used clinically, this sample necessitates a rapid quantitation and data interpretation. The last busulfan PK sample was obtained because busulfan is a glutathione S-transferase substrate which we hypothesized may cause glutathione dysregulation, which is associated with GVHD.[7]

A total of 230 metabolomic samples were obtained: 64 participants had samples available from all three time points; 18 participants had samples from two time points; and 2 participants had samples from one time point.

The 2-week prebusulfan, prebusulfan, and last busulfan PK samples were immediately refrigerated at 4°C, stored for up to 4 hours from time of collection, centrifuged to plasma, and immediately stored at -80°C. The samples underwent at most one freeze-thaw cycle before metabolomic analysis (i.e., the analysis was conducted after the first or the second thaw).

Global pharmacometabonomics analysis

Metabolite profiling of plasma was completed by Metabolon (Durham, North Carolina, USA). The samples were shipped on dry ice to Metabolon's facility and stored at -80°C upon receipt. Samples were divided into 5 aliquots; one was held in reserve while each of the others was analyzed by one of four different mass spectrometry methods. Raw data were extracted, peak-identified, and QC processed; then Metabolon's proprietary software was used to confirm the consistency of peak identification across the various samples. Compounds were identified by comparison of the processed data to Metabolon's library entries of purified standards or recurrent unknown entities. Library matches for each compound were checked for each sample and corrected if necessary. Using the criteria established by the Chemical Analysis Working Group as part of the Metabolomics Standards Initiative,[15] most EMCs met the level 1 standards for metabolite identification with the remainder meeting the level 2 standards per the same criteria. ANCOVA contrasts were performed to examine differences in metabolite levels between the samples at each time point. See Supplemental Methods for more details about metabolite quantitation and quality control.

Statistical analysis

All data transformations and analyses were carried out using R version 3.5.[16]

Of the 763 EMCs (Table S3) measured, 332 (44%) had a detectable signal in all samples; 666 (87%) had detectable signal in three quarters of the samples; and 741 (97%) had detectable signal in at least half the samples. Values were normalized to the sample volume extracted and missing values, if any, were imputed with the minimum observed value for each EMC. These results were transformed using the centered log-ratio (CLR)[17,18] to account for the relative nature of the abundance measures and to approximate a normal distribution prior to analysis. Analyzing the CLR-transformed data amounts to taking the log of all measures and then normalizing each sample by its mean (log) abundance. A permutation MANOVA (perMANOVA) test was performed to check for an association between the set of all EMCs and relapse and between the set of all EMCs and acute GVHD.

Principal component analysis (PCA) was used to visualize differences in metabolomics profiles. The profiles were somewhat affected by age (Supplemental Figure 1, Figure S1); thus, age was included as a covariate in subsequent analyses. The plots revealed a clear separation between the 2-week prebusulfan and prebusulfan metabolomics profiles, collectively, and the last busulfan PK metabolomics profiles (Figure S2).

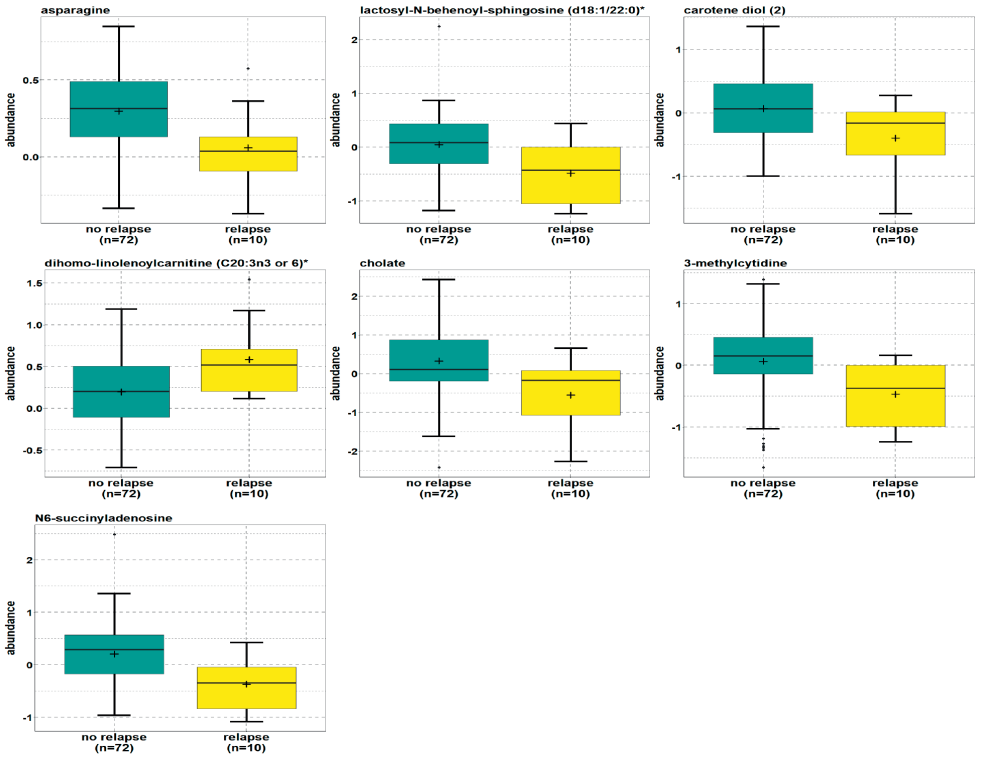


Figure 1. Box plots of prebusulfan plasma EMCs associated with relapse. Box plots represent mean (interquartile range) of centered-log ratio (CLR)-transformed EMC abundances in relapse and non-relapse groups. EMCs shown had a FDR-adjusted p value of <.5. * indicates a compound identified at a lower level of confidence, as described in Results.
Abbreviations. EMC: endogenous metabolomic compounds

Univariate analysis: Each EMC in the set of prebusulfan samples was individually tested for an association with relapse and with acute GVHD. Generalized linear models were fit for each EMC to test its association with both end points including age as a covariate. For acute GVHD, donor type (relation of donor to the recipient) and Human Leukocyte Antigen (HLA) category (which HLAs are matched between donor and recipient) were also included as covariates. The Benjamini-Hochberg (BH) procedure was used to control the false discovery rate for the large number of tests performed.[19] In view of the large number of tests, we designated a significance level of 0.1 for BH-corrected p values.

Pathway analysis: To evaluate whether groups of EMCs were associated with relapse or acute GVHD, pathway analyses integrating pathway enrichment analysis and pathway topology analysis were carried out with MetaboAnalyst 4.0 using CLR-transformed EMC measures from the prebusulfan samples.[20,21] Relapse (yes or no) and acute GVHD (grade 0-1 or 2-4) were evaluated as discrete outcomes, and only prebusulfan samples were considered in the pathway analyses. 270 EMCs out of our total 763 EMCs were not

present in the MetaboAnalyst compound library and were therefore not included in the pathway analysis. We tested pathway-defined sets of EMCs for their association with relapse and acute GVHD using the Global test[22] within MetaboAnalyst. We also applied MetaboAnalyst to perform pathway enrichment analysis to exploit potential information in pathway topology. For this, we used relative betweenness centrality (number of shortest paths passing through a node), based on EMC centrality in a given metabolic network, to calculate EMC importance.[23] Pathway impact was calculated as the sum of the importance measures of the pathway-specific EMCs, normalized by the sum of the importance measures of all EMCs in each pathway.[24] For pathway-level testing, we only used pathways for which our measured EMCs represent at least 5% of the total number of pathway metabolites (60 pathways).

RESULTS

Patient characteristics & clinical outcomes

The pre-transplant characteristics of the 84 participants are given in Table 1. The median age was 53 y (range 1.7 – 66.2), and slightly more participants were male (62%). Seventy-six (90%) participants received an HLA-identical graft, and 57 (68%) participants received a graft from an unrelated donor. Of the patient characteristics, only age impacted the plasma metabolome (Figure S1). PCA of metabolite abundances revealed a moderate separation of metabolic profiles according to age. Overall, the separation was proportional to the difference in age. Thus, age was included as a covariate in subsequent analyses.

All patients received targeted busulfan over 4 days; the majority of the participants (n=77, 92%) received daily (every 24 h) administration of busulfan. For targeted busulfan, an initial dose of busulfan based on body weight or body surface area was chosen (see FAQ6 of Palmer & McCune et al[8]) and administered on the first day of targeted busulfan. Next, sequential pharmacokinetic samples were drawn before the subsequent busulfan dose in order to estimate a patient's busulfan exposure. These pharmacokinetic samples must be drawn over an acceptable time period that accounts for the half-life of busulfan (2-3 h), the dosing frequency, and the need to obtain samples quickly enough to personalize subsequent doses of busulfan. Pharmacokinetic sampling was typically completed within 4 hours for a 2-h busulfan infusion and every 6 h (Q6H) dosing and within 8 h for a 3-h busulfan infusion and every 24 h (daily) dosing. The doses of targeted busulfan given on the second, third, and fourth days of targeted busulfan are adjusted based on the patient's busulfan exposure, as estimated from the pharmacokinetic samples. The busulfan clearance is calculated from the administered busulfan dose and the resulting busulfan exposure (AUC). The majority (n=51, 61%) of participants received CY (60 mg/kg/day on each of two sequential days) followed by targeted busulfan (on each of four sequential days).[9] The remaining participants (n=21, 25%) received targeted busulfan followed by CY (60 mg/kg/day $\times$ 2 days) and total body irradiation; and fludarabine with targeted busulfan $\pm$ CY (n=12, 14%). Prophylaxis of busulfan-induced seizures consisted of phenytoin (n=75), unknown (n=7), or levetiracetam (n=2). In a Center for International Blood and Marrow Transplant analysis, no differences were found in relapse-free survival

or increased risks of relapse or acute GVHD with the use of alternative antiepileptic medications as compared to phenytoin.[25]

Ten patients experienced relapse. Regarding acute GVHD, 30 participants had grade 0 (n=25) or grade 1 (n=5) acute GVHD which were grouped together. The remaining participants were grouped together, with grade 2 (n=46), grade 3 (n=5), and grade 4 (n=3) acute GVHD.

Busulfan administration alters the plasma EMCs

Principal component analysis of all samples revealed a clear separation in metabolic profiles between the 2-week prebusulfan and prebusulfan samples, collectively, and the last busulfan PK sample (Figure S2). This separation indicates that the metabolic profiles of HCT patients are altered following busulfan administration. The PCA plot failed to show any visible difference between the 2-week prebusulfan and the prebusulfan time points, suggesting that the primary changes in the plasma metabolome are associated with busulfan administration rather than with merely the passage of time. Changes in a large number of EMCs were observed between prebusulfan and last busulfan PK samples (by ANCOVA contrast, 542 EMCs exhibited an unadjusted $P<0.05$).

Pharmacometabonomics

In addition to the association with busulfan administration, perMANOVA testing found that metabolite levels in all (i.e., 2-week prebusulfan, prebusulfan, and last busulfan PK) samples collectively were associated with relapse ($p =0.005$) and with acute GVHD ($p =0.001$). Samples from the same participant within the combined 2-week prebusulfan and prebusulfan group tended to be more similar to each other than to samples from other individuals in the combined prebusulfan group. These results suggest high inter-participant variability in the plasma metabolome.

Although our statistical analyses showed limited results for an association between the difference in EMC profiles and relapse (Figure 1) or acute GVHD (Figure 2), Partial Least Squares-Discriminant Analysis (PLS-DA) plots provide some evidence that a supervised selection of components can partially distinguish profiles based on these two outcomes (Figure 3 and 4, respectively).

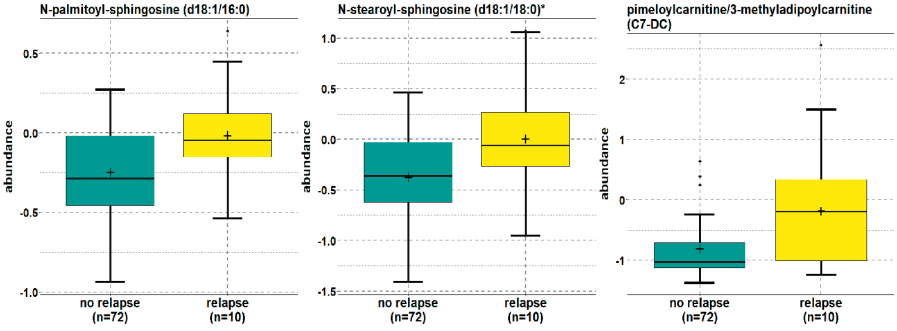
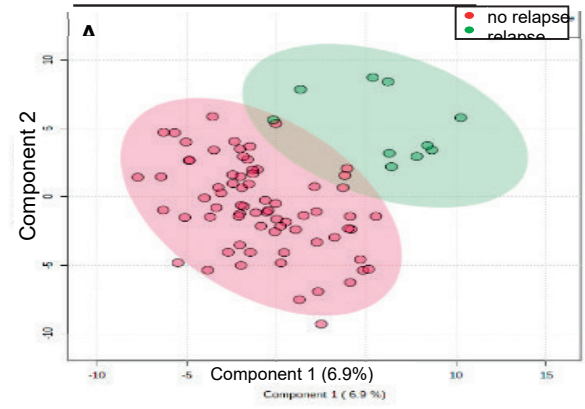


Figure 2. Box plots of prebusulfan plasma EMCs associated with acute GVHD. Box plots represent mean (interquartile range) of centered-log ratio (CLR)-transformed EMC abundances in grade 0–1 acute GVHD event and grade 2–4 acute GVHD event groups. EMCs shown had a FDR-adjusted p value of <.2. * indicates a compound identified at a lower level of confidence, as described in Results. Abbreviations. EMC: endogenous metabolomic compound; GVHD: graft-versus-host disease



In the univariate analysis for relapse, 31 EMCs in the prebusulfan samples exhibited an unadjusted p value less than .05 (12 in the positive and 19 in the negative direction; Figure 3; Table S4, Figure S3); however, none of these EMCs were significant after correcting for FDR. In similar analyses for acute GVHD, 53 EMCs in the prebusulfan samples exhibited an unadjusted P value less than .05 (16 positively and 37 negatively; Figure 4, Table S5 and Figure S4).

The pathway enrichment analysis was performed on the prebusulfan samples considering the EMCs in each pathway together. The top two pathways exhibited FDR-adjusted *P* =.500 (unadjusted *P* <.05) for an association with relapse, and both of them had a pathway impact factor > 0.5 (Table 2, Figure 5).

Table 2. Pathway enrichment analysis of relapse and acute GVHD: Top pathways, significance, and impact from pathway enrichment analyses, sorted by increasing *P*-values. Only those pathways with a p-value <.05 that have over 5% of the pathway matched within our dataset are shown. Data also shown in Figures 5 and 7.

Outcome	Number of outcome events[a]	Pathway Name	Total EMCs[b]	Matched EMCs[c]	P-value	-log(P)[d]	FDR P[e]	Impact[f]
relapse	10	Cysteine and methionine metabolism	56	15	0.0186	3.98	0.500	0.54
relapse	10	Glycine, serine and threonine metabolism	48	17	0.0434	3.14	0.500	0.53
acute GVHD	54	D-Arginine and D-ornithine metabolism	8	3	0.0278	3.58	0.907	0.50
acute GVHD	54	Arginine and proline metabolism	77	20	0.0497	3.00	0.907	0.52

[a] Outcome events are relapse and grade 2-4 acute GVHD
[b] Total number of EMCs in the pathway
[c] Number of matched EMCs, explained in Statistical Analysis section
[d] -log(*P*) is the negative natural log of the *P* value for each pathway shown in Figures 5 and 7
[e] False Discovery Rate (Benjamini-Hochberg)-adjusted *P*-value
[f] Impact is the pathway impact value on relapse calculated from pathway topology analysis

Fifteen EMCs from our analysis were included in the top pathway, cysteine and methionine metabolism (Figure 6). This result is consistent with the fact that cysteine, methionine, and several related EMCs underwent significant changes in abundance as a result of busulfan administration. Two pathways, the arginine and ornithine metabolism pathway and the arginine and proline metabolism pathway, showed some evidence of an association with acute GVHD (each exhibited pathway impact ≥.5 and raw P<.05) but neither met our criteria for FDR-adjusted significance (Table 2, Figure 7, Figure 8).

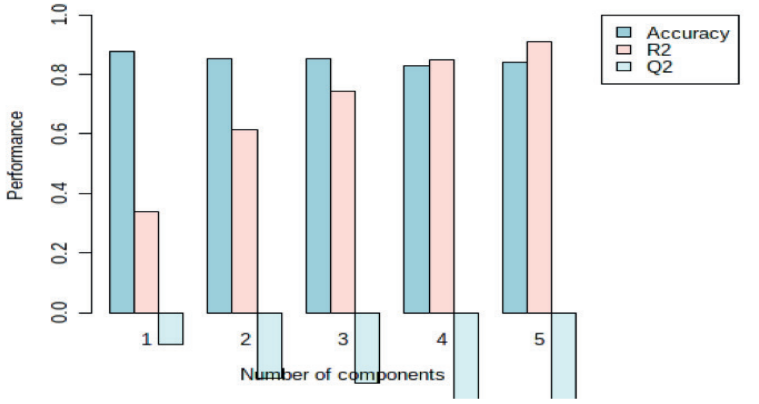


Figure 3: Partial Least Squares-Discriminant Analysis (PLS-DA) of relapse. A) 2D scores plot of the first two components calculated with PLS-DA for predicting relapse using prebusulfan samples. The variance explained by each component is given in parentheses. B), Parameters of the PLS-DA model with 1, 2, 3, 4, and 5 components. PLS-DA was performed using MetaboAnalyst version 4.0. EMC abundance data were centered log-ratio (CLR) transformed prior to PLS-DA. Abbreviations. EMC: endogenous metabolomic compound

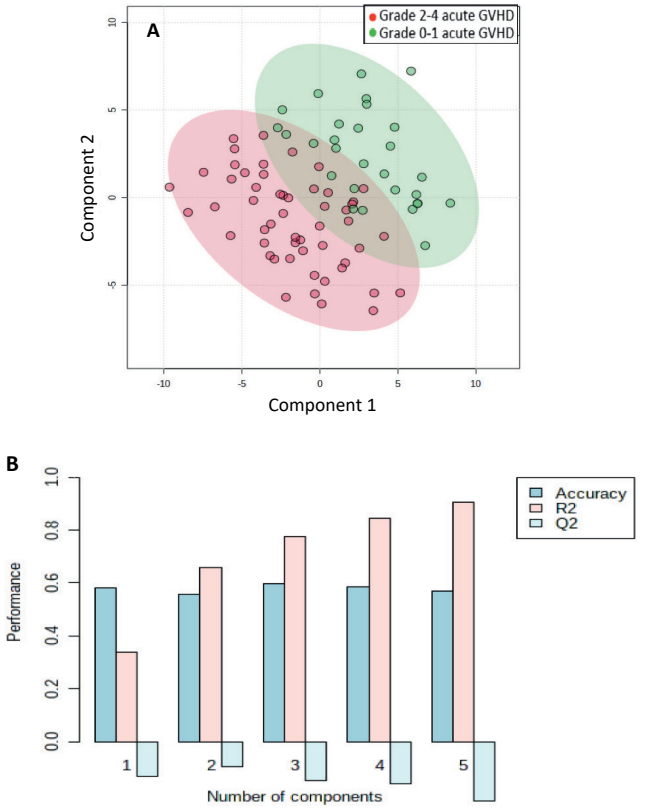


Figure 4: Partial Least Squares-Discriminant Analysis (PLS-DA) of acute GVHD. A) 2D scores plot of the first two components calculated with PLS-DA for predicting acute GVHD grade using pre-busulfan samples. The variance explained by each component is given in parentheses. B), Parameters of the PLS-DA model with 1, 2, 3, 4, and 5 components. PLS-DA was performed using MetaboAnalyst version 4.0. EMC abundance data were centered log-ratio (CLR) transformed prior to PLS-DA. Abbreviations. EMC: endogenous metabolomic compound

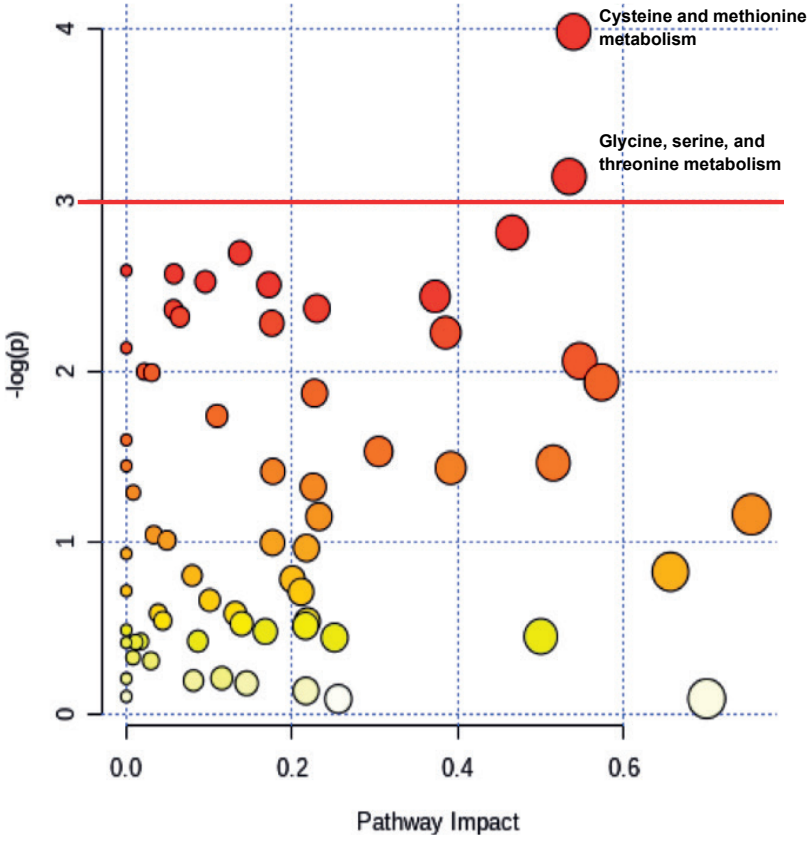


Figure 5. Pathway enrichment analysis of relapse. All dots represent matched pathways from pathway topology analysis. Pathways are colored according to their P values from pathway enrichment analysis, with gradations from yellow – having the largest P – to red – having the lowest P (exact *P* values are given in Tables 2 and S3). Pathways above the horizontal red line correspond to $p<.05$. Pathway impact is indicated on the x-axis. All pathways with $p<.05$ and a pathway impact value >0 are labeled. None of these pathways included EMCs with missing values in this dataset.

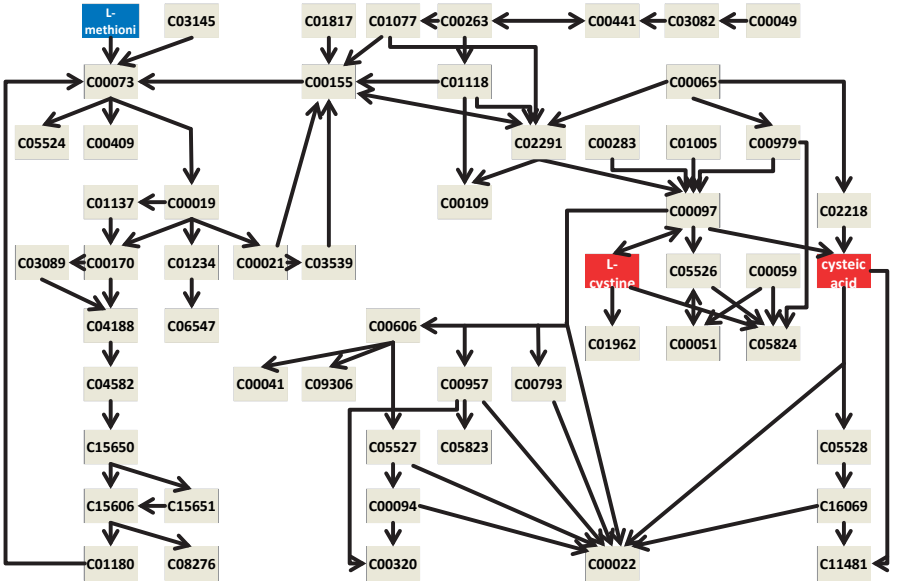


Figure 6. Cysteine and Methionine Metabolism pathway, the pathway with the strongest association with relapse

Each box represents an EMC in the KEGG pathway. Colored EMCs were significant in the pathway analysis with $P < .05$. Red boxes indicate EMCs that were elevated in the relapse group; blue boxes indicate EMCs that were decreased in the relapse group. C00019: S-adenosylmethionine; C00021: S-adenosylhomocysteine; C00022: pyruvic acid; C00041: L-alanine; C00049: L-aspartic acid; C00051: glutathione; C00059: sulfate; C00065: L-serine; C00073: L-methionine; C00094: sulfite; C00097: L-cysteine; C00109: 2-ketobutyric acid; C00155: L-homocysteine; C00170: 5′-methylthioadenosine; C00263: L-homoserine; C00283: hydrogen sulfide; C00320: thiosulfate; C00409: Methanethiol; C00441: L- aspartate-semialdehyde; C00606: 3-sulfinoalanine; C00793: D-cysteine; C00957: 3-mercaptopyruvic acid; C00979: O-acetylserine; C01005: phosphoserine; C01077: O-acetyl-L-homoserine; C01118: O-succinyl-L-homoserine; C01137: S-adenosylmethioninamine; C01180: 2-oxo-4-methylthiobutanoic acid; C01234: 1-aminocyclopropane-1-carboxylate; C01817: DL-homocystine; C01962: thiocysteine; C02218: 2-aminoacrylic acid; C02291: L-cystathionine; C03082: L-aspartyl-4-phosphate; C03089: 5-methylthioribose; C03145: N-formyl-L-methionine; C03539: S-ribosyl-L-homocysteine; C04188: 5-methylthioribose 1 phosphate; C04582: 5-methylthioribulose 1 phosphate; C05524: aminoacyl-L-methionine; C05526: S- glutathionyl-L-cysteine; C05527: 3-sulfinylpyruvic acid; C05528: 3-sulfopyruvic acid; C05823: 3-mercaptolactic acid; C05824: cysteine-S-sulfate; C06547: ethylene; C08276: 3-methylthiopropionic acid; C09306: sulfur dioxide; C11481: hydrogen sulfite; C15650: 2,3-diketo-5-methylthiopentyl-1-phosphate; C15651: 2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate; C15606: 1,2-dihydroxy-3-keto-5-methylthiopentene; C16069: 3-sulfolactate.

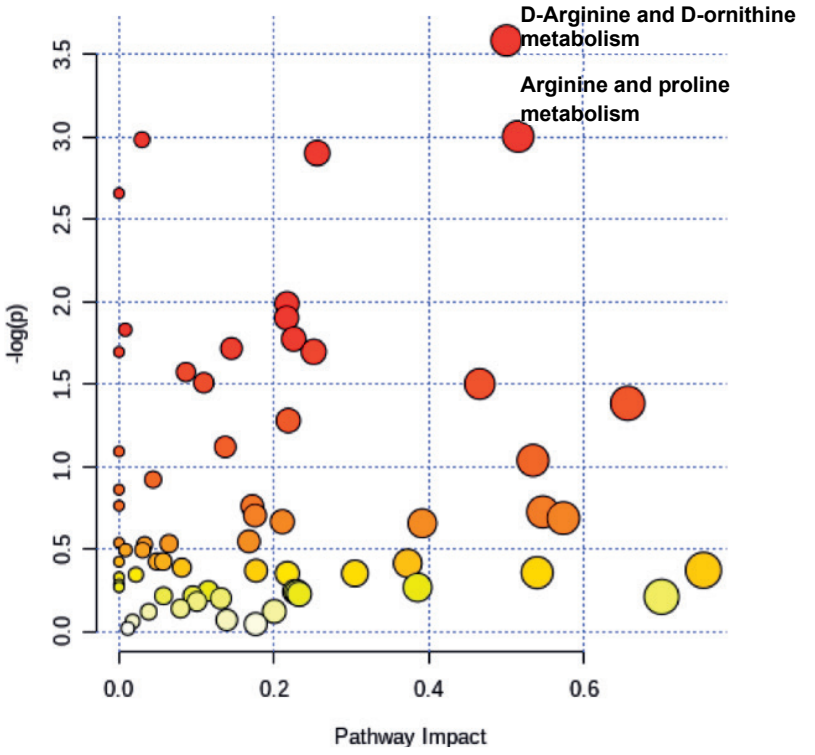


Figure 7. Pathway enrichment analysis of acute GVHD, comparing grade 0–1 and grade 2–4 acute GVHD groups. All dots represent matched pathways from pathway topology analysis. Pathways are colored according to their P values from pathway enrichment analysis, with gradations from yellow – having the largest P – to red – having the lowest P (exact *P* values are given in Tables 2 and S4). Pathways above the horizontal red line correspond to $p<.05$. Pathway impact is indicated on the x-axis. All pathways with $p<.05$ and a pathway impact value >0 are labeled. This pathway did not include EMCs with missing values in this dataset.

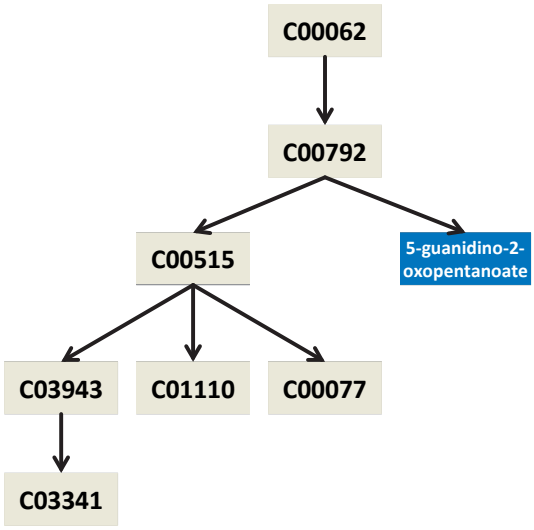


Figure 8: D-arginine and D-Ornithine metabolism pathway, the pathway with strongest association with acute GVHD
Each box represents an EMC in the KEGG pathway. Colored EMCs were significant in the pathway analysis with $P < .05$. Red boxes indicate EMCs that were elevated in the grade 2-4 acute GVHD group; blue boxes indicate EMCs that were decreased in the grade 2-4 acute GVHD group. C00062: L-arginine; C00077: ornithine; C00515: D-ornithine; C00792: D-arginine; C01110: 5-amino-2-oxopentanoic acid; C03341: 2-amino-4-oxopentanoic acid; C03943: (2R,4S)-2,4-diaminopentanoate

DISCUSSION

The key findings of this analysis are, of the 60 pathways with sufficient EMCs for analysis: 1), the cysteine/methionine pathway and the glycine, serine, and threonine metabolism pathway exhibited the strongest association with relapse; 2) the D-arginine and D-ornithine metabolism pathway and the arginine and proline metabolism pathway exhibited the strongest association with acute GVHD. Although these pathways did not exhibit statistical significance, our analysis suggests they deserve further investigation. In this study, we took a first step towards identifying plasma EMCs associated with clinical outcomes with the long-range goal of personalizing the choice of the HCT conditioning regimen, IV busulfan doses, or GVHD prophylaxis using biomarkers identified via pharmacometabonomics.

"Pharmacometabonomics" is the concept of personalized drug treatment using predose metabolite profiling to predict drug response in individuals.[26,27] We chose to focus on the alkylating agent busulfan because of its frequent use in HCT conditioning and its narrow therapeutic index. Recent discoveries demonstrate that metabolomics is an important piece of the puzzle of personalized medicine and that EMCs influence organ function, immune function, nutrient sensing, and gut physiology.[28] In 75 HCT recipients,[29] altered pretransplant levels of several immunoregulatory EMCs, including BCAA and tyrosine derivatives, were found among those who later developed GVHD. This led Reikvam et al to

hypothesize that these EMCs may be involved in developing GVHD and suggests that HCT recipients with high levels of these EMCs may benefit from a stronger immunosuppressive regimen. These studies, as well as our own evaluating the association of the plasma metabolome with IV busulfan clearance,[30,31] suggest pharmacometabonomics may improve clinical outcomes in HCT recipients. Collectively, this work could lead to system-wide perspective of the allogeneic HCT biology wherein EMCs, proteins, and genes are understood to interact synergistically to modify the functions within the allogeneic HCT recipient.

In the present study, we analyzed 763 EMCs representing over 60 pathways. We sought a global metabolomic assay that had sufficient representation of EMCs within glutathione and related pathways. For acute GVHD, pathway enrichment analysis revealed that D-arginine and D-ornithine metabolism and arginine and proline metabolism were the top pathways associated with grade 2 – 4 acute GVHD (Table 2, Figure 7 and Table S5). This contrasts the data in mouse models of HCT showing that early GVHD is associated with accumulation of cysteine, cystathione, and cysteinylglycine .[7] For relapse, pathway enrichment analysis revealed that cysteine and methionine metabolism and glycine, serine and threonine metabolism were the top two pathways associated with post-transplant relapse (Table 2). Although cysteine and methionine metabolism is amongst 10 pathways suggested for further investigation in FMS-like tyrosine kinase 3-internal tandem duplication acute myeloid leukemia, no statistically significant associations between relapse and the cysteine and methionine metabolism pathway were found.[32] No statistically significant associations between the glycine, serine, and threonine metabolism and relapse were found in pathway enrichment analyses. Glycine is an important component of glutathione, and glutathione is involved in busulfan metabolism.[33] Boxplots of plasma EMCs associated with relapse are shown in Figure 1, without a clear delineation in the EMC abundance between those who did or did not relapse.

Strengths of this work include the large population of over 75 HCT participants, a contemporary patient population receiving targeted busulfan, and the global panel providing high accuracy of EMC identification and relative abundances within relevant pathways. However, there are limitations worth noting. Importantly, there were few relapse events (N=10) and few patients with grade 0-1 GVHD (n=24). Future studies with larger sample sizes (and thus, more relapse and GVHD events) are needed, as they would allow for inclusion of risk factors for relapse (e.g., cytogenetics) or GVHD (e.g., HLA). These results show the feasibility of conducting metabolomics studies in allogeneic HCT, with the hope of gaining mechanistic insight into the pathophysiology of relapse and/or GVHD while improving clinical outcomes.

CONCLUSIONS

This work suggests that prebusulfan plasma levels of EMCs in the cysteine and methionine metabolism pathway and the glycine, serine and threonine metabolism pathway may be associated with relapse in HCT patients receiving IV busulfan-based conditioning while EMCs in the D-arginine and D-ornithine metabolism pathway and the arginine

and proline metabolism pathway may be associated with acute GVHD in HCT patients receiving IV targeted busulfan -based conditioning. Further studies, including those that subsequently interrogate the glutathione pathway in larger patient populations, are needed to substantiate these results which may improve the prospect of personalizing the HCT conditioning regimen and potentially improve clinical outcomes.

ACKNOWLEDGEMENTS

This publication was supported by the National Institutes of Health under the Award Numbers: R01CA182963, P01CA18029, 5P30CA015704 and UL1 TR002319. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We are grateful to the patients who participated in this study. We are also grateful to the physicians, nurses, physician assistants, nurse practitioners, pharmacists, and support staff caring for our patients. We are also grateful to the research staff (Kaylene Au, Meagan Bemer, Genecelle Delossantos, Michael Donahue, Alex Men, Brian Phillips, Christine Quinones, Linda Risler, Meron Shiferaw, and Laura Shireman) involved in sample acquisition and transport.

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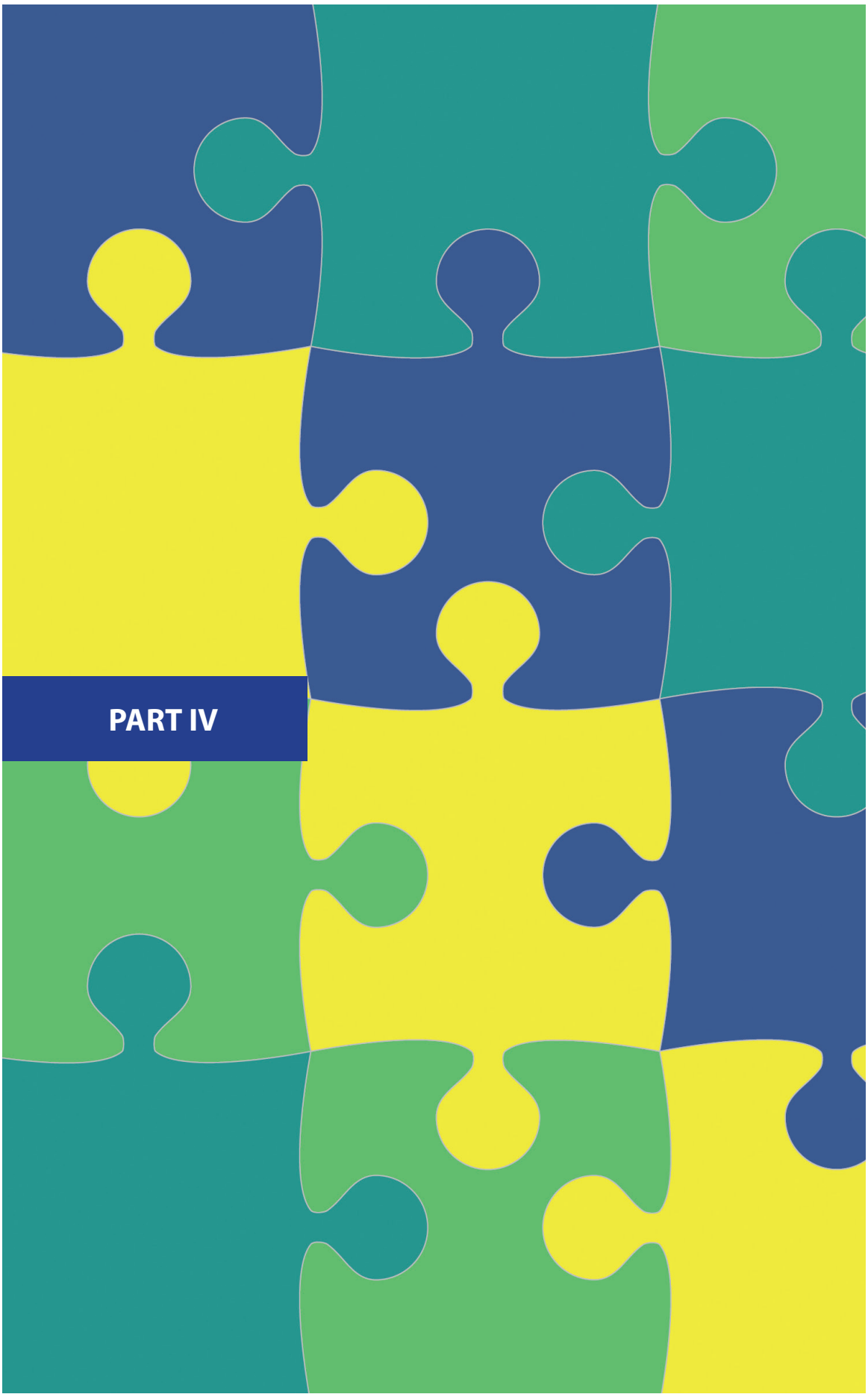

PART IV

Conclusions and Future Perspectives

CONCLUSIONS AND PERSPECTIVES

This thesis focuses on improving outcomes for allogeneic hematopoietic cell transplant (HCT) recipients conditioned with high doses of busulfan, a bi-functional alkylating agent. Over the past 70 years, its use has evolved to, at present, short courses of high-dose busulfan (e.g., 3.2 mg/kg/day intravenously (IV) for four days, termed busulfan hereafter). Busulfan is currently used in many conditioning regimens for allogeneic HCT. The goal of an allogeneic HCT is to cure the patient – termed the host or recipient – of their underlying disease by replacing their hematopoietic cells with cells from a healthy donor. To achieve this cure, a delicate balance must be maintained between the host's immune system and the donor stem cells (graft) that were infused into the host.[10] The transplantation of donor cells that are not genetically identical (i.e., allogeneic) can result in bi-directional immunologic reactions.[13] In allogeneic HCT, grafting of cells from one individual to another provokes immunologic reactions involved in engraftment of the donor cells, graft-versus-host disease (GVHD), control of a malignancy (termed graft versus tumor, GVT), the development of tolerance, and immune reconstitution.[13] These immunologic reactions are influenced by the conditioning regimen, the type and source of the donor graft, and the postgraft immunosuppressive regimen (Supplemental Table 1), all of which are essential components of the allogeneic HCT procedure.

The substantive heterogeneity in the conditioning regimen, type of donor graft, and postgraft immunosuppression, combined with variability in the recipient's characteristics, create challenges to completing adequately powered biomarker studies in allogeneic HCT patients. Despite these challenges, a busulfan area under the plasma concentration-time curve (AUC) has been associated with various important clinical outcomes in HCT patients.[14] Busulfan has a narrow therapeutic index, with small changes in the AUC being associated with increased toxicity (mainly liver toxicity) or decreased efficacy (rejection of the allograft or relapse of the underlying malignancy).[16] Thus, busulfan AUC is a predictive biomarker. Using the definition of the FDA-NIH Biomarker Working Group, "A predictive biomarker is used to identify individuals who are more likely to respond to exposure to a particular medical product or environmental agent. The response could be a symptomatic benefit, improved survival, or an adverse effect."[19] Busulfan doses are often personalized to a specific plasma AUC using the individual patient's clearance.[20] This process is referred to as pharmacokinetic (PK)-guided busulfan (^{PK}busulfan) hereafter.

Supplemental Table 1. Basics of HCT and how it's heterogeneity can affect the relationship between busulfan AUC and clinical outcomes or the exposure – response (E – R). The sources of heterogeneity are in the HCT patient population and the HCT process.

HCT days	Step	Rationale	Heterogeneity
-14 to -2	Conditioning regimen administration	-Initially, with high-dose (myeloablative), gain benefit from steep dose-response curve for alkylating agents and radiation,[5] suppressing the host immune system, and creating space in the marrow compartment to facilitate engraftment.[6,7]	At least 24 different conditioning regimens to treat AML – Slide 52, CIBMTR 2021 Summary[11]
		-At present, lower dose, reduced intensity conditioning (RIC) relies more on the GVT effect via the immune-mediated assistance from donor lymphocytes for the complete eradication of malignant cells.[8]	
0	Allograft infusion	-Initially, to circumvent dose-limiting myelosuppression with a stem cell infusion	At least 8 different donor types – Slide 4, CIBMTR 2021 Summary[11]
		-Allograft is progenitor cells that are self-renewing and provide a lifetime source of blood cells.[13]	Three different graft sources – Slide 17, CIBMTR 2021 Summary[11]
-3 to +180	Postgraft immunosuppression administration	-To ensure engraftment and prevent the development of GVHD while maintaining GVT.	At least 9 different combinations – EBMT[15]
		-Immunosuppressants inhibit and minimize the activity of donor T-cells.	Variation in discontinuing immunosuppressants[17]

1. Allogeneic HCT is used to treat nonmalignant and malignant diseases.
   a. This leads to wide range of ages and comorbidities. Patients with nonmalignant diseases are often infants while patients with malignant diseases are typically older.
   b. Examples of how patient-related factors may influence E – R relationships
      i. Thymus-dependent regeneration of $CD4^+$ and $CD8^+$ cells after chemotherapy occurs primarily in children[2,3]
      ii. Engraftment kinetics after nonmyeloablative (i.e., low dose) conditioning differ between children and adults[4]
2. The HCT procedure is a multi-step procedure, with considerable heterogeneity in each step such that over 5000 combinations are possible.
   a. Above is a summary of each step in the HCT procedure, its rationale, and its heterogeneity.
   b. Examples of how the HCT procedure can influence E – R relationships
      i. The additional drugs used with busulfan and their administration sequence can influence the E – R relationship of busulfan AUC with clinical outcomes.[18]
      ii. Graft source affect engraftment, acute GVHD, chronic GVHD, and immune recovery, and can affect the E – R relationship of immunosuppressant AUC with clinical outcomes.[21-24]
      iii. The type of and adherence to postgraft immunosuppression influence GVHD risk.[10]

Table 1. Summary of Key points

Key point #1:	A framework should be built to use real-world data (RWD) to improve standard of care (SOC) personalized dosing.
Key point #2:	^{MIPD}busulfan should replace ^{PK}busulfan.
Key point #3:	^{PK}busulfan cannot be replaced with omics tools, but they may provide insight into pharmacokinetic variability.
Key point #4:	Pharmacometabolomics offers mechanistic insight into the pharmacodynamics of busulfan.

Key point #1. A framework should be built to use real-world data (RWD) to improve standard of care (SOC) personalized dosing.

With personalized dosing using any precision medicine tool (PMT), the goal is to personalize an individual patient's dose to sufficiently minimize the interpatient variability and, therefore, maximize that individual's chance of survival. Within the context of using busulfan-based conditioning before allogeneic HCT, the goal of ^{PK}busulfan dosing is to decrease relapse while maintaining low rejection and low toxicity. Pharmacokinetics is the oldest of the available PMT based on patient characteristics. ^{PK}busulfan was implemented over 30 years ago because it reduced the frequency of two fatal outcomes – i.e. hepatotoxicity[25-27] and graft rejection.[26] Over that extended time period, there has been a 'laissez faire' approach to improving ^{PK}busulfan. In **Chapter 1,** two pitfalls are identified from this laissez-faire approach. The first pitfall is that the E – R relationships cannot be updated to current treatment regimens. The HCT conditioning regimens and the characteristics of allogeneic HCT patients have changed over the 30 years since ^{PK}busulfan started. However, the published literature regarding the association of E – R (i.e., association of busulfan AUC with clinical outcomes) was too heterogeneous and lacked adequately powered and sufficiently controlled studies to provide evidence-based target AUCs for these contemporary patients.[18] Thus, Chapter 1 revealed two substantive challenges: 1. An inability to reach a consensus for choosing the target busulfan AUC for current treatment regimens; 2. The ongoing use of outdated pharmacokinetic modeling methods to estimate busulfan clearance, despite changes in pharmacokinetic (mathematical) modeling in phase I to III drug development.[28,29]

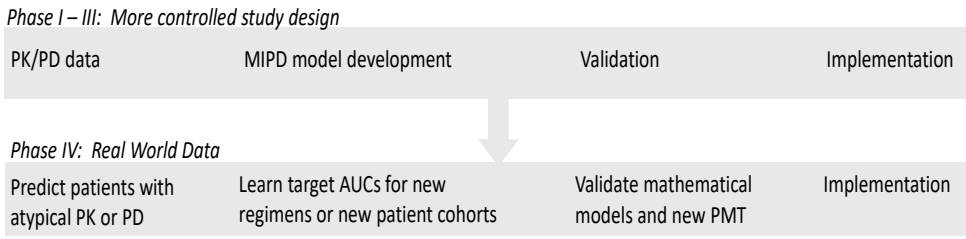

Figure 1. Conceptual framework for 'predict, learn, confirm, implement cycle'[1] for phase I – III drugs undergoing drug development and Phase IV drugs already EMA or FDA approved

We need to understand how the E – R relationship changes as clinical care changes, yet there are few ideas regarding *how* to collect sufficiently detailed data to do so. Figure 1 conceptual differences in the "predict, learn, confirm, implement cycle" for drugs undergoing development (i.e., phase I – III) and those drugs with personalized dosing after regulatory approval (i.e., phase IV). As the use of PMT continues to expand, it is increasingly important to develop a framework to refine these tools as clinical care and PMT evolve.[30,31] With the extended history of ^{PK}busulfan, many of the lessons learned from the laissez-faire approach lead us to propose a framework for using the 'predict, learn, confirm, implement cycle' framework for precision dosing.[1] The steps listed in Table 2 can be used for other drugs and other PMT. Table 2 merely describes busulfan as the exemplar drug, PK as the exemplar PMT, and MIPD as the exemplar mathematical modeling tool. However, a substantial challenge with enacting such a framework is no clear funding source to support such activities. Thus, regulatory bodies may need to create metrics for successfully implementing ^{PK}busulfan or ^{MIPD}busulfan for accreditation of their HCT center.

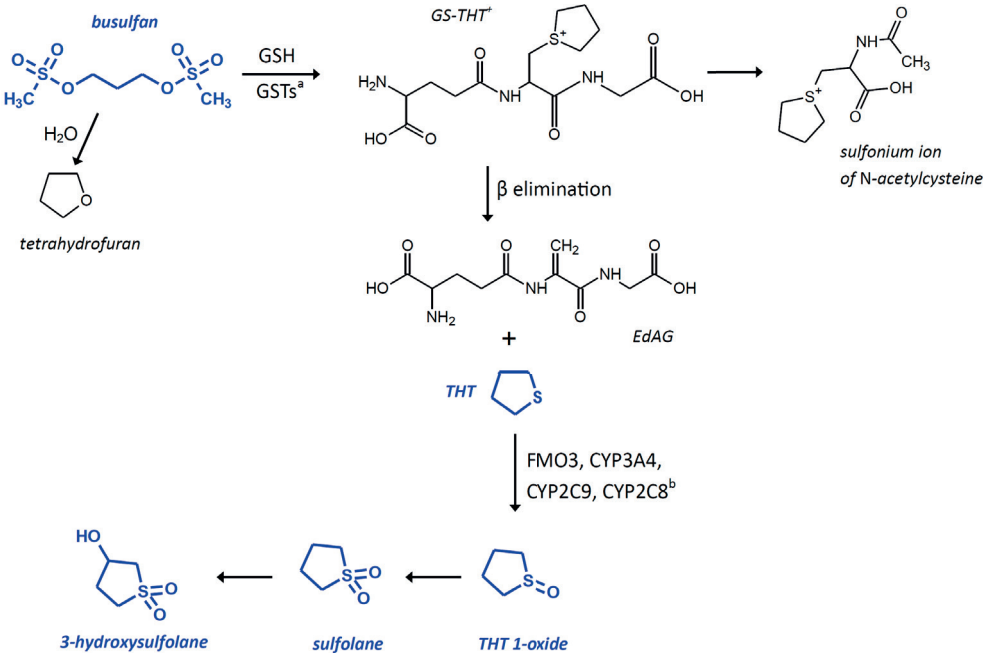

Figure 2. Busulfan metabolism. Blue font represents analytes that can be reliably quantitated in HCT recipients.
[a]GSTs involved in busulfan metabolism, (highest to lowest):[9] GSTA1-1, M1-1, P1-1
[b]THT intrinsic clearance, μL/min/mg protein (highest to lowest)[12]: FMO3 (highest), CYP3A4, 2C9, 2C8, 2C19, 2B6, 2D6, 4A11, 1A2, 1A1, 2E1. Abbreviations: CYPs: Cytochrome P450 enzymes; EdAG: γ-glutamyldehydroalanylglycine; FMO: Flavin-containing monooxygenase; GSG: 2-amino-5-[[3-[2-[[4-amino-5-hydroxy-5-oxopentanoyl]amino]-3-(carboxymethylamino)-3-oxopropyl]sulfanyl-1-(carboxymethylamino)-1-oxopropan-2-yl]amino]-5-oxopentanoic acid; GSH: glutathione; GST: glutathione S-transferase; GS+THT: γ-glutamyl-β-(S-tetrahydrothiophenium)alanylglycine; THT: tetrahydrothiophene

Table 2. Proposed framework to improve SOC [PK]busulfan.

Process	Thesis Section	
1. Convene a multi-disciplinary group regarding busulfan, and its current PMT of pharmacokinetics	Chapter 1	Learn
1.1. Share process for [PK]busulfan	Chapter 1, Appendix	Implement
1.2. Is busulfan being used in new treatment regimens or new patient cohorts?	Conclusions	Learn
1.3. Is personalized dosing with the current PMT tool still appropriate, or should a new PMT be used?		
2. Develop consensus regarding quality assurance for [PK]busulfan and [MIPD]busulfan		Implement
2.1. Harmonize to one way of reporting busulfan AUC results	Chapter 2	
2.2. Create an international quality assurance program for [PK]busulfan	Chapter 3	
2.3. Decide if compliance metrics should be part of HCT center accreditation	NA	
3. Use existing RWD to		Learn
3.1. Create a mathematical model generalizable to the majority of the patient population	Chapter 4	
3.2. Learn if a different PMT – i.e., pharmacogenomics and pharmacometabolomics are associated with relevant parameters from the current PMT tool	Chapters 5 to 7	
4. Build or reinforce multicenter existing infrastructure for data-sharing and population pharmacokinetic-dynamic modeling[32]	NA	Predict
4.1. Data upload from academia		
4.2. Use CIBMTR database infrastructure to link to clinical outcomes to allow for multicenter busulfan AUC – outcomes association studies		
4.3. If that is not feasible, then anonymize the raw dataset		
4.4. Data handling with tracking		
4.5. Data merge with tracking		
4.6. Population pharmacokinetic – dynamic modeling		
4.7. Data extraction and sharing of model and parameters		
5. Transition from [PK]busulfan to [MIPD]busulfan	NA	Implement
5.1. Decide timeline for implementing MIPD		
5.2. Decide expectations for compliance with these metrics		
6. Continued learning – confirming	NA	Implement
6.1. At pre-specified times, repeat the steps 1 to 3		
6.2. Conduct routine (at least annually) evaluations of [MIPD]busulfan		
6.3. Establish centralized experts (at least 1/continent) on busulfan and [MIPD]busulfan		
6.4. Establish a training program to ensure expertise is maintained		
7. Confirm encouraging RWD with prospective cohorts		Confirm
7.1. Validate and expand mechanistic findings of pathways associated with busulfan clearance	Chapter 8	
7.2. Evaluate if clinical outcomes are associated with data from novel PMT tools, specifically pharmacometabolomics	Chapter 9	

Abbreviations: AUC – area under the plasma concentration-time curve; CIBMTR – Center for International Blood and Marrow Transplant Research; MIPD – model informed precision dosing; NA – Not available; PMT – precision medicine tool; RWD – real-world data.

Key point #2: [MIPD]busulfan should replace [PK]busulfan

For the past 30 years, the majority of HCT centers have conducted [PK]busulfan using the following process: 1. choosing the target AUC specific for that patient, their conditioning regimen, and their underlying disease; 2. administration of a busulfan dose based on weight or body surface area, 3. intensive pharmacokinetic sampling, 4. quantitation of busulfan concentrations, 5. pharmacokinetic modeling of the resulting concentration-time data to estimate the individual-specific busulfan clearance, and 6. use that clearance to personalize the dose to achieve the target AUC. **Chapters 1 to 3** describe how to improve this process; **Chapter 4** describes how a population pharmacokinetic (popPK) model, created from a large and heterogeneous patients population, can be used to estimate the initial busulfan dose (i.e., the "right-dose-first-time" paradigm[33]) and be used to enable model informed precision dosing of busulfan ([MIPD]busulfan).

Population pharmacokinetic models[34] can be used to address relevant hurdles by accounting for variability and mitigating the resource intensity of PK-guided dosing. PopPK models mathematically describe typical drug pharmacokinetics while simultaneously accounting for between-subject variability, residual unknown variability,[35] and the role of covariates (e.g., age or gender) responsible for or related to this variability. **Chapter 4** describes an age- and size-dependent model well-described the pharmacokinetics of busulfan over a wide age continuum (0.1 to 68 years of age). This popPK model was built using RWD from 51 different HCT centers, proving that the "predict-learn-confirm-implement" paradigm is possible for phase IV drugs (Figure 1). This popPK model has several uses. The first use is to predict the optimal initial busulfan dose. This model can accurately estimate the initial busulfan dose, hopefully improving upon the current initial dosing practices in which only 24% of children achieve the individual-specific therapeutic window of busulfan exposure.[36] Simulations suggested that using the initial doses predicted from this popPK model would lead to a higher proportion of initial doses achieving the therapeutic window (72%) compared with dosing recommended by the U.S. Food and Drug Administration (57%) or the European Medicines Agency (70%). The second use of this model is [MIPD]busulfan, specifically real-time Maximum A posteriori Probability (MAP) Bayesian estimation of the individual's pharmacokinetic parameters, incorporating a blend of individualized pharmacokinetic data and a population parameter priors. The final use of this popPK model is to provide insight into the ontogeny of busulfan metabolism. Busulfan is metabolized in the liver through glutathione (GSH) conjugation by glutathione S-transferase (GST) enzymes. This process depletes hepatocyte GSH stores. Conjugation of busulfan with GSH forms an unstable S-glutathione sulfonium conjugate (GS+THT). The subsequent metabolism results in the formation of a tetrahydrothiophenium ion (THT+) in a GST-catalyzed reaction. GSTA1-1 is the most active human form of GST for busulfan conjugation; GSTM1-1 and GSTP1-1 also mediate IV busulfan conjugation, but their estimated *in vivo* contributions to IV busulfan conjugation are ~5% and 0.2%, respectively, after accounting for their lower activity for busulfan conjugation and lower hepatic expression relative to GSTA1.[9,37] To our knowledge, the popPK model described in **Chapter 4** is the first to describe the maturation of IV busulfan clearance; our data modeling indicates that at 2.5 years of age IV busulfan clearance is essentially that (95th percentile) of adults. Thus, these data suggest that children 2.5 years of age and older have similar hepatic GSTA1-1 activity to adults, although In vitro data is needed to confirm these data.

Thus, the ontogeny of hepatic GSTA1-1 activity appears to differ from intestinal GSTA1-1 because children less than 4 years old have enhanced intestinal GSTA1-1 expression and formation clearance of THT^+ in patients receiving oral busulfan.[38,39] This popPK model provides mechanistic insight into hepatic GST, predominantly GSTA1, activity.

Future work should focus on incorporating this popPK model into a decision support system that includes relevant clinical data in a user-friendly interface (e.g., InsightRx or NextDose[40]) to communicate the optimal busulfan dose based on the ^{MIPD}busulfan. This model can also facilitate the development of pharmacokinetic sampling schedules for outpatient ^{MIPD}busulfan. For outpatient ^{MIPD}busulfan to be feasible, an individual's busulfan clearance and volume must be predicted with this popPK model and pharmacokinetic sampling over eight hours from the start of the three-hour infusion.[41] We propose that this popPK model can be used to accomplish this goal by facilitating the development of an outpatient sampling schedule to decrease the time needed for the required blood sampling. At present, IV busulfan is typically administered as a three-hour infusion once daily. Creation of an outpatient (i.e., maximum of 8 hours after the start of the three-hour infusion) limited sampling schedule for daily IV busulfan administration could allow for outpatient administration of daily IV ^{MIPD}busulfan with doses personalized to achieve the target busulfan AUC. Because current pharmacokinetic sampling requires inpatient admission, this could result in significant cost savings. Such an outpatient sampling schedule would reduce the need for clinical resources (i.e., nursing and laboratory staff time) and may increase patient convenience. In addition, greater convenience is likely to increase patient accrual in subsequent studies, facilitating their experimental design and more rapid identification of novel E – R relationships.

Key point #3: ^{PK}busulfan cannot be replaced with omics tools, but they may provide insight into pharmacokinetic variability

Busulfan conditioning has a short (2 to 4 day) duration, necessitating on-site busulfan quantitation for dose personalization shortly thereafter. Predicting busulfan clearance before the start of busulfan administration would be preferred at some (e.g., those without an on-site busulfan quantitation) HCT centers. Thus, we evaluated if preemptive pharmacogenomics and pharmacometabolomics – i.e., PMT that can be evaluated weeks before busulfan administration is started – could predict IV busulfan clearance.

Over the past few decades, technical advances have led to the creation and/or refinement of a multitude of PMT along the '-omic cascade'[42], which is: genome, epigenome, transcriptome, proteome, metabolome, lipidome, and microbiome. Each of these has the potential to serve as PMT to explain pharmacokinetic variability. Pharmacogenomics seeks to identify germline genetic variants that contribute to an individual's unique drug response.[43] A sizable percentage of cancer patients could benefit from preemptive pharmacogenomics-guided dosing (PGx-guided dosing). In preemptive PGx-guided dosing, genotyping is performed such that its results are available within the patient's electronic health record. These results can be used to personalize which chemotherapy and/or chemotherapy dose before a relevant high-risk chemotherapy is prescribed.[44] Reizine et al. found that nearly one-third of > 1,500 cancer patients have genetic variations

that could change the recommendations of which chemotherapy, or how much of, chemotherapy should be given to a patient.[44]

Although preemptive PGx-guided dosing holds promise for many patients, it is not a PMT for every drug. Turning now to busulfan, the main focus of pharmacogenomics studies has been the candidate gene approach focusing on the different glutathione S-transferases encoding for the enzymes that metabolize busulfan. None of the genes associated with GSTs have demonstrated a consistent effect on the pharmacokinetics of busulfan (**Chapter 1**, FAQ 10, and **Chapter 5**). As stated earlier, GSTA1-1 is the most active human form of GST, with GSTM1-1 and GSTP1-1 having lower contributions to busulfan conjugation. The inconsistent GST genotype to IV busulfan clearance phenotype is not surprising for a few reasons. First, multiple GSTs involved in busulfan clearance: another GST could still metabolize busulfan if a GST is poorly expressed. Second, busulfan clearance is influenced by nongenetic factors, including young age (**Chapter 4**) and drug-drug interactions (**Chapter 1**, FAQ 9). Third, the association of GST polymorphisms with various clinical phenotypes, ranging from tobacco-related diseases to response to chemotherapy treatment for various solid tumor malignancies, has not been replicated.[45]

Clearly, alternative PMTs were needed. In addition to the clinical studies, preclinical models can provide biological insight. A problem in asking the question – are GST polymorphisms important in mediating the clinical phenotype of IV busulfan clearance? – is that we do not know the effect of gene function of each polymorphism. The GSTs are very wide expressed, and their expression is tightly controlled during human development. Although GST knockout mice have been generated for GSTA4, GSTP1, and GSTZ1, these models often overexpress the Alpha-, Mu- and Pi-class GSTs.[45] Thus, these preclinical models have limited relevance to the GST genotype to the IV busulfan clearance phenotype question. We also recognize that proteomics can provide insight regarding the ontogeny of hepatic uptake and efflux drug transporters[46] or the drug-drug interaction potential.[47] However, a pre-HCT liver biopsy for proteomics of GSTs in an individual is not clinically feasible PMT. Thus, we turned to plasma metabolomics.

Metabolomics, which is the study of small-molecule metabolite profiles in biological samples, is a promising PMT for allogeneic HCT recipients. In hopes of avoiding confusion between the metabolites of busulfan and the metabolites from metabolomics quantitation, we use the term 'endogenous metabolomic compounds' (EMCs) to refer to the compounds quantitated using the more sensitive liquid chromatography-mass spectrometry methods of metabolomic quantitation. The compounds that are drugs or known drug metabolites are removed from the dataset. Pharmacometabolomics (PMx) is defined as an "enhanced understanding of mechanisms for drug effect and increased ability to predict individual variation in drug response phenotypes, based on using both baseline EMC profiles prior to treatment and also effects of drug treatment over time ('longitudinal' metabolomic profiles)".[48,49]

Chapters 5 to 8 are amongst the first pharmacometabonomic studies in allogeneic HCT. The first two pharmacometabonomic studies (**Chapter 6 and 7**) were in a retrospective cohort focusing on known pathways (targeted metabolomics, **Chapter 6**)

or completely unknown EMCs (global metabolomics, **Chapter 7**). **Chapter 6** describes our targeted metabolomics analysis; we found that the EMCs glycine, N-acetylglycine, 2-hydroxyisovaleric acid, creatine, serine, and tyrosine were significantly associated with IV busulfan clearance. The pathway analyses revealed that the glycine, serine, and threonine pathway was associated with IV busulfan clearance. Glycine is a component of glutathione, which is conjugated to busulfan during detoxification, indicating the biological plausibility of our findings in predicting busulfan clearance. In **Chapter 7,** our global pharmacometabolomic analysis revealed found that tertiles of increasing IV busulfan clearance were associated with 21 ions ($R^2 \geq 0.3$). We sought to identify those 21 ions or EMCs, but only two could be identified. The identified EMCs included linoleic acid and the bile acids deoxycholic acid and/or chenodeoxycholic acid. Bile acids have been shown to inhibit GSTs *in vitro*.[50] This also provided biological plausibility to **Chapter 7**'s findings.

In **Chapter 8,** we sought to validate these previous global[51] or targeted[52] pharmacometabonomic analyses of EMCs associated with IV busulfan clearance in a separate cohort of prospectively collected samples. We also sought to discover additional EMCs and pathways. Using pathway enrichment analysis, 18 pathways were statistically significantly associated with IV busulfan clearance. Lysine degradation followed by steroid biosynthesis was significantly associated with IV busulfan clearance. There are at least three avenues for future exploration. First, these novel EMCs and pathways should be validated *in vivo*, specifically in future cohorts of prospectively collected EMC samples. Second, the most promising EMCs should be validated *in vitro*. To our knowledge, a paradigm for validating EMCs associated with drug clearance *in vitro* has yet to be established. An in vitro paradigm for validating these EMCs is likely more rapid than *in vivo* validation. In addition, in vitro studies could allow for novel mechanistic insight. We propose to use the *in vitro* to *in vivo* extrapolation (IVIVE) tools, such as human liver microsomes or human hepatocytes, established for accurately predicting the hepatic clearance of new drugs.[53] Using an EMC from our global pharmacometabolomic analysis (**Chapter 7**) as an example, we propose to compare IV busulfan clearance from sandwiched human hepatocytes with and without physiologic concentrations of deoxycholic acid. The first question to be addressed is – which *in vitro* model should be used? We propose a whole-cell system, such as a human hepatocyte model, is needed. In addition to drug detoxification and degradation of tyrosine, GSTs are involved in the biosynthesis of leukotrienes, prostaglandins, testosterone, and progesterone. The GSTs may alter signaling pathways. Thus, it is desirable to use an intact cellular system to maintain these regulatory systems as close to the *in vivo* system. In addition, a human model is preferred because of differences in drug metabolism by species.[54] Specific to this example, bile acid redox metabolism is species-dependent,[55] providing further support for using a human *in vitro* model. The second question is – what type of study design should be used? We are trying to understand EMCs that predict busulfan clearance, and thus, establishing these experiments similar to drug-drug interaction studies and not hepatoxicity studies seems optimal. Metabolomics can offer mechanistic insight into the hepatoxicity of drugs;[56,57] however, our focus is on predicting busulfan clearance. Thus, we propose evaluating busulfan clearance in hepatocytes with or without deoxycholic acid to determine if deoxycholic acid affects busulfan clearance. The third question is – what concentrations of busulfan should be used in the human

hepatocyte experiments? The relationship of the quantitated plasma concentrations to the unattainable hepatic concentrations is unknown for any drug. To predict hepatic busulfan concentrations, we propose using IVIVE extrapolation equations for predicting drug clearance. Those extrapolated concentrations should be used for human hepatocyte experiments. The fourth question is – what are the hepatic concentrations of deoxycholic acid? The IVIVE equations could also be used here. However, deoxycholic acid has been studied in human hepatocytes,[58] so there should be an overlap in the deoxycholic acid concentrations between the previous experiments and the deoxycholic acid–busulfan experiments. Conducting such *in vitro* experiments can provide mechanistic insight regarding if each specific EMC alter busulfan clearance and guide which EMCs should be included in the future metabolomic panels for the subsequent pharmacometabonomic studies in allogeneic HCT patients.

After validating the EMCs associated with busulfan clearance, the following steps should include the prediction of IV busulfan clearance using the EMCs (**Chapter 8**) and the popPK model (**Chapter 4**). Specifically, for each individual, we propose to incorporate the covariates for IV busulfan clearance (currently age and NFM) into a multiple regression model for the association between IV busulfan clearance and EMC. We will then have a prediction model to estimate IV busulfan clearance by combining the EMC with the popPK model covariates (i.e., EMC/popPK method or $^{PMx\text{-}MIPD}$busulfan). Subsequently, each individual could have an estimated IV busulfan clearance from the EMC/popPK prediction model, which will be evaluated using a predefined validation criterion. This criterion is based on the FDA Guidance for Bioequivalence of Generic Drugs. [59] We will consider the EMC/popPK prediction successful if, using 90% confidence intervals, the estimated clearance is anywhere between 80% and 125% (the inverse of 80%) of the observed clearance used for clinically used ^{PK}busulfan dosing. In addition, this estimated EMC/popPK clearance should be used with the individual-specific target busulfan AUC chosen by the physician to provide an EMC/popPK predicted IV busulfan dose. This estimated dose will be compared to the IV busulfan dose actually administered, which is weight-based for the first dose and personalized for subsequent doses (i.e., ^{PK}busulfan). The predicted dose will be considered successful if it is anywhere between 80% and 125% (i.e., 80%<(predicted/observed)<125%) of the personalized ^{PK}busulfan dose for over 45% of the subjects since the current weight-based method for dose 1 of IV busulfan[36] dosing results in only 24%[36] of children and 23%[60] of adults achieving their target busulfan AUC.

Key point #4: Pharmacometabolomics provides mechanistic insight into the pharmacodynamics of busulfan

Although ^{PK}busulfan is often used clinically, it fails to provide a rich, mechanistic description of the E – R relationship[61] that could advance our understanding of why certain allogeneic HCT patients experience adverse outcomes. There is substantial enthusiasm for the –omics technologies, specifically genomics, proteomics, and metabolomics, to identify patients at higher risk of adverse effects. One major challenge for the –omics tools is the interference from confounding factors.[62,63] PopPK models can identify confounding factors associated with aberrant busulfan AUC; thus, our proposed popPK/EMC model may also provide insight into the pharmacodynamics of busulfan-based conditioning regimens.

With the nonspecific nature of alkylating agents, it is not surprising that candidate genes focusing on busulfan pharmacodynamics (i.e., its effectiveness or its toxicity) have yet to be found (see **Chapter 1**, Supplemental Table 1). The association of GST polymorphisms with numerous clinical phenotypes has not been replicated; [45] There is encouraging data that proteomics-based biomarkers can predict outcomes in allogeneic HCT. An acute GVHD-specific urinary proteome classifier correctly identified patients developing severe acute GVHD 14 days before any clinical signs and did so with acceptable predictive value (82.4% sensitivity and 77.3% specificity).[64] The classifier, consisting of 17 peptides derived from albumin, β_2-microglobulin, CD99, fibronectin, and various collagen α-chains, indicated inflammation, T-cell activation, and changes in the extracellular matrix as early signs of GVHD-induced organ damage.[64] Similarly, a panel of six protein biomarkers – IL-2 receptor-α; tumor necrosis factor receptor-1; hepatocyte growth factor; IL-8; elafin, a skin-specific marker; and REG3a, a gastrointestinal tract–specific marker – relevant to GVHD treatment has been identified using proteomics discovery and validation strategies.[65] It is hoped that these proteomics-based GVHD panels will be used for the early identification of allogeneic HCT recipients at high or low risk for not responding to GVHD treatment or death.[65]

Recent discoveries demonstrate that metabolomics is an important piece of the puzzle of personalized medicine and that EMCs are associated with organ function, immune function, nutrient sensing, and gut physiology.[66] In 75 HCT recipients,[67] altered pre-HCT levels of several immunoregulatory EMCs, including BCAA and tyrosine derivatives, were found among those who later developed GVHD. This finding led Reikvam et al. to hypothesize that these EMCs may be involved in developing GVHD and suggests that HCT recipients with high levels of these EMCs may benefit from a stronger immunosuppressive regimen. Finally, we started to interrogate metabolomic pathways to identify predictors of busulfan response (**Chapter 9**) using longitudinal sample collection. Alkylating agents are cell-cycle nonspecific and, to date, only busulfan AUC predicts outcomes in allogeneic HCT patients. Using the global metabolomics panel in a subset of the prospective cohort of **Chapter 8**, we found that 1) the cysteine/methionine pathway and the glycine, serine, and threonine metabolism pathway exhibited the strongest association with relapse; 2) the D-arginine and D-ornithine metabolism pathway and the arginine and proline metabolism pathway exhibited the strongest association with acute GVHD. We took a first step towards identifying plasma EMCs associated with clinical outcomes with the long-range goal of personalizing the choice of the HCT conditioning regimen, IV busulfan doses, or GVHD prophylaxis using biomarkers identified via pharmacometabolomics. Collectively, this work could lead to a system-wide perspective of the allogeneic HCT biology wherein EMCs, proteins, and genes are understood to interact synergistically to modify the functions within the allogeneic HCT recipient.

Pharmacometabolomics is also advantageous because it can reflect the effects of busulfan and its metabolites. As described earlier (Key point #1), conjugation of busulfan with GSH forms an unstable GS+THT. GS+THT undergoes b-elimination to form g-glutamyldehydroalanylglycine (EdAG). EdAG reacts with human glutaredoxins (Grx)-1 or Grx-2; this was the first demonstration of the reactivity of any busulfan metabolites with intact proteins. These recent data suggest that GSH-binding sites containing thiolates

are most susceptible to EdAG, which may contribute to the narrow therapeutic index of busulfan through various mechanisms.[68,69] Although EdAG cannot be reliably quantitated in the plasma obtained from HCT patients receiving busulfan, the subsequent metabolites THT^{+},[39] THT 1-oxide, sulfolane, and 3-OH-sulfolane[70,71] have been reported in HCT recipients. Future work should characterize the pharmacokinetics of these metabolites and evaluate if the metabolite's AUCs are associated with clinical outcomes.

Beyond the E – R of busulfan's metabolite AUCs with clinical outcomes, a quantitative framework may improve our ability to achieve the delicate balance of the grafting of the donor cells to the HCT recipient.[72,73] We propose that quantitative systems pharmacology (QSP) models be built in allogeneic HCT patients receiving busulfan-based conditioning. QSP is a rapidly expanding area that merges the traditional focus of pharmacokinetic/dynamic studies with a mechanistic understanding of disease pathways.[74] QSP combines computational modeling and experimental data to examine relationships between a drug, biological system, and disease processes.[74] It integrates in vitro, preclinical, and clinical data to create a QSP model that can subsequently use real-time clinical data to generate discoveries regarding existing therapies and attributes of disease progression. Systems immunology models are built using multi-omics technologies to characterize response mechanisms and identify powerful biomarkers of response to other immunotherapies (e.g., checkpoint inhibitors, CAR-T cell therapy). However, as allogeneic HCT is less specific than these newer immunotherapies, it's continued use creates an imperative to build QSP models to better understand and predict outcomes in allogeneic HCT patients.

CONCLUSION

The goal of these chapters is to decrease relapse while maintaining low rejection and low toxicity to busulfan-based conditioning. We have developed a framework for improving the clinical use of ^{PK}busulfan using RWD that may be expanded to other drugs and other PMT. We demonstrated that IV busulfan clearance could be predicted with popPK modeling or pharmacometabolomics, but not pharmacogenomics. We have begun identifying metabolomic pathways associated with response (relapse) and toxicity (GVHD) to busulfan-based conditioning for allogeneic HCT.

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SUMMARIES

Summary

In patients with cancer, chemotherapy can be personalized based on the characteristics of the tumor and the patient. This thesis focuses on using patient characteristics to personalize an old chemotherapy drug, specifically busulfan, in allogeneic hematopoietic cell transplant (HCT) recipients. For the past 30 years, busulfan doses are often personalized to a target plasma area under the concentration-time curve (AUC) using an individual patient's pharmacokinetic characteristics, a process termed pharmacokinetic-guided busulfan dosing (^{PK}busulfan). Because of this narrow therapeutic index necessitating ^{PK}busulfan and the extended time after initial Food and Drug Administration (FDA) approval (~70 years), busulfan is a unique exemplar of adapting the 'learn – confirm' paradigm used in phase I – III drug development to the 'predict, learn, confirm – implement cycle' for model informed precision dosing (MIPD) of a phase IV drug. We used real-world data (RWD) combined with population pharmacokinetic modeling (Part 1) and various -omics tools (Parts 2 and 3) to improve the effectiveness and toxicity of busulfan after its regulatory approval.

Part 1 focuses on improving ^{PK}busulfan. In the early 1990s, ^{PK}busulfan decreased graft rejection and hepatotoxicity in hematopoietic cell transplant (HCT) patients conditioned with high-dose busulfan. Over the past 30 years, the relationship between exposure-response (i.e., busulfan AUC – clinical outcomes) may have changed as contemporary HCT regimens were developed. In addition, the actual process of ^{PK}busulfan has minimally changed over the past 30 years and should evolve to ^{MIPD}busulfan. In this context, the present models available suggest that ^{MIPD}busulfan would be real-time Maximum A posteriori Probability (MAP) Bayesian estimation of the individual patient's pharmacokinetic parameters, incorporating a blend of individualized pharmacokinetic data and a population parameter priors.

Chapter 1 was a 'call to action,' as it summarized the lack of evidence-based decisions for several aspects of ^{PK}busulfan. We led a multidisciplinary and international group (i.e., the Practice Guidelines Committee of the American Society of Blood or Marrow Transplantation (ASBMT, now American Society of Transplantation and Cellular Therapy or ASTCT) in addressing ^{PK}busulfan topics of practical relevance to HCT clinicians. Using recently published studies (2008-2016), we sought to grade relevant data according to criteria set forth by the Steering Committee for Evidence-Based Reviews from ASTCT. Unfortunately, the published literature was too heterogeneous and lacked adequately powered and sufficiently controlled studies for this to be feasible. Despite this observation, the continued interest in this topic led us to develop a list of most frequently asked questions (FAQs) regarding personalized busulfan dosing. Chapter 1 highlighted the paucity of rigorous published data regarding most of the factors influencing ^{PK}busulfan, such as the initial busulfan dose, how the busulfan clearance is estimated, and the target AUC for patients receiving contemporary HCT regimens.

Table 1. Frequently Asked Questions (FAQs) from Chapter 1

FAQ1. Why does personalized busulfan dosing need to be considered during HCT?

FAQ2. Is personalized busulfan dosing always necessary?

FAQ3. When should conditioning utilize [PK]busulfan?

FAQ4. Is oral or IV busulfan preferred?

FAQ5. How should personalized busulfan dosing be achieved?

FAQ6. How is the initial busulfan dose best selected?

FAQ7. What is the optimal dosing frequency of busulfan?

FAQ8. What is the best method for predicting busulfan clearance?

FAQ9. How do other medications affect busulfan pharmacokinetics?

FAQ10. Should the initial busulfan dose be personalized based on genetic polymorphisms?

Chapter 2 sought to harmonize the worldwide HCT community to one busulfan plasma exposure unit (BPEU). Unfortunately, five different BPEU evolved over the 30 years of [PK]busulfan. This variability in busulfan BPEU is a barrier to data capture by HCT registry databases. However, the registry databases could overcome the lack of adequately powered and sufficiently controlled studies regarding the busulfan AUC to clinical outcomes relationship, thus addressing FAQ1 to 3 in Chapter 1. Using the Delphi consensus methodology of iterative surveys to numerous relevant clinical stakeholders, all respondents conceptually agreed on the ideal properties of a BPEU and to adopt a harmonized BPEU. Because the respondents were equally divided between two BPEUs, the busulfan AUC in mg×h/L was finally selected as the harmonized BPEU because it satisfied most of the survey-determined ideal properties for the harmonized BPEU and is easily understood in the clinical practice environment.

Chapter 3 created an international proficiency program for each step in [PK]busulfan. Using the unique resources within the Netherlands of the KKGT, we discovered inaccuracies in busulfan quantitation, pharmacokinetic modeling, and dose recommendations for busulfan. These rounds of proficiency testing demonstrated the need for additional educational efforts and proficiency rounds to ensure accurate [PK]busulfan. Such efforts can address FAQs 1, 2, 3, 5, and 8 in Chapter 1.

Chapter 4 summarizes a busulfan population pharmacokinetic (popPK) model built using RWD from 1,610 patients over the entire age continuum (0.1 – 66 years). This popPK model can be used to estimate the initial intravenous (IV) busulfan dose and be used to enable [MIPD]busulfan. We accounted for differences in age and body size by using post-menstrual age and normal fat mass, respectively. Addressing FAQ6 in Chapter 1, simulations from this popPK model suggested that using the initial doses predicted from this popPK model would lead to a higher proportion of initial doses achieving the therapeutic window (72%) compared with dosing recommended by the U.S. Food and Drug Administration (57%) or the European Medicines Agency (70%). Furthermore, this popPK model can modernize the estimation of busulfan clearance in an individual patient. The process of [PK]busulfan has

minimally evolved over the past 30 years, with rapid quantitation and pharmacokinetic modeling of one patient's concentration-time data to estimate their busulfan clearance to estimate their personalized busulfan dose. The creation of this model over the entire age continuum can facilitate its clinical use in all HCT patients and thus, help the field evolve to ^{MIPD}busulfan.

Part 2 describes our efforts to use novel precision medicine tools (PMT) to predict IV busulfan clearance. Although ^{PK}busulfan is feasible, the short 4-day duration of busulfan dosing makes it challenging to estimate the busulfan clearance quickly enough to personalize the dose in those HCT centers without an on-site method to quantitate concentrations. Three methods may predict IV busulfan clearance: 1. administering a pre-HCT test dose; 2. Constitutional pharmacogenomics; 3. pharmacometabonomics, using pre-dose endogenous metabolomic compound (EMC) profiling.

In **Chapter 5**, we used the candidate gene approach to evaluate whether IV busulfan clearance was associated with polymorphisms in the genes regulating the predominant metabolizing enzymes involved in busulfan conjugation, specifically glutathione S-transferase (GST) isoenzymes A1 (GSTA1) and M1 (GSTM1). In 58 HCT patients, IV busulfan clearance was not associated with *GSTA1* (p=0.21) or *GSTM1* (p=0.99). Therefore, this chapter addresses FAQ8 and supports Chapter 1 FAQ10's conclusion that pharmacogenomics-guided dosing of IV busulfan is not recommended.

We then evaluated if IV busulfan clearance could be predicted with EMCs. The EMCs reflect the influence of the patient's genotype and environmental factors (e.g., nutritional status). We adapted the concept of pharmacometabonomics, which is using pre-dose EMC profiling to predict drug response, to using pre-dose EMC profiling to predict IV busulfan clearance. This series of pharmacometabonomic chapters addressed FAQ8 in Chapter 1.

Chapter 6 used a retrospective cohort of 106 plasma samples from HCT patients to evaluate if IV busulfan clearance could be predicted with a targeted panel of 200 identified EMCs. Univariate analysis revealed that glycine, N-acetylglycine, 2-hydroxyisovaleric acid were statistically significantly associated with IV busulfan clearance at $P<0.05$ and a false discovery rate (FDR) of $q<0.1$. Using pathway enrichment analysis, the glycine, serine, and threonine metabolism pathway was statistically significantly associated with IV busulfan clearance at $P<0.05$ and $q<0.1$, and a pathway impact >0.1. Glycine is a component of glutathione, which is conjugated with busulfan via GST enzymes.

Using the same cohort of samples, **Chapter 7** describes if IV busulfan could be predicted with a global panel of 1885 unknown ions (i.e., the chemical structure of these ions was not known). First, we evaluated which ions were associated with IV busulfan clearance. Subsequently, we tried to determine which drug metabolite or EMC those ions were. Of the 21 ions associated with IV busulfan clearance tertiles ($r^2 \geq 0.3$), the identified EMCs were linoleic acid or deoxycholic acid and/or chenodeoxycholic acid. Unfortunately, we could not find any published literature evaluating the effect of linoleic acid or these bile acids upon busulfan metabolism. However, bile acids inhibit human GST activity in vitro.

In **Chapter 8**, we sought to validate the findings of Chapters 6 and 7 and identify new pathways associated with IV busulfan clearance in a new cohort of prospectively collected EMC and busulfan pharmacokinetic data. In a cohort of 138 patients, we chose a different global panel that included some of the EMCs previously identified. The chosen EMC panel was also more extensive, allowing for the exploration of additional pathways. A total of 783 EMCs were quantitated in 228 longitudinal blood samples before IV busulfan administration. Nineteen pathways were statistically associated with IV busulfan clearance, including the glycine, serine, and threonine pathway identified (Chapter 6) and the linoleic pathway (Chapter 7).

Part 3 addresses if additional biomarkers associated with relapse (the effectiveness of busulfan and the allograft) or graft versus host disease (GVHD, a significant contributor to nonrelapse mortality in allogeneic HCT patients), Busulfan is hepatically metabolized through glutathione (GSH) conjugation by GST enzymes; this process depletes hepatocyte GSH stores in murine hepatocytes in vitro. Dysregulation of GSH and accumulation of cysteine, cystathionin, and cysteinyl glycine are associated with GVHD in experimental murine models of HCT.

In **Chapter 9**, we sought to identify additional biomarkers associated with relapse (the effectiveness of busulfan) or graft versus host disease (toxicity to HCT) in a prospective cohort of 84 patients receiving ^{PK}busulfan. A total of 763 EMCs were quantitated in 230 longitudinal blood samples before, during, and shortly after IV busulfan administration. The cysteine/methionine pathway and the glycine, serine, and threonine metabolism pathway were most associated with relapse. The latter be explained by the fact that glutathione-S-transferases conjugate both busulfan and glutathione, which contains glycine as a component. The D-arginine and D-ornithine metabolism pathway and arginine and proline metabolism pathway were most associated with acute GVHD, but none were significant after correcting for FDR. Although larger studies are needed to substantiate these findings, the results show that EMCs may be used as predictive biomarkers in HCT patients.

To conclude, adopting the 'learn and confirm' paradigm to busulfan requires a multi-pronged approach with population pharmacokinetic modeling (**Chapter 4**) combined with multidisciplinary collaboration (**Chapter 1**), RWD (**Chapters 2-4**), and -omics tools to predict busulfan pharmacokinetics (**Chapters 5-8**), toxicity and effectiveness (**Chapter 9**).

Nederlandse samenvatting

De behandeling van kankerpatiënten behandeld met chemotherapie, kan gepersonaliseerd worden gebaseerd op de karakteristieken van de tumor én van de patiënt. Dit proefschrift is gericht op het personaliseren van het al lang gebruikt klassiek cytostaticum, busulfan, voor allogene hematopoëtische stamceltransplantatie ('*allogeneic hemapoietic cell transplant (HCT)*'). Al 30 jaar worden busulfan doseringen gepersonaliseerd tot een streefwaarde van de plasma oppervlakte onder de concentratie-tijd curve (''*area under the concentration-time curve (AUC)*') gebruik makend van de farmacokinetische karakteristieken van de individuele patiënt, een proces wat ook wel farmacokinetisch-gestuurd busulfan doseren (^{PK}busulfan) genoemd wordt. ^{PK}Busulfan is vereist vanwege de erg smalle therapeutische index van dit middel. De enorm lange tijd na de eerste goedkeuring door de Food and Drug Administration (FDA) (~70 jaar geleden) en het implementeren van ^{PK}Busulfan is een uniek voorbeeld van de toepassing van het '*learn - confirm*' paradigma dat wordt gebruikt in fase 1 tot 3 van geneesmiddelontwikkeling. Hier is dit toegepast voor een reeds geregistreerd geneesmiddel en uitgebreid naar een '*predict, learn, confirm – implement*' cyclus leidend tot *'Model Informed Precision Dosing (MIPD)'*.. Data gegenereerd in de zorg voor patiënten (*'Real World Data (RWD)'*) in combinatie met populatie-farmacokinetisch modelleren (Deel 1) en verschillende '*-omics'* technologieën (Deel 2 en 3) is gebruikt om de effectiviteit en toxiciteit van busulfan te verbeteren.

Deel 1 van dit proefschrift is gericht op het verder verbeteren van ^{PK}busulfan. Door de introductie van ^{PK}busulfan in het begin van de jaren 90, verminderde de afstoot van transplantaten en de levertoxiciteit in HCT-patiënten behandeld met hoge doseringen van busulfan. Door gebruik van nieuwe HCT schema's in de afgelopen 30 jaar is de relatie tussen blootstelling (gemeten als AUC) en response mogelijk veranderd. Het proces van ^{PK}busulfan is minimaal veranderd in de afgelopen 30 jaar terwijl het logisch zou zijn dat dit proces zich ontwikkeld tot ^{MIPD}busulfan. De beschikbare farmacokinetische modellen maken ^{MIPD}busulfan mogelijk waarbij een real-time Maximum-A-Posteriori-(MAP) Bayesiaanse schatting van de blootstelling van de individuele patiënt gemaakt kan worden op basis van beperkte individuele farmacokinetische data.

Hoofdstuk 1 beschreef een '*call to action*', waarin het ontbreken van bewijs voor verschillende aspecten van ^{PK}busulfan werd samengevat. Hierbij hebben onder de vlag van een multidisciplinaire en internationale groep, Practice Guidelines Committee of the American Society of Blood or Marrow Transplantation (nu American Society of Transplantation and Cellular Therapy or ASTCT), alle onderdelen van ^{PK}busulfan die praktisch relevant zijn voor HCT-clinici onderzocht. Hieruit bleek dat de literatuur naar ^{PK}busulfan heterogeen is en er onvoldoende goed opgezet en gecontroleerde onderzoek is. Desondanks, wordt ^{PK}busulfan belangrijk geacht en daarom werdeen lijst voor meest gestelde vragen ('*frequently asked questions*') over gepersonaliseerde busulfan doseringen opgesteld met vragen rond de initiële busulfan dosis, hoe de busulfan klaring bepaald dient te worden, en het AUC-target voor patiënten die gelijktijdig HCT regimen ontvangen.

Hoofdstuk 2 beschrijft een poging om internationaal naar afstemming tot één busulfan plasma blootstellings eenheid ('*busulfan plasma exposure unit (BPEU)*') te komen. Helaas zijn er in de afgelopen 30 jaar van [PK]busulfan vijf verschillende BPEU ontwikkeld. Dit vormt een serieuze belemmering in het vastleggen van data in databases en maakt vergelijking van data en literatuur ingewikkeld en foutgevoelig. Gebruikmakend van de Delphi methode, werd overeenkomst bereikt over de ideale eigenschappen van een BPEU. Overeenstemming voor een finale BPEU werd niet bereikt, aangezien alle respondent eerlijk verdeeld waren over tweeeenheden. Uiteindelijk werd de AUC van busulfan aangeduid in mgxh/L geselecteerd als de geharmoniseerde BPEU, omdat deze voldeed aan de meeste eigenschappen, bepaald uit de enquêtes.

Hoofdstuk 3 beschrijft de resultaten van een internationaal kwaliteitscontrole programma voor iedere stap in [PK]busulfan. Gebruikmakend van de unieke mogelijkheden binnen de Nederlandse sectie Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie (KKGT) ontdekten we een grote variatie in busulfan kwantificering, farmacokinetisch modelleren, en in doseringsadviezen voor busulfan. Deze rondes toonde de behoefte aan aanvullende educatieve inspanningen en kwaliteitscontroles aan om het adequaat gebruik van [PK]busulfan te garanderen.

Hoofdstuk 4 beschrijft de ontwikkeling van een busulfan populatie farmacokinetisch (popPK) model, waarbij gebruik is gemaakt van data van 1610 patiënten van 0.1 tot 66 jaar. Dit popPK model kan gebruikt worden om de initiële intraveneuze (IV) dosering van busulfan te bepalen en om het gebruik van [MIPD]busulfan mogelijk te maken. We hebben rekening gehouden met de verschillen in leeftijd en lichaamsgrootte door respectievelijk gebruik te maken van de post menstruele leeftijd en de normale vetmassa. Simulaties vanuit dit popPK model suggereren dat het gebruik van initiële doses voorspeld op basis van dit model, ertoe zou leiden dat een groter deel van de initiële doses het therapeutische venster zou bereiken (72%) in vergelijking met de dosering aanbevolen door de Amerikaanse Food and Drug Administration (57%) of het Europees Geneesmiddelenbureau (European Medicines Agency (EMA)) (70%). Daarnaast kan dit popPK-model de schatting van de busulfan klaring bij een individuele patiënt verbeteren. De ontwikkeling van dit model over het gehele leeftijdscontinuüm kan het klinische gebruik ervan bij alle HCT-patiënten vergemakkelijken en zo het veld helpen ontwikkelen naar [MIPD]busulfan.

Deel 2 beschrijft nieuwe methodes om de klaring van IV busulfan te voorspellen. Hoewel [PK]busulfan haalbaar is in de meeste centra, blijft dit een logistieke uitdaging vooral voor centra waar busulfan niet lokaal bepaald kan worden. Het vooraf voorspellen van busulfan klaring zou daarvoor een oplossing kunnen zijn. Hiervoor zijn drie methoden beschikbaar: 1. het toedienen van een pre-HCT testdosis; 2. Doseren op basis van genotype van relevante metaboliserende enzymen; 3. '*metabolomics*' , gebruikmakend van pre-dosis endogene metabolomische verbindingen ('*Endogenous Metabolomic Compound (EMC)*') profilering.

In **Hoofdstuk 5** hebben we onderzocht of de IV-busulfan klaring geassocieerd was met polymorfismen in de genen die de belangrijkste metaboliserende enzymen reguleren

die betrokken zijn bij busulfan conjugatie, met name glutathion S-transferase (GST) isoenzymen A1 (GSTA1) en M1 (GSTM1). Bij 58 HCT-patiënten was de IV-busulfan klaring niet geassocieerd met GSTA1 (p=0,21) of GSTM1 (p=0,99).

Hoofdstuk 6 gebruikte een retrospectieve cohort van 106 HCT-patiënten om te evalueren of de IV-busulfan klaring kon worden voorspeld door middel van een gericht panel van 200 EMCs. Een univariate analyse liet zien dat glycine, N-acetylglycine en 2-hydroxyisovaleriaanzuur statistisch significant geassocieerd waren met de IV-busulfan klaring met een P<0,05 en een false discovery rate (FDR) van q<0,1. Met behulp '*pathway enrichment analysis*'werd gezien dat serine en threonine statistisch significant geassocieerd met IV-busulfan klaring met een P<0,05 en q<0,1, en een impact op de route van >0,1. Glycine is een bestanddeel van glutathion, dat via GST-enzymen met busulfan wordt geconjugeerd.

Gebruikmakend van gegevens uit hetzelfde cohort, werd in **Hoofdstuk 7** beschreven of IV busulfan voorspeld kan worden met een wereldwijd panel van 1885 niet geïdentificeerde verbindingen. Eerst evalueerden we welke verbindingen geassocieerd waren met IV-busulfan klaring. Vervolgens probeerden we te bepalen welke geneesmiddelmetaboliet of EMC deze verbindingen waren. Van de 21 verbindingen die geassocieerd waren met IV-busulfan klaring tertielen (r2 ≥ 0.3), waren de geïdentificeerde EMC's linolzuur of deoxycholzuur en/of chenodeoxycholzuur. Helaas konden wij geen gepubliceerde literatuur vinden waarin het effect van linolzuur of deze galzuren op het metabolisme van busulfan werd geëvalueerd. Wel remmen galzuren de menselijke GST-activiteit in vitro.

In **Hoofdstuk 8** hebben we getracht de bevindingen van de Hoofdstukken 6 en 7 te valideren en nieuwe routes te identificeren die geassocieerd zijn met IV-busulfan klaring in een nieuw cohort van prospectief verzamelde EMC en farmacokinetische gegevens van busulfan. In een cohort van 138 patiënten hebben we een ander wereldwijd panel gekozen dat enkele van de eerder geïdentificeerde EMC's omvatte. Het gekozen EMC-panel was ook uitgebreider, waardoor aanvullende routes konden worden onderzocht. In totaal werden 783 EMC's gekwantificeerd in 228 longitudinaal verzamelde bloedmonsters vóór IV-busulfan toediening. Inclusie van de 37 statistisch significante EMC's in een enkel regressiemodel verklaarde 88% van de variabiliteit in IV-busulfan klaring (R^2 =0,88). Negentien routes waren statistisch geassocieerd met IV-busulfan klaring, inclusief de geïdentificeerde glycine, serine, en threonine route (Hoofdstuk 6) en de galzuur route (Hoofdstuk 7).

Deel 3 beschrijft of aanvullende biomarkers geassocieerd zijn met terugkeer van de onderliggende ziekte of graft-versus-host-ziekte ('*graft versus host disease (GVHD)*'. Busulfan wordt hepatisch gemetaboliseerd door glutathion (GSH) conjugatie door GST-enzymen; dit proces put de hepatocytaire GSH-voorraden in hepatocyten in vitro uit. Ontregeling van GSH en accumulatie van cysteïne, cystathionine, en cysteinylglycine zijn geassocieerd met GVHD in experimentele HCT-muismodellen.

In **Hoofdstuk 9** probeerden we aanvullende biomarkers te identificeren die geassocieerd zijn met terugkeer van onderliggende ziekete of graft-versus-host-ziekte (toxiciteit voor

HCT) in een prospectief cohort van 84 patiënten die [PK]busulfan kregen. Een totaal van 763 EMC's werden gekwantificeerd in 230 longitudinale bloedmonsters voor, tijdens en kort na IV-busulfan toediening. De cysteïne/methionine-route en de glycine-, serine- en threonine-metabole route waren het meest geassocieerd met terugval. Dit laatste wordt verklaard door het feit dat glutathion-S-transferasen zowel busulfan als glutathion conjugeren, wat glycine als component bevat. De metabolismeroute van D-arginine en D-ornithine en de metabole route van arginine en proline waren het meest geassocieerd met acute GVHD, maar geen enkele was significant na correctie voor FDR. Hoewel grotere studies nodig zijn om deze bevindingen te onderbouwen, tonen de resultaten aan dat EMC's mogelijk kunnen worden gebruikt als voorspellende biomarkers bij HCT-patiënten.

Concluderend, het toepassen van het '*learn and confirm*' paradigma voor busulfan vereist een meervoudige benadering met populatie farmacokinetisch modelleren (hoofdstuk 4) gecombineerd met multidisciplinaire samenwerking (hoofdstuk 1), RWD (hoofdstukken 2-4) en '*-omics*' technologieën om farmacokinetiek (hoofdstukken 5-8), toxiciteit en effectiviteit (hoofdstuk 9) van busulfan te voorspellen.

APPENDIX

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List of publications

PEER-REVIEWED ARTICLES

1. Palmer J*, **McCune JS***, Perales M-A, Marks D, Bubalo J, Mohty M, Wingard JR, Paci A, Hassan M, Bredeson C, Pidala J, Shah N, Shaughnessy P, Majhail N, Schriber J, Savani BN, Carpenter PA. Personalizing Busulfan-based Conditioning: Considerations from the American Society for Blood and Marrow Transplantation Practice Guidelines Committee. *shared first authorship. Biol Blood Marrow Transplant. 2016 Nov;22(11):1915-1925. PMID: 27481448.

2. **McCune JS**, Quinones CM, Ritchie J, Carpenter PA, van Maarsveen EM, Yeh RF, Anasetti C, Boelens JJ, Hamerschlak N, Hassan M, Kang HJ, Kanda Y, Paci A, Perales MA, Shaw PJ, Seewaldt VL, Savani BN, Hsieh A, Poon B, Mohty M, Pulsipher MA, Pasquini M, Dupuis LL. Harmonization of Busulfan Plasma Exposure Unit (BPEU): A Community-Initiated Consensus Statement. Biol Blood Marrow Transplant, 2019 Sep;25(9):1890-1897. PMID: 31136799.

3. **McCune JS,** Punt AM, Yeh RF, Dupuis LL, Kweekel DM, Franssen EJF, Ritchie JC, van Maarseveen E, Huitema ADR. Quality Control of Busulfan Plasma Quantitation, Modeling, and Dosing: An Interlaboratory Proficiency Testing Program. Ther Drug Monit. 2021 Oct 1;43(5):657-663. PMID: 33675302.

4. **McCune JS**, Bemer MJ, Baker KS, Gamis A, Holford NHG. Busulfan in infant to adult hematopoietic cell transplant recipients: A population pharmacokinetic model for initial and Bayesian dose personalization. Clinical Cancer Research. 2014 Feb 1;20(3):754-63. doi: 10.1158/1078-0432.CCR-13-1960. Epub 2013 Nov 11. PMID: 24218510.

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7. Lin YS, Kerr SJ, Randolph T, Shireman L, Senn T, **McCune JS**. Prediction of intravenous busulfan clearance by endogenous plasma biomarkers using global pharmacometabolomics. Metabolomics. 2016 Oct;12(10). pii: 161. doi: 10.1007/s11306-016-1106-6. PMID: 28827982.

8. **McCune JS**, McKiernan JS, van Maarseveen E, Huitema ADR, Randolph TW, Deeg HJ, Nakamura R, Baker KS. Prediction of Acute Graft versus Host Disease and Relapse by Endogenous Metabolomic Compounds in Patients Receiving Personalized Busulfan-Based Conditioning. J Proteome Res. 2021 Jan 1;20(1):684-694. PMID: 33064008.

Dankwoord

Ik ben ontzettend dankbaar dat ik mogelijkheid heb gekregen mijn promotie te doen in een Nederlands traject aan de universiteit van Utrecht. Ik realiseer mij dat het dankwoord normaliter in het Nederlands geschreven is. Echter, omdat de meeste genoemden in deze sectie geen Nederlands spreken of begrijpen, zal ik mijn dankwoord in het Engels schrijven.

Almost thirty years ago, I had my first opportunity to take care of patients with cancer. That rotation led to my first experience in cancer research, which inspired me to be where I am today. I will never forget the kind woman who talked to me about her experience with ovarian cancer and (then) experimental paclitaxel. That conversation one afternoon inspired me to become a clinical pharmacist. With each subsequent cancer patient, I was inspired to improve my research skills in hopes of better helping them and each subsequent patient. These chapters were made possible by the numerous cancer patients who donated their precious time and gave samples willingly in the hopes that what we learned from them could help tomorrow's patients. Each one of them has inspired me. I am also grateful to their family members for supporting their loved ones. Furthermore, the health care providers caring for these patients and the multidisciplinary research staff supporting these studies were essential for this work. Thank you all.

Turning toward this thesis, I am eternally grateful to Prof. Dr. Alwin D.R. Huitema. His dedication to using model-informed precision dosing to improve patient care has always inspired me. I cannot thank him enough for all his time and thoughtful comments, especially to a non-traditional student like myself. He is a brilliant mentor who challenges his team members to do better. I am most thankful that he took the time to 'adopt' me as one of his Ph.D. students after the tragic passing of Dr. Erik van Maarseveen. Erik's contribution to this thesis cannot be overstated. He was incredibly supportive despite his health challenges and my delays resulting from me chairing a National Institute of Health (NIH) study section. Furthermore, Erik was phenomenally enthusiastic about international collaborations focusing on applying model-informed precision dosing to patient care.

I would also like to thank my committee members for their time and thoughtfulness: Prof. Dr. Jos Beijnen, Dr. Lidwien Hanff, Dr. Stefan Nierkens, Prof. Dr. Donald Mager, Prof. Dr. Josef Vormoor. I am also grateful to the NONMEM journal club for their willingness to offer suggestions, share code, and genuine kindness.

And to the faculty and staff at Utrecht University, each of whom was direct, professional, and kind, my gratitude. I appreciate the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) and its support of Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology (KKGT). The KKGT's infrastructure is essential for ensuring accurate and precise drug quantitation, pharmacokinetic modeling, and dose recommendations for any drug that can be dosed based on concentration-time data.

Words cannot sufficiently express my gratitude to Prof. Dr. Danny D. Shen. Over many years, Danny always shared his extensive knowledge about pharmacokinetics, building an NIH-funded research program, and being a thoughtful and reliable colleague. Despite having substantive administrative responsibilities, he always listened and provided thoughtful advice to me and all the members of the University of Washington/Fred Hutchinson Cancer Research Center Pharmacokinetics Laboratory (PK Lab). The PK Lab is a phenomenal team that I was honored to co-direct with Danny. In addition, I am indebted to its alumni, especially Linda Risler. Linda is a pragmatic scientist who always ensured that our PK Lab results were as reproducible as possible within the constraints of the chemically reactive analytes. I remember well my first day entering the PK Lab and being astonished at how organized and efficient it was. I quickly learned that this infrastructure was due to Linda and her efforts. I am honored that she remains a long-time and dear friend now that she is retired.

I am also grateful for the mentorship and collaborations of Prof. Dr. Kenneth Thummel, Prof Dr. Brenda Sandmaier, and Prof. Dr. K. Scott Baker. At various times throughout my career, each provided sage advice that propelled my career and research program forward. I would also like to thank my colleagues who were non-traditional students that inspired me to pursue my PhD: E. Beth Devine, L. Lee Dupuis, Cara L. McDermott, Sandi L. Navarro, Paul V. O'Donnell, and Karin Steere. Finally, I am also grateful to Leslie Sandberg and Virginia (Ginny) Schmith, as they provided a supportive ear while I was pursuing my PhD, working, and having a family. Thank you.

To my husband, Kurt Round, for always understanding that I am eternally curious and love learning. And to my children – Krista and Andrew. You each supported me and my passion for learning in your own way without me asking. I love you 'right up to the moon and back'. I am proud of you both for who you are. You both approach life with curiosity to learn, kindness for our world, and compassion for those in it. I am so honored to be your Mom.

Curriculum Vitae

Jeannine McCune was born on August 18th 1970 in Pittsburgh, Pennsylvania, United States of America. In 1993 she graduated *Magna Cum Laude* with a Bachelor's Degree in Pharmacy from the University of Pittsburgh. Subsequently, she obtained a Doctor of Pharmacy (PharmD) degree at the University of North Carolina at Chapel Hill. After graduating in 1995, she completed a residency in hematology/oncology pharmacotherapy and a research fellowship in the drug metabolism and pharmacokinetics of anti-cancer drugs. In 1998, she joined the faculty at the University of Washington (UW) School of Pharmacy and Fred Hutchinson Cancer Research Center (Fred Hutch), both in Seattle, Washington. She was sequentially promoted to tenured Associate Professor and Professor at the UW and Associate Member and Member at the Fred Hutch. While in Seattle, she also served as the Clinical Director for Seattle's Busulfan Pharmacokinetic Laboratory for 7 years before completing a sabbatical in pharmacometrics at Prof. Dr. Donald E. Mager's laboratory at the University of Buffalo. Supported by the mentorship of Prof. Dr. Danny D. Shen, her NIH-funded research program focuses on model-informed precision dosing of anti-cancer agents and immunosuppressive drugs. Dr. Shen and she also co-directed the UW/Fred Hutch Research Pharmacokinetics Laboratory, which created novel liquid chromatography – mass spectrometry assays and combined them with population pharmacokinetic models to break new ground in model-informed precision dosing. Since 2017, she served as a Professor in Hematologic Malignancies Translational Medicine at the Beckman Research Institute of the City of Hope in Los Angeles, California.