# PHARMACOKINETIC, PHARMACODYNAMIC AND PHARMACOGENETIC FACTORS ASSOCIATED WITH BUSULFAN CLINICAL PHARMACOLOGY

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*For Bharat Bansilal Trivedi*

I hereby declare that this submission is my own work and to the best of my knowledge it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any degree or diploma at The University of Sydney or any other institution, except where due acknowledgement is made in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in this project's design and conception or in style, presentation and linguistic expression is acknowledged.

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Parth J. Upadhyay

January 2019

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# ACKNOWLEDGEMENTS

**“ If you want to walk fast, walk alone, but if you want to go far, walk together”**

**– Ratan Tata**

Just as the journey from milk to butter cannot be described in a single step, a PhD thesis too, requires the full cream milk of rich ideas, a culture of experience from mentors and a rigorous churning through experiments, all in the hopes of an outcome forms the basis (or for the lack of a better pun, bread and butter) of future practice.

Perhaps a milk-based allegory does form a suitable start to what might seem like a cheesy acknowledgement section, but like all journeys walked in life, none would be complete without the interactions and influences of the people who walk the journey with you. The pursuit of an academic career is a test of resilience not only for the candidate but also for their family, friends and colleagues all of whom form the safety net for every failed leap and crash in a PhD- to not acknowledge them would a crime. I will divide my acknowledgement in two sections, the cultures- who have supported this thesis, and the vessels- who have protected me from spilling into disaster.

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**"You have one spark of madness… you must never lose it"**

**– Robin Williams**

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With God I start and in God I end.

# PRESENTATIONS ARISING FROM THIS THESIS

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***Population Pharmacokinetics of Busulfan in Patients Receiving Bone Marrow and Haematopoietic Stem Cell transplants at the Children's Hospital, Westmead.***

Parth J Upadhyay[1], Janna Duong[1], Christa Nath[2], Peter Shaw[2] and Alan V Boddy[1]. [1]University of Sydney, NSW, Australia; [2]The Children's Hospital at Westmead, NSW, Australia

2016 SCRN Symposium- Sydney, Australia. Poster Presentation

***Quantifying n,n-dimethylacetamide In Patient Plasma Samples Using LCMS***

Parth J Upadhyay[1], Janna Duong[1], Christa Nath[2], Peter Shaw[2] and Alan V Boddy[1]. [1]University of Sydney, NSW, Australia; [2]The Children's Hospital at Westmead, NSW, Australia

2017 KCA Post-Graduate Symposium- Sydney, Australia. Poster Presentation

***The Relationship Between Busulfan AUC and the Incidence of Sinusoidal Obstruction Syndrome in Haematopoietic Stem Cell Transplants.***

Parth J Upadhyay[1], Janna Duong[1], Christa Nath[2], Peter Shaw[2] and Alan V Boddy[1]. [1]University of Sydney, NSW, Australia; [2]The Children's Hospital at Westmead, NSW, Australia

2017 APSA-ASCEPT- Brisbane, Australia. Oral Presentation

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***Analysing Targeted Busulfan Therapy and the Incidence of Sinusoidal Obstruction Syndrome in Paediatric Haematopoietic Stem Cell Transplantations***

Parth J Upadhyay[1], Janna Duong[1], Christa Nath[2], Peter Shaw[2] and Alan V Boddy[1]. [1]University of Sydney, NSW, Australia; [2]The Children's Hospital at Westmead, NSW, Australia

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Parth J Upadhyay[1], Janna Duong[1], Christa Nath[2], Peter Shaw[2], David Gottlieb[3], Stephen Larsen[4], John Gibson[4], Andrew Grigg[5], Ashish Bajel[6], Eric Wong[6], David Ritchie[6], Simon Harrison[7], Philip Campbell[8] and Alan V Boddy[1].

[1] University of Sydney, NSW, Australia; [2]The Children's Hospital at Westmead, NSW, Australia, [3]Westmead Hospital, [4]Royal Prince Alfred Hospital, [5]Austin Hospital, [6]Royal Melbourne Hospital, [7]Peter MaCallum Cancer Care Centre, [8]Geelong Hospital

# ABSTRACT

Busulfan is a bifunctional alkylating agent used in combination with other chemotherapeutics for the ablation of dysfunctional bone marrow prior to haematopoietic stem cell transplantation. Busulfan use is complicated by a large inter-individual and inter-occasion pharmacokinetic variability. Furthermore, the exposure of busulfan (estimated as a cumulative area under the curve) has been associated with dose-limiting toxicities such as sinusoidal obstruction syndrome, which can lead to multi-organ failure and death if left untreated.

The research presented in this thesis retrospectively explores SOS incidence in 337 HSCT patients over an eleven-year study period (2006 – 2017) across seven institutions in Australia. Out of 344 busulfan-based conditioning occasions, there were 64 cases of SOS reported. A population pharmacokinetic analysis was developed to explore the pharmacokinetic variability present. Association studies were conducted post hoc and non-parametric and Cox proportional hazards models were developed to better understand the development of SOS post-busulfan use. Lastly, an exploration of the influence of patient genotypes on clinical outcome was conducted.

A total of 3241 observations informed the selection of a one-compartment pharmacokinetic model. Inter-occasion and inter-individual variability were characterized for both clearance and volume. Adjusted-ideal bodyweight (kg) and a sigmoidal $E_{max}$, maturation function were incorporated as covariates. A post hoc analysis into concomitant medications found a significant decrease in busulfan CL for patients co-administered metronidazole (difference in median $CL_{NORM}$ = 0.05 L/h/kg, n = 17, $P < 0.0001$), and a marginal increase with dexamethasone (difference in median $CL_{NORM}$ = 0.01 L/h/kg, n = 49, $P < 0.01$).

Overall, cAUC was not significantly associated with SOS, although median $C_{max}$ observed on Day 1 of busulfan therapy was higher in patients with SOS (2 µg/mL vs. 2.61 µg/mL, $P < 3.7 \times 10^{-5}$). A multivariate Cox proportional hazard model characterized the hazard of developing SOS as a combination of risk factors: low AIBW, low pre-transplant albumin, younger age and high $C_{max}$.

A linear regression analysis on an ADME panel of 67 SNPs in a sub-cohort (223 patients) found found no significant effect of SNPs on busulfan clearance. A logistic regression analysis of the same number of patients over the panel of enzyme-related SNPs failed to report any significant genetic associations with SOS. A separate analysis of 189 patients for polymorphisms of GSTA1 (an enzyme previously associated with busulfan metabolism) showed a 14% – 18% lower clearance (difference in mean $CL_{NORM}$ = 0.03 L/h/kg, $P$ = 0.004 and 0.04 L/h/kg, $P$ = 0.0003) in patients heterozygous and homozygous for the *B allele, respectively. A further categorisation of GSTA1 polymorphisms into activity-based groups was of no additional benefit.

In conclusion, the high degree of inter-occasion and inter-individual variability in busulfan pharmacokinetics was reconfirmed through this thesis. Patient specific factors such as GSTA1 polymorphisms affected pharmacokinetic variability, but cAUC was not associated with risk of SOS.

# ABBREVIATIONS

| | |
|---|---|
| **A** | Adenosine |
| **ABC** | ATP-Binding Cassette |
| **ADME** | Absorption-Distribution-Metabolism-Elimination |
| **AGE** | Age (y) |
| **AGRF** | Australian Genomic Research Foundation |
| **AIBW** | Adjusted Ideal Bodyweight (kg) |
| **AIC** | Akaike Information Criterion |
| **ALL** | Acute Lymphoblastic Leukaemia |
| **AML** | Acute Myeloid Leukaemia |
| **AUC** | Area Under the Curve |
| **AUS** | Austin Health |
| **AYA** | Adolescents and Young Adults |
| **BMT** | Bone Marrow Transplantation |
| **BSA** | Du Bois calculated Body Surface Area (m2) |
| **Bu-Cy** | Busulfan followed by cyclophosphamide |
| **Bu-Mel** | Busulfan followed by melphalan |
| **Busulfan** | 1,4-butanediol-dimethylsulfonate |
| **cAUC** | cumulative Area Under the Curve |
| **CHW** | Children's Hospital Westmead |
| **CL** | Clearance (L/h) |
| **$Cl_i$** | Individual Clearance |
| **$CL_{NORM}$** | Clearance normalised to bodyweight (L/h/kg) |
| **$Cl_{pop}$** | Population Value for Clearance |
| **$C_{max}$** | Maximum concentration post infusion |
| **$C_{min}$** | Minimum concentration post infusion |
| **CML** | Chronic Myeloid Leukaemia |
| **CSP** | certified service provider |
| **$C_{ss}$** | Concentration at steady state |
| **CWRES** | Conditionally Weighted Residuals |
| **Cy-Bu** | Cyclophosphamide followed by Busulfan |

| | |
|---|---|
| **CYP** | Cytochrome P450 enzyme |
| **Cy-TBI** | Cyclophosphamide followed by total body irradiation |
| **DNA** | deoxyriboneucleic acid |
| **dOFV** | difference in Objective Function Value |
| **EDTA** | Ethylene-diamine-tetraacetic acid |
| **EMA** | European Medicines Agency |
| **$E_{MAX}$** | Maximal Effect |
| **F** | bioavailability |
| **f(t)** | Probability Density Function |
| **FDA** | Food and Drug Administration |
| **Flu-Bu** | Fludarabine concomitantly administered with busulfan |
| **$F_{mat}$** | Factor for Maturation |
| **$F_{size}$CL** | Factor for Bodysize on Clearance |
| **$F_{size}$V** | Factor for Bodysize on Volume |
| **G** | Guanosine |
| **GEE** | Geelong Hospital |
| **GST** | Glutathione-S-transferase |
| **GSTA1** | Glutathione-S-transferase-alpha 1 |
| **GSTM1** | Glutathione-S-transferase-mu 1 |
| **GSTP1** | Glutathione-S-transferase-pi 1 |
| **GSTT1** | Glutathione-S-transferase-tau 1 |
| **h(t)** | Base Hazard Function |
| **H(t)** | Cumulative Hazard Function |
| **HGT** | Height (cm) |
| **HILL** | The gradient of maturation |
| **HL** | Hodgkin's Lymphoma |
| **HLA** | Human Leukocyte Antigen |
| **HLAMATCH** | HLA-matched transplantation |
| **HOSP** | Hospital |
| **HSC** | Haematopoietic Stem Cells |
| **HSCT** | Haematopoietic Stem Cell Transplantation |
| **HWE** | Hardy-Weinberg Equilibrium |

| | |
|---|---|
| **IIV** | Inter-Individual Variability |
| **$IIV_{CL}$** | Inter-Individual Variability on Clearance |
| **$IIV_{V}$** | Inter-Individual Variability on Volume |
| **IOV** | Inter-Occasion Variability |
| **$IOV_{CL}$** | Inter-occasion Variability on Clearance |
| **$IOV_{V}$** | Inter-occasion Variability on Volume |
| **IPRED** | Individual Predicted Concentration |
| **IV** | Intravenous |
| **LLOQ** | Lower limit of Quantitation |
| **Mel-Bu** | melphalan followed by Busulfan |
| **Mel-TBI** | melphalan followed by total body irradiation |
| **MOF** | Multi-Organ Failure |
| **MTFR** | methylene-tetrahydrofolate-reductase |
| **NAT** | N-acetyltransferase |
| **NATA** | National Association of Testing Authorities |
| **NCA** | Non-Compartmental Analysis |
| **NHL** | Non-Hodgkin's Lymphoma |
| **NLME** | Non-Linear Mixed Effects |
| **OFV** | Objective Function Value |
| **OS** | Overall Survival |
| **PFS** | Progression Free Survival |
| **PK** | Pharmacokinetic |
| **PMA** | Post Menstruation Age |
| **PMCC** | Peter MacCallum Cancer Centre |
| **PNA** | Post Natal Age |
| **pop-PK** | population-pharmacokinetic |
| **PRED** | Population Predicted Concentration |
| **PSN** | Perl Speaks NONMEM |
| **RMH** | Royal Melbourne Hospital |
| **RPA** | Royal Prince Alfred Hospital |
| **RSE** | Residual Standard Errors |
| **S(t)** | Survival Function |

| | |
|---|---|
| **SCM** | Step-wise Covariate Modeling |
| **SNP** | Single Nucleotide Polymorphism |
| **SOS** | Sinusoidal Obstruction Syndrome |
| **TBI** | Total Body Irradiation |
| **TDM** | Therapeutic Drug Monitoring |
| **Thymo** | Thymoglobulin |
| **TM50** | Age at which maturation of a function is half that of an Adult |
| **TXTYPE** | Transplant type (Autologous, Allogeneic or Syngeneic) |
| **ULOQ** | Upper Limit of Quantitation |
| **V** | Volume of Distribution (L) |
| **$V_i$** | Individual Volume |
| **VOD** | Veno-Occlusive Disease (synonymous with Sinusoidal Obstruction Syndrome) |
| **VPC** | Visual Predictive Check |
| **$V_{pop}$** | Population Value for Volume |
| **WGT** | weight (kg) |
| **WMD** | Westmead Adults Hospital |
| **WRES** | weighted residuals |

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# Chapter 1.

## BUSULFAN USE IN HAEMATOPOIETIC STEM CELL TRANSPLANTATION

## 1.1 INTRODUCTION

Busulfan or 1,4-butanediol-dimethylsulfonate[1] is a bifunctional alkylating agent[2] of the alkylsulfonate class of drugs.[3] The main mechanism of action of busulfan involves the release of methanesulfonate groups that produce carbonium ions, which can alkylate DNA by forming intrastrand cross-links at 5'-GA-3' and 5'-GG-3', halt DNA replication and induce cellular senescence.[4-6] There have also been studies on busulfan activity in forming DNA-protein crosslinks.[7] Busulfan appears to target haematopoietic stem cells (HSC) more selectively than mature lymphocytes, possibly due to a greater action during the $G_0/G_1$ phase of the cell cycle.[3] In the body, busulfan undergoes extensive phase II metabolism through glutathione conjugation along with a range of other minor pathways producing sulfolane, tetrahydrothiophene-1-oxide and N-acetylcysteine-sulfonium.

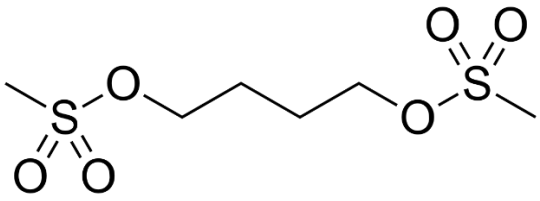

***Figure 1-1*** *Chemical structure of busulfan*

## 1.2 BUSULFAN THERAPY

Haematopoietic stem cell transplants (HSCT), including bone marrow transplants (BMT), are used as a curative treatment option in several malignant, haematological and immunological conditions and inherited diseases.[3 8 9] This form of treatment is used to deplete bone-marrow using high-dose chemotherapy with or without total body irradiation, followed by a rescue with HSC from the patient or from donor(s) to restore immune functionality.[3 8] Several chemotherapeutic agents in multiple dose forms and regimens

have been successfully utilised to deplete stem cells, thereby conditioning a patient prior to transplant. Busulfan is an integral part of many of these conditioning regimens and has become a common replacement for total-body irradiation since the first documented use in 1953 for chronic myeloid leukaemia (CML).[10 11]

Today, busulfan is primarily administered as an intravenous (IV) infusion in combination with other cytotoxic agents such as cyclophosphamide, melphalan or fludarabine. The pharmacokinetics (PK) of busulfan are highly variable and for myeloablation, busulfan also has a narrow therapeutic window associated with dose-limiting toxicities such as sinusoidal-obstruction syndrome (SOS), seizures and gonadal toxicities.[12 13] Therefore, busulfan administration is accompanied with toxicity prophylaxis through concomitant phenytoin, levetiracetam or benzodiazepines for seizures,[14 15] and ursodeoxycholic acid and, more recently, defibrotide for SOS.[16 17] Considerable effort has been made to personalise busulfan use for optimum outcomes in transplant patients. However, despite the implementation of several interventions, such as therapeutic drug monitoring (TDM), PK-guided dosing, and toxicity prophylaxis, optimal busulfan therapy continues to be a challenge. Furthermore, there is still no consensus amongst the transplant community with regards to either the ideal busulfan dosing regimen, or the most appropriate target ranges for PK-guided dosing and TDM.

This chapter explores the developments and present-day challenges of IV busulfan therapy, nearly twenty years after FDA approval, including the persistent issues of PK variability and SOS. Busulfan TDM is performed routinely in Australian patients, although the target ranges can vary according to conditioning regimen, diagnosis and tolerability. The appropriateness of a uniform target for PK monitoring has recently been debated and, in this review, the changing definition of the PK target range is summarised. A brief history

of transplantation and the evolving use of busulfan in HSCTs provides a suitable starting point.

### 1.3 Brief History on the Development of Busulfan-Based Transplant Regimens

The story of transplantation begins from the first documented, prolonged engraftment of allogeneic transplantations that resulted from an opportunistic rescue of six physicists exposed to near lethal doses of radiation.[18] This serendipitous discovery gave rise to bone marrow transplantation, where high doses of radiation were used to annihilate cancer cells from the body, followed by HSC rescue from either the patient or a donor to restore immune function.[19] [20] For the first time, a curative treatment option for cancers like Hodgkin's lymphoma and lymphosarcomatosis was offered through BMTs.[20]

Total body irradiation (TBI), although effective at myeloablation (the process of depleting bone-marrow), was severely toxic to patients and not readily available at all institutions.[21] [22] Cyclophosphamide, an alkylating agent, was first introduced to the BMT regimen in 1971 as an alternative to TBI.[23] While cyclophosphamide had a safer toxicity profile than TBI, relapse was still a major concern. A combination therapy of cyclophosphamide and TBI (Cy-TBI) was later shown to successfully reduce the rate of relapse.[24]

Busulfan was initially introduced as a last-resort treatment option for resistant patients with CML due to its myelosupressive properties.[25] With longer remission times and markedly reduced toxicities, busulfan became a preferred replacement for nitrogen mustards, arsenic and urethane in the treatment of myeloproliferative diseases.[26] [27] In earlier trials, busulfan therapy was generally limited to lower doses of between 2 mg and 6 mg per day to avoid haematological toxicites such as neutropaenia and thrombocytopaenia, and also

pulmonary fibrosis, popularly known as busulfan lung.[26] [28] [29] Depending on disease severity, doses of up to 16 mg daily were also used in other myeloproliferative conditions.[30]

The incorporation of busulfan in preparative regimens for transplantation was first suggested by George Santos in the 1970s as an alternative to TBI. Services for TBI were not readily available in all institutions prompting research into alternative methods of myeloablation.[21] An *in vivo* exploration of busulfan activity on haematopoietic organs in rats demonstrated effective myeloablation without affecting lymphoid cells. Further investigation by Santos in animal models confirmed these findings[31] and later successfully demonstrated recovery, after conducting a syngeneic bone marrow infusion in rats following a lethal dose of busulfan.[32]

The use of busulfan in transplants however was limited to syngeneic bone marrow infusions, due to the lack of immunosuppressive activity. Cyclophosphamide is a potent immunosuppressive, and the combination with busulfan was expected to synergise the activity of the preparative regimen in ablating both HSC and mature lymphocytes. This combination demonstrated rapid engraftment of allogeneic stem cells in rats, which was not observed with cyclophosphamide alone,[33] thus proving the concept. The first use of busulfan in humans as a combination with cyclophosphamide (busulfan followed by cyclophosphamide, Bu-Cy) was reported by Santos *et al*. for the treatment of acute leukaemia.[34] Busulfan, at high doses (8 and 20 mg/kg over four to eight days), was expected to increase the activity of cyclophosphamide in ablating both HSC and mature lymphocytes, whilst being a safer alternative to total body irradiation.[35] The Bu-Cy regimen resulted in accelerated engraftment and a markedly-reduced relapse rate and was as effective as Cy-TBI.[35] [36]

Whilst melphalan and busulfan were used independently at lower doses in CML in the 1950s,[10 25 37] the use of a combined busulfan and melphalan (busulfan followed by melphalan, Bu-Mel) conditioning regimen was first reported for an autologous transplant in 1987.[38] In subsequent studies, Bu-Mel was also used successfully in BMT for relapsed non-Hodgkin's Lymphoma in children[39] and also for autologous transplants in high-risk neuroblastoma.[40] The combination of Bu-Cy followed by melphalan was also tried successfully for haematological malignancies in autologous[41] and allogeneic transplants in patients with no prior exposure to melphalan.[42 43]

The introduction of fludarabine, a purine analogue, in the 1990s revolutionised the field of HSCTs, as it allowed for successful engraftment of HSC through immunomodulation, without the need for complete myeloablation. Since the first documented use in Fanconi's anaemia,[44] fludarabine paved the way for new types of transplant regimens that would not require the same high doses as myeloablative conditioning regimens. The resultant low(er)-dose conditioning regimens, which unlike myeloablative conditioning regimens induced reversible cytopaenia, were referred to as non-myeloablative and reduced-intensity conditioning regimens, although definitions have further evolved as specified in other sections of this chapter. Reduced intensity conditioning regimens opened transplantation for malignant and immune-related conditions to a range of patients, such as the elderly, who were not considered fit for myeloablative conditioning regimens.[45-47]

Busulfan is most commonly used in combination with fludarabine, cyclophosphamide or melphalan, although there are other notable combinations used routinely in practice. The addition of thiotepa to busulfan-fludarabine or busulfan-cyclophosphamide improves relapse rates and has greater anti-leukaemic effect, at the cost of higher non-relapse mortality and risk of infection in a range of haematological malignancies such as acute

myeloid leukaemia (AML) and non-Hodgkin's lymphoma (NHL).[48-52] The combination of busulfan and etoposide, a topoisomerase II poison, has also been used recently for autologous transplantations in AML.[53]

The use of busulfan has therefore evolved greatly over the past four decades into a range of myeloablative and reduced intensity conditioning regimens for a myriad of diagnoses and indications. However, therapy is complicated by various dosing and monitoring challenges and, therefore, busulfan has been studied extensively over many years. The resulting recommendations have been adopted inconsistently at various institutions, as highlighted in a large-retrospective analysis of busulfan TDM practices by Bartelink *et al*. Therapeutic concentrations monitored and achieved at the different institutions varied significantly and that study indicated the need for a harmonisation of global practices to ensure optimal outcomes for all patients.[54]

### 1.4 BUSULFAN - CURRENT ISSUES

In the past 10 years, the IV formulation of busulfan has largely replaced oral administration, following evidence of improved survival, lower inter-individual pharmacokinetic variability and lower incidence of SOS. [55] [56] Neither pharmacokinetic variability nor SOS have been completely resolved by the use of IV busulfan, and institutions have sought to develop further improvements in busulfan therapy, but without any global consistency in approach, as described below:

### 1.4.1 Busulfan dosing - Variability in Practice

Variation in practice begins from the starting point of determining the first busulfan dose - either through a test dose, nomogram or dose-calculator (Bayesian or otherwise). During therapy, differing therapeutic drug monitoring practices, using either steady state concentrations or area under the curve (AUC, per dose and cumulative) and also, the methods of estimating exposure (AUC) can also contribute to significant variability in results. This is illustrated in Figure 1-2, [54] where AUC is shown for three methods of estimation: using a pharmacokinetic model or trapezoidal rule (either 0 – τ or 0 – ∞).

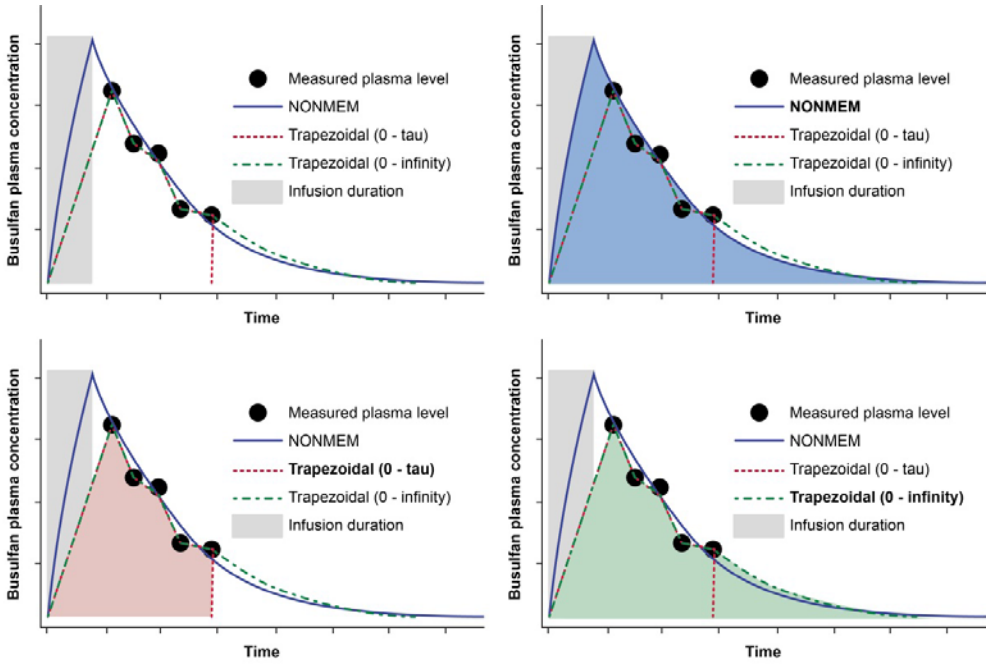


***Figure 1-2*** *The differences in estimating area under the curve using either a pharmacokinetic model, trapezoidal rule (0 – t) or trapezoidal rule (0 – inf) as supplied in the appendix by Bartelink et al.*[54]

The relationship between busulfan pharmacological effect and exposure in plasma has been demonstrated many times, providing an imperative to overcoming inter-individual variability so as to optimise outcome and reduce toxicity. Over the years, the target plasma

exposures to achieve optimal outcome have also varied between institutions, with some hospitals pursuing narrower therapeutic windows than others.[57 58] The administration of busulfan has shifted from being dose dependent (a cumulative dose of 16 mg/kg) to concentration/ exposure dependent (depending on a range of targets to be achieved). However, new busulfan-based conditioning regimens periodically emerge for new indications, often using the traditional 16 mg/kg dose.[59] As a result, there are clear differences in global practices for transplantation and conditioning, which have led to demands for a harmonised approach for the optimal use of busulfan in HSCT.[60] In considering the harmonization of the multitude of busulfan-based conditioning regimens across the world, certain aspects of busulfan pharmacology must be explored.

### 1.4.2 Pharmacokinetic Variability

Busulfan displays variability in pharmacokinetics, which has a direct impact on accurate dosing, clinical outcome, and the optimisation of definable targets that can help achieve these outcomes. Numerous retrospective investigations have described busulfan PK in the past two decades, using a variety of population PK (pop-PK) approaches. Literature is divided on the pharmacokinetic structure of the pop-PK models.[2 54 61-74] Approved dosing methodologies developed for the FDA and EMA use one-compartment models[70 75], while more recent Bayesian-guided dose calculators have used a two- compartment model.[62] Although pop-PK models have effectively described patient data in both cases, the difference in the resulting first dose is a contributor to variability, along with the subsequent dose adjustment required to achieve target exposure. Beyond the aforementioned differences, however, population pharmacokinetic models have been successful in identifying the main causes of variability in busulfan pharmacokinetics, which are described below:

#### *1.4.2.1 Body-Size*

Differences in body-size between individuals of various ages are often the main underlying cause for variability observed in PK parameters, particularly in paediatrics.[76] For busulfan, the first dose is calculated relative to body-size at a number of institutions, using a dosing nomogram relating to either body weight[70 75] or body surface area (BSA). Most pop-PK models incorporate one measure of body-size on clearance and volume of distribution (V); either weight,[71 77] adjusted ideal bodyweight[63] or BSA.[65]

The relationship between body-size and metabolic clearance is not proportional, and an allometric exponent on increasing body-size is more reflective of the differences observed.[78] An established scaling exponent of $^{3}/_{4}$ for weight and $^{2}/_{3}$ for BSA is commonly used to describe the change in clearance of busulfan from children to adults. However, the exponents are considered insufficient to describe the changes in clearance in preterm and very young children, as they seem to over-predict for infants and under-predict for neonates.[79] This was confirmed for intravenous busulfan, where the allometric exponent estimated for children under nine kilos was substantially higher than $^{3}/_{4}$.[80] Several modifications to allometric scaling such as sigmoidal functions of age,[81] have been attempted to better explain the effect of changes in body-size over the human lifespan, but have not been successfully implemented in pop-PK modeling for busulfan.

#### *1.4.2.2 Age and Maturation*

Neonates and infants have variable clearance, which cannot be entirely explained using an allometric exponent of $^{3}/_{4}$ on bodyweight. The exponent leads to an overprediction of clearance in children under two and an underprediction in neonates[82] and hence alternative allometric exponents have been proposed to counter this discrepancy.[81]

Beyond allometric exponents, however, there is an underlying difference in the maturation of metabolising enzymes in neonates and infants that can affect the CL of drugs. Therefore, functions have been developed to describe the maturation of enzymes in neonates to explain at least in part the variability in clearance.[78]

Busulfan is primarily metabolised by the glutathione- S-transferases (GSTs), mainly GSTA1, which is known to have a different profile in very young children compared to adults. When busulfan was dosed orally, children had a higher apparent oral clearance (clearance divided by bioavailability CL/F),[83] supposedly due to a higher level of enteric activity of GSTA1 enzymes compared to adults.[84]

Even with the IV formulation, which bypasses metabolism by enterocytes, busulfan clearance varies markedly with age.[65] After normalising to a measure of body-size, significant variation in clearance (mL/min/kg or mL/min/$m^2$) persists. Figure 1-3 illustrates changes in clearance over age per a) kg and b) $m^2$.[58] Although clearance standardised to weight (mL/min/kg), appears to be higher in children for IV busulfan, net CL is lower compared to adults, and normalisation of clearance by BSA is better reflective of this. Lower clearance in children is attributed to a lower metabolic activity and has been characterised by various functions describing the process of maturation. The incorporation of a maturation function in populations including infants has also been used effectively incorporated in pop-PK analyses for IV busulfan.[62 69]

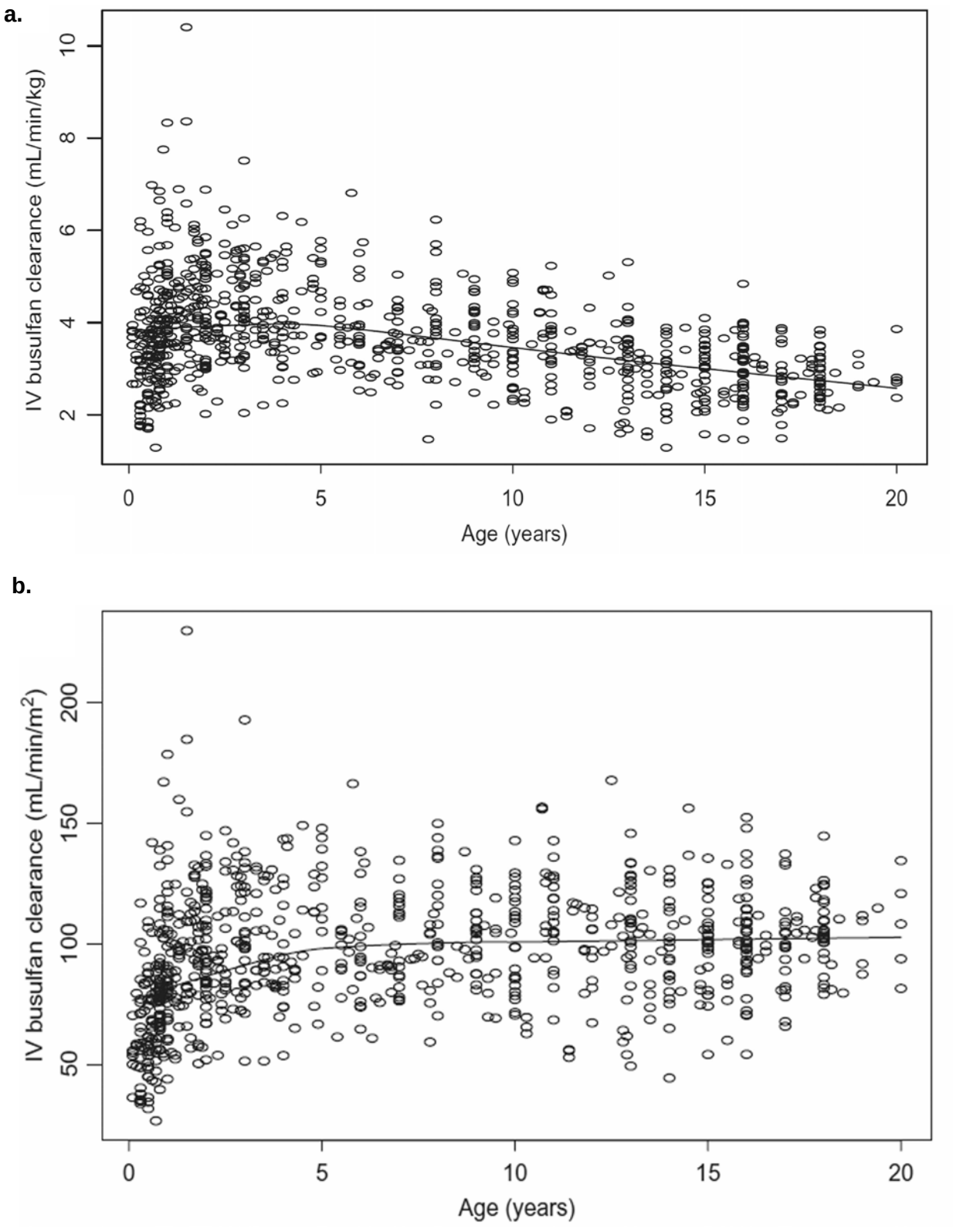


***Figure 1-3*** *Illustration of the change in busulfan clearance over age when standardised by a) weight (mL/min/kg) and b) BSA (mL/min/m$^2$). A moving average of clearances identified as a solid black line describes the change in clearance over age.* [58]

### *1.4.2.3 Metabolism*

1.4.2.3.1 Major and Minor Pathways

Busulfan undergoes extensive phase two metabolism in the liver through glutathione conjugation by the glutathione-S-transferase family of enzymes. Alternative metabolic pathways through N-acetylcysteine conjugation by N-acetyl transferase (NAT) have also been identified for busulfan. The busulfan glutathione-conjugate is further metabolised to tetrahydrothiophene (THT) by cystathionase (CTH) and eventually undergoes metabolism by cytochrome P450 enzymes CYP2C9, CYP2C19, and to a lesser extent CYP3A4 and CYP3A5 to form sulfolane and other inactive metabolites as described in Figure 1-4.

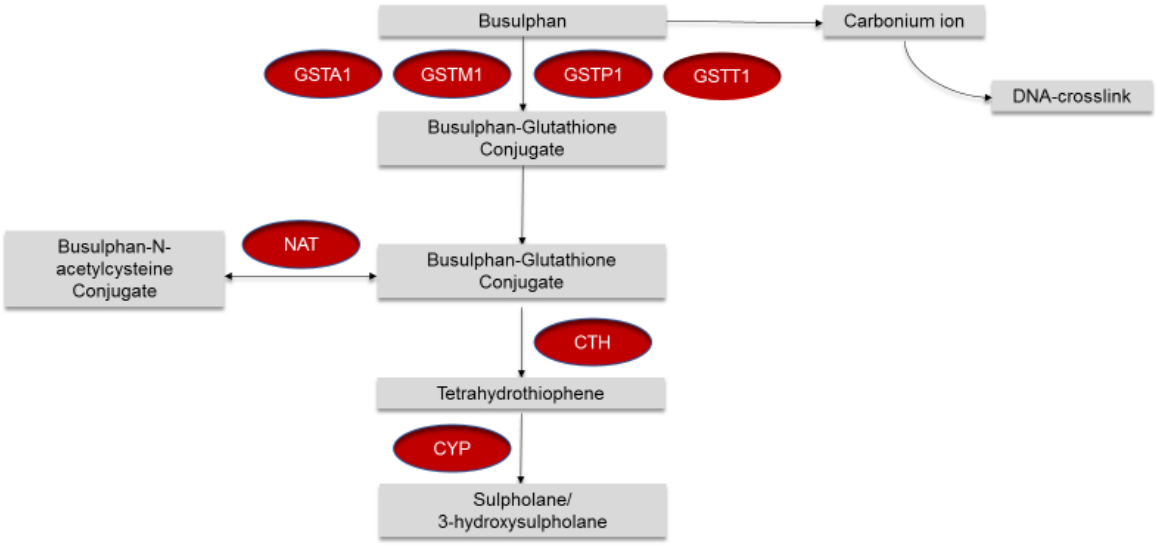


***Figure 1-4*** *The intracellular site of action and metabolic pathways involved in busulfan pharmacology.*[85]

Polymorphisms in the enzymes GSTA1 and GSTM1, and less commonly GSTT1 and GSTP1, which conjugate reactive alkylating species, have been associated with higher Bu plasma concentrations and lower CL.[86-88] [8990] [91]GSTA1 diplotypes may be categorised by enzymatic activity into three groups, with some variants associated with significantly lower

clearance of busulfan.[92] An analysis in 112 children and adolescents receiving IV busulfan found a difference in CL of between 7% and 12% amongst GSTA1 activity groups and reaffirmed the modest impact of GST metabolic pathways on busulfan PK variability.[93] However, studies on these associations are inconsistent, with other studies not finding an association between GST enzymes and busulfan clearance in adults,[89] and the implementation of GST genotyping in dosing guidelines has been limited.[91] Currently, specialty organisations such as the American society for BMTs does not recommend pharmacogenomics- aided dosing of busulfan.[90]

Studies reporting on the ontogeny of the GSTA1 enzyme are currently limited to enteric activity, which was reflected in oral busulfan administration.[84] With increased popularity of the intravenous formulation, the research is still lacking on maturation of GSTA1 in other parts of the body and their effect on busulfan CL.

#### *1.4.2.4 Concomitant Medications*

There have been few systematic studies investigating the potential drug-drug interactions that affect busulfan CL. Anecdotal evidence has been reported on lower busulfan CL associated with metronidazole, the iron chelator deferasirox or antifungal use .[94-97] A small study demonstrated significantly lower CL of busulfan in patients receiving metronidazole,[98] although no similar study has been repeated with the IV formulation. Drugs such as phenytoin and fludarabine affect apparent oral busulfan CL, but there is no evidence for similar interactions with the IV formulation.[99 100] While the product information for busulfan specifies theoretical interactions with paracetamol, due to similar metabolic pathways, there is no clinical evidence to suggest such an interaction.[55]

#### *1.4.2.5 Addressing Inter-Occasion Variability*

Inter-occasion variability is a known, yet unexplained, aspect of busulfan PK analysis. While models quantify IOV on CL and V as 10-15% between occasions, the mechanism of this variation is unknown.[101 102] Using a Michaelis-Menten elimination model, clearance gradually decreases on successive dosing occasions as concentration increases.[103] An alternative model implemented a factor for decreasing CL on each occasion after the first dose of busulfan, resulting in a further 6% reduction above the 12% IOV on CL.[54]

### 1.4.3 Pharmacokinetic Association with Outcome

The implementation of therapeutic drug monitoring relies on an established relationship between a measure of exposure in plasma (usually AUC, $C_{ss}$ or $C_{max}$) and clinical outcome, either efficacy or toxicity. To discuss the influence of PK on outcome, it is first necessary to consider the most appropriate measure of exposure, based on plasma concentrations. There are differences in the methods of estimating and quantifying this exposure. While there has been notable effort made in some reviews to standardise the measure of exposure prior to comparison,[90] there is still ambiguity as to the relevant cut offs for clinical implementation, and how stringently these should be applied. Busulfan exposure is typically estimated as either steady state concentration ($C_{ss}$ ) or area under the curve (AUC) which can be estimated through compartmental or non-compartmental analysis. Steady state concentrations take into account dosing intervals of busulfan when estimating exposure, which can make comparison simpler amongst the various dosing regimens.[104] Estimation of $C_{ss}$ is more relevant in the context of repeated doses with a relatively short dosing interval, and due to increasing popularity of the single-dose daily regimen, the concept of a cumulative exposure of busulfan in one transplant occasion (measured as cumulative AUC, or cAUC) for comparing outcomes, has become more widespread in recent studies.[54] Some studies have also reported the use of maximum

concentration post infusion ($C_{max}$) and trough concentrations ($C_{min}$) to assess outcomes.[105] [106]

Although several drugs display high inter-occasion and inter-individual PK variability, the short-term yet high-dose administration of busulfan makes overcoming PK variability particularly pertinent. Furthermore, there is an accepted relationship between busulfan exposure and transplant outcomes such as disease relapse, overall and progression free survival (OS and PFS, respectively) and toxicities such as SOS.[54] [107] The validation of these pharmacodynamic relationships is however far from consistent.[105]

#### *1.4.3.1 Association Between Busulfan Exposure and Transplant Success*

The first reported exposure-response relationship for the IV formulation of busulfan found a lower OS and PFS in adult patients with busulfan AUCs > 6000 µM.min (per dose, given daily over four doses- equivalent to 98.4 mg.h/L). This was attributed to higher non-relapse mortality (NRM) with no evidence to suggest a lower rate of relapse in patients with higher exposure.[108] Product information guidelines from both the EMA and FDA for IV busulfan cite exposure- response profiles derived from oral busulfan, where transplant success was associated with an AUC > 900 µM.min (per dose of oral busulfan, given Q6H over 16 doses) and an increased risk of SOS with AUC > 1500 µM.min.[109] The guidelines remain unchanged for steady-state concentrations between oral and IV busulfan, where $C_{ss}$ < 600 ng/mL was associated with graft rejection, while $C_{ss}$ > 900 ng/mL results in a greater risk of toxicities.[110] The cut-offs for these measures of exposure are similar in both children and adults.[107]

#### *1.4.3.2 Relationship Between Busulfan and SOS*

Sinusoidal obstruction syndrome can present as a range of symptoms, from a rapid increase in weight due to fluid overload, thrombocytopaenia refractory to platelet infusion, painful ascites and hepatomegaly, followed by elevated liver function tests and bilirubin.[111] The patterns of presentation can vary between patients, with particular differences between children and adults, making SOS difficult to diagnose. Guidelines for diagnosis such as Seattle, modified Seattle and Baltimore guidelines incorporate at least two or more symptoms for diagnosis and retrospective classification.[112] Recent guidelines from the European Society for Bone Marrow Transplants (EBMT) have distinct diagnoses between children and adults and have constructed a scheme for grading SOS during presentation, rather than retrospectively, providing time for treatment.[113] [114] Prevention is key in SOS management. Ursodeoxycholic acid is proven to reduce the severity of SOS and defibrotide has been introduced more recently.[16] [115] Treatment of acute SOS is predominantly achieved through symptom control of fluid retention using diuretics, platelet and albumin transfusions and defibrotide, although the exact mechanism of action of defibrotide in SOS is not fully understood.

Sinusoidal obstruction syndrome is a dose limiting toxicity of busulfan, which was first shown to correlate with busulfan exposure in 1989 by Grochow el al.[13] For treatment with busulfan (dosedQ6H for 16 doses), an AUC > 1500 µM.min or a $C_{ss}$ > 900 ng/mL. is associated with a higher incidence of SOS.[66] [116] A prospective evaluation in adult AML patients using daily IV busulfan with fludarabine found SOS occurs in all patients who were targeted to an overall cumulative AUC of 9000 µM.min (37 mg.h/L), but in only 7% of patients targeted to 7500 µM.min (31 mg.h/L).[117]

### 1.4.4 The Therapeutic Window - Does One Size Fit All?

The therapeutic window to avoid either toxicity or rejection is narrow and difficult to achieve as busulfan displays considerable pharmacokinetic variability amongst patients, and over different occasions of dosing in a single patient. A therapeutic window for AUC of Q6H busulfan administered over 16 doses has commonly been described as 900 to 1500 µM.min, or 900 to1350 µM.min for each dose of administration.[70 75 118] Slight variations of the target range, such as 950 to 1520 µM.min, have also been reported.[119] Expressing the cAUC in alternative units, corresponding to 58 – 86 mg.h/L or 58 – 100 mg.h/L, allows comparison to narrower recommended cumulative AUC targets of 74 – 82 mg.h/L and, a significantly higher 78 – 101 mg.h/L window which have been proposed recently.[54 107] Steady-state concentration monitoring (estimated as AUC divided by dose frequency)[58] is still used in many institutions and the target of 600 – 900 ng/mL has been widely accepted,[120] although a recent investigation has shown a poor correlation between $C_{ss}$ and AUC in individual patients when comparing cross-institutional practices, the reasons for which were not discussed.[54]

The counter argument to a single-therapeutic window for all busulfan dosing is that the intensity of myeloablative conditioning required may vary with the underlying condition, and that the combination of cytotoxics may influence the level of exposure to busulfan required for successful outcome. But even for each condition, the targets ranges can vary significantly, which has the potential to have a direct impact on patient outcomes. The choice of target ranges is often institution-dependent and often based on small studies or institution experience. Figure 1-5 developed by McCune *et al*. describes the heterogeneity in busulfan target AUC ranges used at 51 institutions in a study on the most common indications in 729 children and young adult transplant patients across the United States of America.[58] Each shaded square indicates the number of children treated with busulfan at a

particular target range in acute myeloid leukaemia and myelodysplastic syndrome. The targets used by several institutions are overlapping between 900 μMol.min – 1500 μMol.min, with large numbers of patients targeted between 900 μMol.min – 1300 μMol.min. Beyond the larger squares, there are many smaller squares indicating tighter target ranges for smaller numbers of patients. Analysis of other malignant and non-malignant conditions studied in the same investigation revealed a similar discrepancy in target busulfan therapy amongst all participating institutions in the United states.

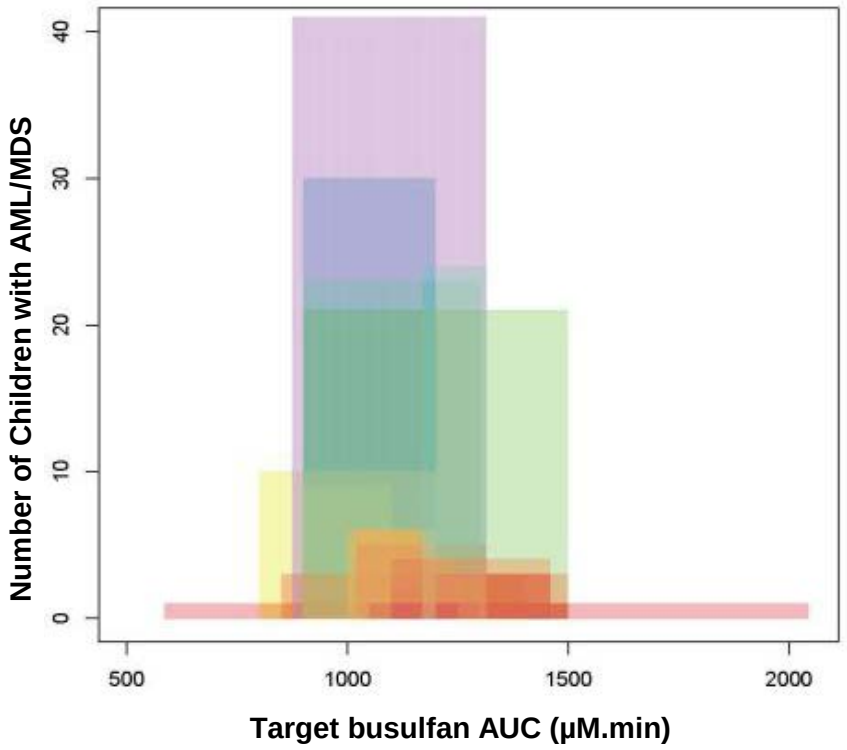


***Figure 1-5*** *The target AUC (μM.min) plotted against the number of children transplanted as shaded rectangles for each of the 51 institutions in the United States of America for acute myeloid leukaemia or myelodysplastic syndrome (AML/MDS).*[58]

### 1.4.5 Dosing

#### *1.4.5.1 The First Dose of Busulfan*

Busulfan dosing for myeloablative conditioning regimens still finds its roots in 16 mg/kg, originally identified as the maximum tolerated dose by Santos *at al*. in 1983.[35] Population pharmacokinetic modeling has informed the dosing beyond the standard 16 mg/kg total dose, with approved dosing regimens developed for the FDA and EMA using one-compartment models[70 75] and more recent Bayesian-guided dose calculators using a two-compartment model.[62] The change in doses over young age are aimed at allowing for the age-related changes in CL (mL/min/kg) as described in section 1.4.1.2. While these dosing nomograms were developed to overcome inter-individual variability, there is still substantial variation that is not accounted for, and PK- guided dose adjustments are a valuable intervention to bring plasma concentrations within the therapeutic window. Busulfan dosing is also modified in special groups such as obese patients where adjusted ideal body weight is often used.[121 122]

In terms of AUC, single daily dose regimens (4mg/kg or 150 mg/m$^2$) were not different to four times a day dosing of either 1mg/kg (cumulative dose of 16mg/kg) or 37.5 mg/m$^2$ (cumulative dose of 600 mg/m$^2$)[123]. A study of once daily IV busulfan showed that large inter-individual PK variability continued to be an issue in children[124] and a novel dosing strategy was introduced whereby the dose was halved and administered as separate doses over two days. Exposure (AUC) was assessed on day one, followed by the second instalment of the halved-dose and dose-adjustments could be performed for doses two to four, thereby avoiding potentially high exposures from the first full dose.[125]

Other strategies to better target busulfan given as daily dosing include the administration of a test dose to estimate individual PK parameters, prior to dose calculation [126]. The test dose (e.g. 0.8mg/kg) has been used to effectively target a busulfan AUC range as narrow as 4800- 5200 µM.min (19.7 - 21.3 mg.h/L) per day of busulfan administration in adult patients.[127] However, age-dependent pharmacokinetic variability is still a persisting issue with test dosing in young children (< 4 years), with six of 18 patients still falling outside the target range (3200 – 4800 µM.min (13.1 – 19.7 mg.h/L) for single dose daily IV busulfan .[128]

Bayesian and PK-guided first-dose calculators have been successfully implemented for accurate dosing of busulfan.[54 62 103 129] With the effort invested in understanding the PK variability of busulfan using pop-PK analyses, and the identification of various contributing factors to inter-individual PK variability, PK-guided calculators should in principle solve the issue of accurate dosing to the target range. These calculators are commercially available,[129 130] but not used consistently amongst all institutions, with many recent pharmacokinetic studies still using body-size as the basis for calculation of first dose.[131] , as is recommended in the product information.

### 1.5 BUSULFAN THERAPEUTIC DRUG MONITORING

The high PK variability, exposure to outcome relationship, and narrow therapeutic window makes busulfan a desirable candidate for therapeutic drug monitoring. Overall, PK monitoring practices have helped to reduce inter-individual PK variability and bring busulfan exposure within target range for patients to optimise transplant outcome and avoid toxicity. Several methods, from extensive plasma sampling, to limited sampling to

dried blood spot sampling techniques[132] have been investigated for optimal therapeutic drug monitoring.

The measurement of several busulfan concentrations over time can be used to estimate the AUC (through various variations of the trapezoidal-rule). A reliable estimation of AUC can require extensive sampling (from as few as four, or up to nine plasma samples per patient post infusion). Although laborious, time-consuming and expensive, more extensive sampling permits reliable estimation of AUC without the need for prior assumptions or knowledge of any patient-relevant factors apart from dose administered. The number of samples required and the duration of sampling post infusion can vary, depending on dosing interval and mode of administration. For instance, sampling up to six hours after start of infusion is adequate for a Q6H dosing regimen of 16 doses. Single dose daily regimens, however, require more extensive sampling up to eleven hours from the start of infusion,[102] despite a log-linear decline that should allow for extrapolation from four concentration time points. Extensive sampling with direct estimation of AUC is still one of the most commonly-used methods for targeting busulfan exposures in several institutions.[54]

Population PK analyses can be used to develop models that utilise rich sampled data from a population, and combine patient specific factors such as genetic traits, body size, and age group, to estimate individual PK parameters.[133] Institutions such as the American Society for BMT (ASBMT) recommend pop-PK guided dosing of busulfan.[90] Models can be used to predict pharmacokinetic behaviour of the drug in a patient and therefore inform limited sampling techniques, which require fewer blood samples (one to two as opposed to four or more) at selected time points to estimate exposure.[129] Limited- sampling processes,

however have not been universally adopted clinically, partially due to the high inter-individual PK variability in busulfan at any given time.[90]

Bayesian models can be implemented for both extensive- and limited-sampling strategies where pharmacokinetic information from patients can be pooled to better predict the PK parameters in future patients.[62 134] Several commercially-available busulfan therapeutic drug monitoring applications work on Bayesian principles and have successfully demonstrated effective AUC control.[54] Therapeutic drug monitoring performed on the first day of busulfan dosing can help to identify an appropriate dose to achieve the target required. Studies on test doses have indicated that a single therapeutic drug monitoring occasion may not suffice and follow-up PK analysis is necessary to ensure the target has in fact been achieved.[135] Variability in practice also persists, as institutions perform therapeutic drug-monitoring on a schedule between one day and all days of busulfan therapy. Given the high inter-individual PK variability, the estimation of cumulative AUC (cAUC) may vary significantly depending on the number of occasions of therapeutic drug monitoring. The accuracy and precision of the AUC calculated has clear implications for the clinical decisions that need to be made for busulfan therapy. With recent dosing and monitoring recommendations suggesting narrower and significantly higher target ranges (78 mg.h/L – 101 mg.h/L as opposed to 56 mg.h/L – 86 mg.h/L), the scope for accepting variability is substantially reduced and the risk of toxicity increased.

## 1.6 SUMMARY

The most recent pop-PK investigations of busulfan combine data from multiple conditioning regimens over several diagnoses. As guidelines for myeloablative conditioning using busulfan vary in the literature, individual physicians need to decide the

appropriate dose and exposure target in the context of the conditioning regimen. The resulting plasma concentrations in the individual are subject to variability depending on patient-specific factors and transplant outcome may be influenced by busulfan exposure, amongst other aspects beyond the scope of this review. The question of whether a single target range is appropriate for all patients continues to be a matter of debate.[136 137] The implementation of Bayesian methods to estimate first dose brings a sound theoretical rationale, but is in conflict with institutionalised practices resisting change. Therapeutic drug monitoring has found a place in therapy, but interpatient variability and toxicity persist, with some evidence for a greater risk of toxicity in patients where pharmacokinetic interventions are implemented.[138] Clearly, there is still more to be done and more proactive strategies that need implementation beyond current practices.

## 1.7 STUDY AIMS

While this thesis may not be able to resolve the complete gamut of issues identified above, the data and analysis presented address a wide range of concerns associated with busulfan therapy. This thesis presents the current practices of busulfan therapeutic drug monitoring as observed in hospitals participating in the multi-institutional retrospective analysis of Australian transplant patients across New South Wales and Victoria and aims to address the most pertinent issues related to busulfan therapy. Currently, research is lacking on the impact of various concomitant medications on busulfan clearance. Clinical practice informed by theoretical drug-drug interactions or anecdotal case studies, for example with paracetamol or metronidazole, also need to be analysed further. The retrospective nature of the analysis should also provide an opportunity to observe and assess any changes in practice intended to optimise therapy at the various institutions. Furthermore, recent analysis of GSTA1 polymorphisms has shown promise in

characterising variability in busulfan clearance in paediatrics. However, no similar study exists to date for the adult population.

The impact of the pharmacokinetic interventions on toxicity will also be of interest. Given the unpredictable nature of SOS incidence post busulfan use (with or without therapeutic drug monitoring), and a requirement for timely access to therapy, there is an unmet need to identify patient predisposition for SOS beyond the pharmacokinetic cut-off for over-exposure. Therefore, through a variety of experimental techniques this thesis aims to:

1. Characterise pharmacokinetic variability in the study population using population pharmacokinetic analysis
2. Analyse the efficacy of current TDM practices in trying to achieve the desired AUC targets for busulfan.
3. Characterise the influence of various transplant-related factors (including concomitant medications) on PK variability.
4. Explore the incidence of SOS in a study population of patients receiving busulfan, as a primary outcome of the TDM practices
5. Identify predictors of SOS and develop a model to predict SOS incidence post commencement of busulfan therapy, using non- semi- and parametric time-to-event analyses

# Chapter 2.

## THE PATIENTS- A DETAILED ANALYSIS OF THE PATIENT POPULATION RECRUITED FOR ANALYSIS

## 2.1 INTRODUCTION

Chapter One described the evolution of busulfan use in BMTs and HSCTs, and described pertinent problems of present-day busulfan therapy. Despite the associated challenges in overcoming pharmacokinetic variability and adverse effects, busulfan has become an integral part of the transplantation process. The research conducted in this thesis aims to characterise the present-day situation of busulfan therapy with regards to the role of therapeutic drug monitoring in overcoming pharmacokinetic variability, through an extensive retrospective analysis of transplant patients in the previous decade. This chapter introduces the study, the recruited population and also the types of analyses performed in subsequent chapters to investigate the pharmacokinetic, pharmacogenetic and pharmacodynamic aspects of busulfan therapy.

## 2.2 THE STUDY

### 2.2.1 Protocol

The data for this retrospective analysis was collated from three clinical trials over the study periods of 2006-2010, 2010-2015 and 2015 to 2020 (the last still ongoing). All studies labelled “PK BMT” were intended for the analysis of several common chemotherapeutic drugs routinely used in transplants under chief investigator Dr Christa Nath and principal investigators Professor Peter Shaw, Professor Andrew Grigg, and Associate Professor David Ritchie. This study was planned, performed and evaluated in compliance with GCP-Guidelines and local regulatory and ethical requirements. The basis of this study is the Declaration of Helsinki, 1964 and subsequent amendments. More details can be found on www.australianclinicaltrials.gov.au under the trial ID ACTRN12612000544875.

The research described in this thesis focused on a sub-population of the PK BMT study from seven institutions (Austin Health (AUS), Children's Hospital at Westmead (CHW), Geelong Hospital (GEE), Peter MacCallum Cancer Centre (PMCC), Royal Melbourne Hospital (RMH), Royal Prince Alfred Hospital (RPA) and Westmead Hospital (WMD)), comprised of patients who received busulfan prior to transplant. Data were collected on busulfan concentrations in patient plasma over time analysed for therapeutic drug monitoring, along with transplant-related information such as HLA-matching, autologous or allogeneic transplant, type of cells and source of stem cells or marrow, and also information on concomitant medications during busulfan therapy. Details of toxicity and transplant outcome were collected from patient medical records at each institution. Information regarding the onset of symptoms leading up to the diagnosis of sinusoidal obstruction syndrome were also collected.

### 2.2.2 Dosing and Sample Collection

The first dose of busulfan was calculated according to body-size, either bodyweight or BSA depending on protocol. In adult patients where actual bodyweight (ABW) was 20% greater than ideal body weight (IBW), patients were dosed according to Adjusted Ideal Bodyweight (AIBW)[122] calculated as:

*Equation 2-1*
$$AIBW = IBW + 0.25 \times (ABW - IBW)$$

Institutions collected PK blood and transported them to Dr Nath for therapeutic drug monitoring. To make clinically-relevant decisions in time for the patients, a dosing regimen of two divided doses for Day 1 was implemented. Blood samples from the first half-dose were sent to CHW for therapeutic drug monitoring (TDM) and analysed by the following

day (Day 2) when the patient was administered the second half of the Day 1 dose, so subsequent doses could be adjusted from Day 3. According to the study, all participants required monitoring on Day 1 and a follow up TDM was performed for a full dose administered on Days 3, 4 or 5 to ensure that the targeted AUC was achieved. From 2012, paediatric patients were monitored on every day of busulfan administration

Busulfan infusion rate was maintained at 3.2 mg/kg over 3 hours. Samples were collected in lithium-heparinised tubes from the opposite lumen to where busulfan was administered, at the end of infusion and over the following time points and windows specified:

T = 0 hours

T = 1 – 2 hours

T = 3 – 4 hours

T = 4 – 8 hours

Additional samples at T = 11 hours and pre-transplant samples were also collected for paediatric patients after 2012. Busulfan concentrations were analysed immediately upon receipt and exposure (estimated as $AUC_{0\text{-}inf}$ (μM.min)) was reported to clinicians by Dr Nath. Doses were adjusted assuming linear pharmacokinetics using the following equation:

Equation 2-2

$$New\ Dose\ (mg) = \frac{Dose\ Administered\ (mg) \times Target\ AUC\ (\mu M.min)}{AUC\ Estimated\ (\mu M.min)}$$

As busulfan is unstable at room temperature, care was taken to avoid sample degradation by immediately placing the samples on ice and storing them at -40 ºC until transportation. Remaining plasma was curated and stored at -80 ºC for further research as approved for the study.

In a sub-section of patients, blood samples were collected in EDTA tubes prior to the commencement of busulfan therapy for genotyping analysis. DNA was extracted from fresh blood samples using a MagNA Pure™ Compact Instrument at CHW whenever possible or at first thaw upon receipt from other institutions.

### 2.2.3 Therapeutic Drug Monitoring

Busulfan concentration was measured in plasma samples by Dr Nath at CHW using gas-chromatography with electron capture detection (GC-ECD).[123] The method has been validated and approved by the National Association of Testing Authorities, Australia. All plasma concentrations used for busulfan pharmacokinetic analysis in this thesis were generated by Dr Nath. All data on plasma concentrations of busulfan for every occasion of busulfan TDM were stored on the hospital network, compliant with ethical approval.

Busulfan exposure was estimated as area under the curve (AUC) using the linear trapezoidal rule in the pharmacokinetic software Kinetica® (Version 4.0). Dose adjustments were recommended assuming linear pharmacokinetics, but left at the discretion of the physician.

Busulfan AUC was calculated as µM.min, although AUC values calculated with units of mg.h/L have also been described, particularly in the case of cAUC. For the purposes of this thesis, both AUC and cAUC have been reported in mg.h/L, unless reporting guidelines specified units of µM.min. Conversion from µM.min to mg.h/L was performed using the following equation:

*Equation 2-3*

$$AUC\ (mg/L\,.h) = \frac{AUC\ (\mu M.min) \times 246.31\ (g/mol)}{60\ (min) \times 1000}$$

## 2.3 THE PATIENTS

### 2.3.1 The Population Demographics

The dataset comprised of 344 conditioning episodes of busulfan in 337 individual patients aged 44 days to 70.1 years, who received a transplant between February 2006 to June 2017. Of these, three patients did not progress to transplant due to complications, and four patients received a repeat transplant with busulfan-based conditioning following disease relapse.

A histogram of post-natal age in the study population revealed a non-normal distribution, with a large proportion of infants and children under four years of age. Median age was 30.2 years. Post menstrual age (PMA) has been suggested to be a useful measure of enzymatic maturation in patients,[62] but was not recorded for patients in this study.

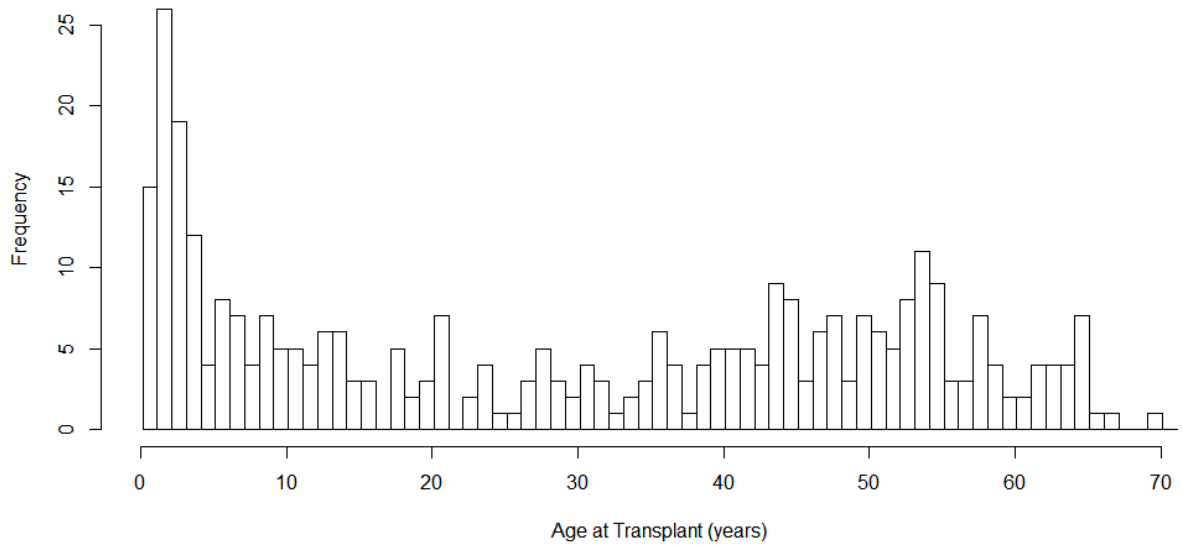


***Figure 2-1*** *Frequency distribution of patients over the age range, where bins were separated per year of age.*

Patients were further divided for categorical analysis into four age bands: infants and toddlers, children, adolescents and young adults, and adults. A geriatric category was not possible due to the small number of patients over the age of sixty-five. Table 2-1 summarises the number of patients in each age category for the study population.

*Table 2-1 Number of patients categorised into infants or toddlers, children, adolescents and young adults and adults.*

| **Age Category** | **n** |
|---|---|
| Infants and toddlers (1 month to < 2 years) | 38 |
| Children (2 years to <10 years) | 68 |
| Adolescent and Young Adults (AYA, 10 years to <25 years) | 50 |
| Adults (25 years and above) | 188 |

Patient weight and height were recorded on the day of hospital admission, and BSA was calculated using the Du Bois Formula. Ideal bodyweight (IBW) and AIBW were calculated separately for all patients over 152 cm. Table 2-2 summarises patient height, weight, BSA and AIBW for all patients.

*Table 2-2 Median and range patient characteristics in the study population.*

| **Characteristics** | **Median (Range)** |
|---|---|
| Age (years) | 30.2 (0.12 – 70.1) |
| Weight (kg) | 63.9 (2.9 – 150) |
| Height (cm) | 163 (48 – 197) |
| BSA ($m^2$) | 1.96 (0.2 – 2.53) |
| BMI ($kg/m^2$) | 2.91 (12.6 – 50.1) |
| Adjusted Ideal Body weight (kg) (Used to adjust dose in 47 patients) | 73 (2.9 – 127.5) |

### 2.3.2 Transplant-Related Information

Busulfan was administered for 263 allogeneic and 78 autologous transplant occasions. One patient received a syngeneic transplant. Within the 263 allogeneic transplants, 160 were HLA-matched and 90 were HLA-mismatched. Information on HLA-matching was not available for 13 patients. A total of 35 patients received cord blood transplants (single or multiple), 95 patients received stem cells from a related donor and 125 from single or multiple, unrelated donors.

### 2.3.3 The Diagnoses

There was marked heterogeneity in the diagnoses of the study population, with over 50 different cancer, immunological and haematological diagnoses. Acute Myeloid Leukaemia (AML) was the largest subgroup of 115 patients, followed by the grouped Non-Hodgkin's Lymphomas (NHL), consisting of 52 patients.

*Table 2-3 Summary of patients transplanted for various types of cancer, with median age and range.*

| **Cancer Diagnoses** | **n** | **Median Age (range)** |
|---|---|---|
| Acute Myeloid Leukaemia | 115 | 36.8 (1.4 – 70.1) |
| Non-Hodgkin's Lymphomas | 52 | 54.4 (1.7 – 66.9) |
| Myelodysplastic Syndrome | 25 | 41.4 (1.4 – 64.7) |
| Acute Lymphocytic Leukaemia | 21 | 10.9 (0.6 – 64.7) |
| Neuroblastoma | 19 | 3.2 (1.4 – 8.3) |
| Other cancers | 14 | 30.2 (5.1 – 58.4) |
| Multiple Myeloma | 11 | 47.2 (30 – 61.8) |
| Hodgkin's Lymphoma | 9 | 26.4 (18.7 – 36.6) |
| Myelofibrosis | 9 | 47.2 (28.5 – 60.3) |
| Chronic Myeloid Leukaemia | 7 | 47 (8.7 – 60.1) |
| Juvenile Myelomonocytic Leukaemia | 6 | 2.2 (1.2 – 5.4) |

Non-malignant diagnoses accounted for 54 patients, 51 of which were children. Table 2-4 describes the demographics of all the patients transplanted for non-malignant diagnosis. All but three patients from this cohort were paediatric patients. The three adult patients were treated for chronic granulomatous disease, pyruvate kinase deficiency and

adrenoleukodystrophy. There was large heterogeneity in the non-malignant conditions, with the largest category of patients combined as “other”. The other category consisted of adreno- and metachromatic leukodystrophy (n = 4 and n = 2, respectively), severe combined immune deficiency (n = 4), Omenn syndrome (n = 3), aspartylglucosaminuria (n = 3), severe congenital neutropaenia (n =2) and single cases of other haematological and immune conditions. No patients in the study population were treated for β-thalassaemia.

*Table 2-4 Summary of patients transplanted for various types of non- cancer related illnesses, with median age and range.*

| **Non-Cancer Diagnoses** | **n** | **Median Age (range)** |
|---|---|---|
| Mucopolysaccharidoses | 10 | 1.5 (1.1 – 4.1) |
| Wiskott-Aldrich Syndrome | 7 | 0.8 (0.3 – 9) |
| Chronic Granulomatous Disease | 5 | 14.6 (1.4 – 28) |
| Haemophagocytic Lymphohistocytosis | 5 | 2.4 (1.7 – 13.4) |
| Other | 27 | 4.7 (0.1 – 4.7) |

### 2.3.4 Conditioning Regimens

Eight principal conditioning regimens were used prior to transplantations. Regimens were also separated according to the sequence of chemotherapeutic agents administered, due to documented differences in efficacy and toxicity profiles.[139] The administration of other cytotoxic medicines beyond the core conditioning regimen, such as clofarabine, gemcitabine and thiotepa are also notated for the regimens.

*Table 2-5 Number of patients per conditioning regimen used prior to transplants.*

| **Conditioning Regimen** | **n** |
|---|---|
| Bu-Cy[•] | 58 |
| Bu-Mel[†] | 74 |
| Cy-Bu | 42 |
| Flu-Bu[§] | 80 |
| Concomitant Flu-Bu-Alkylating agent (either Cy/Mel or Thiotepa[ɣ)] | 43 |
| Complete Flu followed by Bu | 17 |
| Mel-Bu | 11 |
| RIC Flu-Bu[×] | 19 |

[•]three patients had concomitant administration of etoposide, [†]one patient had concomitant administration of gemcitabine, [§]concomitant administration of thiotepa in five patients and clofarabine in two other patients, [ɣ]one patient was administered concomitant clofarabine, [×]five patients received concomitant thiotepa.

The doses of other chemotherapeutics in the conditioning regimen depended on the type of conditioning regimen used. For a fludarabine and busulfan regimen, patients received 1.6 mg/kg of fludarabine, where as in Flu-Bu-alkylating agent regimens a lower dose of 30 mg/m$^2$ was used. Cyclophosphamide was dosed at 50 mg/kg and melphalan doses ranged from 70 mg/m$^2$ in the Flu-Bu-Mel regimens to 140 mg/m$^2$ in the Mel-Bu or Bu-Mel regimens. Protocols have not yet been implemented for individualised fludarabine, melphalan or cyclophosphamide doses for transplantation in any of the participating institutions.

### 2.3.5 Toxicity Prophylaxis

All patients were administered either clobazam (0.25mg/kg/day) or clonazepam (0.5 mg twice daily) during busulfan therapy for seizure prophylaxis. Mesna (60 mg/kg/day) was co-administered for all cyclophosphamide-containing conditioning regimens to reduce the risk of haemorrhagic cystitis. Antifungal prophylaxis was provided using fluconazole (8 mg/kg) and also liposomal amphotericin (Ambisome, 1 mg/kg/day). GvHD prophylaxis was provided using methotrexate (dose dependent on diagnosis and graft source) with folic acid, mycophenolate mofetil (15 mg/kg) or cyclosporin (trough level monitoring at 300 ± 50 ng/mL).

Forty-nine patients from RMH, 40 from WMD, and all patients from CHW were treated with ursodeoxycholic acid (12 mg/kg/day) during the transplantation process for SOS prophylaxis. Royal Prince Alfred hospital were prescribed enoxaparin as SOS prophylaxis between 2010- 2014. This practice has ceased since and no other toxicity prophylaxis has been added to the transplant regimen. A small cohort of 14 patients at CHW received defibrotide (100 µg/kg/day), along with ursodeoxycholic acid as SOS prophylaxis.

### 2.3.6 The Institutions

Patients were transplanted at seven institutions across New South Wales and Victoria. Treatment at each institution varied depending on diagnosis, conditioning regimen and type of transplant. All patients from AUS and PMCC received autologous transplants for various types of non-Hodgkin's Lymphomas. Fifty-one patients from CHW and only three adult patients from RPA and RMH were transplanted for non-cancer related diagnoses.

All patients were administered once daily doses of busulfan, except for a handful of paediatric patients from CHW who received a dosing regimen of Q6H busulfan

adminisrered over 16 doses. The choice of conditioning regimen also varied across the transplant centres. Institutions AUS and PMCC consistently used the Bu-Mel conditioning regimen, except for one PMCC patient who received Bu-Cy. All patients at RMH receiving autologous transplants were also conditioned with Bu-Mel, and allogeneic patients mainly received Bu-Cy with or without thymoglobulin; two patients received Flu-Bu with thymoglobulin. All patients at WMD underwent allogeneic transplantation using Cy-Bu with or without thymoglobulin, except for three patients who received a Flu-Bu reduced-intensity conditioning regimen. Patients at CHW received over 27 different busulfan-containing protocols for autologous and allogeneic transplantation.

#### *2.3.6.1 Physician Discretionary Factors*

Some trends in clinician practices are worth noting here prior to analysis. The implementation of AIBW-calculated doses was highest in AUS and RMH (62% and 33%, respectively). While doses for all adult institutions were aimed to achieve a daily target of 5000 µM.min (20.5 mg.h/L), there was no consensus amongst institutions of treating the target as a maximal or minimal exposure. Busulfan doses were generally escalated in AUS to achieve a target of 20.5 mg.h/L or higher, while RMH and PMCC consistently decreased or left the doses unchanged, so as to not surpass the daily 20.5 mg.h/L target. Preferences of conditioning regimens and dose modifications on busulfan are summarised in Table 2-6.

As busulfan pharmacokinetic variability is known to be highest in children, a change in TDM practice was also observed during the study aimed at better achieving target cAUCs. After 2012, patients received daily TDM for busulfan doses in CHW allowing more opportunities to achieve the target cAUC through dose changes. Furthermore, clinician reported target cAUCs were also observed to increase (cAUC +5 mg.h/L) in the children population for certain regimens.

*Table 2-6 Patient characteristics from each institution participating in the PK BMT study. Dose modifications were calculated as a net mg/kg alteration in busulfan dosing for each transplant occasion.*

| Hospital | Number of Patients | Median Age (Years, Range) | Median Weight (Kg, Range) | Conditioning Regimens | Number of Dose Modifications (Inc /Dec / Unadjusted) |
|---|---|---|---|---|---|
| Austin Health (AUS) | 29 | 54 (19 – 67) | 85 (49 – 129) | Bu-Mel | 20 / 6 / 3 |
| Children's Hospital Westmead (CHW) | 133 | 4 (0.1 – 18) | 16 (3 – 109) | Bu-Cy, Bu-Mel, Flu-Bu, Flu-Bu-Alkylating agent, Consecutive Flu-Bu, Mel-Bu, RIC Flu-Bu | 26 / 26 / 86 |
| Geelong Hospital (GEE) | 2 | 50 (48 – 51) | 99 (82 – 116) | Bu-Mel | 0 / 0 / 2 |
| Peter MacCallum Cancer Centre (PMCC) | 12 | 55 (20 – 70) | 82 (57 – 110) | Bu-Cy, Bu/ Mel | 0 / 5/ 7 |
| Royal Melbourne Hospital (RMH) | 54 | 43 (18 – 65) | 76 (50 – 116) | Bu-Cy, Bu-Mel, Flu-Bu | 2 / 12 / 40 |
| Royal Prince Alfred Hospital (RPA) | 62 | 49 (21 – 65) | 74 (40 – 122) | Bu-Cy, Bu-Mel, Flu-Bu, RIC Flu-Bu | 19 / 9 / 36 |
| Westmead Hospital (WMD) | 45 | 40 (20 – 64) | 75 (40 – 150) | Cy/ Bu, Flu-Bu, RIC Flu-Bu | 4 / 9 / 32 |

Busulfan exposure targeting varied amongst institutions. All adult institutions principally targeted 20.5 mg.h/L for each full day of busulfan administered. Exposure at RMH was targeted to 22.6 mg.h/L for Flu-Bu based regimens. Also at RMH, in patients with extremely high or low Day 1 AUCs, a cAUC target of 82 mg.h/L or 90 mg.h/L for Flu-Bu was prescribed. All institutions aimed to avoid AUCs greater than 24 mg.h/L per dose. Exposure targeting was based on a combination of published targets and clinical impressions, and varied substantially between diagnoses and conditioning at CHW as summarised in Table 2-7.

*Table 2-7 Busulfan based conditioning regimen used at the Children's Hospital at Westmead, corresponding to diagnosis and target AUC*

| **Protocol** | **Diagnosis** | **Targets** |
|---|---|---|
| Thiotepa/Flu-Bu[140] | ALL | 70mg.h/L +/- 5 |
| Flu-Bu/Clofarabine | ALL/NHL | 98.5 mg.h/L |
| Flu-Bu | AML | 85 mg.h/L +/- 5 |
| Flu-Bu-Mel | AML | 75 mg.h/L +/- 5 |
| Bu-Flu-Cy | Fanconi Anaemia (2 bd for 2 days) | Nil Target |
| Flu-Bu | CGD | 55-60 mg.h/L |
| Flu-Bu | Gen Non-Malignant | 80 mg.h/L +/- 5 |
| Flu-Bu-Thymo | MPS | 80 mg.h/L +/- 5 |
| Flu-Bu-Cy | Non-Malignant HR | 80 mg.h/L +/- 5 |

### 2.3.7 Targets for TDM

Guidelines and targets for busulfan concentrations at CHW were principally between 55 mg.h/L – 100 mg.h/L for all patients. The analysis of busulfan targets could not be simply divided according to the various conditioning regimens in children, as targets were revised for various conditions over the ten-year study period. Other studies such as Bartelink *et al*. have used a standard target of 56 mg.h/L – 86 mg.h/L and provided a uniform target recommendation of 78 mg.h/L – 101 mg.h/L across all busulfan containing regimens.[141] Most patients in the study population were targeted for a cAUC between 55 mg.h/L – 90 mg.h/L, with two patients treated with the Flu-Bu/Clo conditioning regimen, which requires a higher (98.5 mg/L) cut-off. Therefore, the published target range described as the "historical target" by Bartelink *et al*. was used as the uniform target range for the rest of this thesis. Further discussion on the target cAUC is provided in Chapter three where the pharmacokinetic variability in busulfan has been described in detail using population pharmacokinetic analysis.

# Chapter 3.

## CHARACTERISING PHARMACOKINETIC VARIABILITY IN THE BUSULFAN STUDY POPULATION USING POPULATION-PHARMACOKINETIC ANALYSIS

## 3.1 INTRODUCTION

Busulfan therapy is associated with large inter-individual and inter-occasion pharmacokinetic variability (IIV and IOV, respectively) as explained in Chapter One. While the exact causes of this pharmacokinetic variability are unclear, several contributing factors such as body size, immature metabolic function in infants and concomitant administration of medications such as paracetamol and metronidazole have been investigated in patients receiving high-dose busulfan. This chapter explores the observed variability in busulfan pharmacokinetics using a population approach, and the contributions of various other transplant-related factors are examined using a *post hoc* statistical analysis.

### 3.1.1 Rationale

Chapter 2 described in detail the demographic composition of the study population. In keeping with the broad indication for transplant, the large number of patients, participating across different institutions, varied in age (40 days to 70 years), weight (2.1 – 150 kg), diagnoses and conditioning regimen used prior to transplantation. All patients received a variety of essential medications during their transplant, such as antibiotics, chemoprotective agents, chemotherapeutics and therapies for other comorbidities, guided by institutional protocols. The nature of the transplant itself (autologous, allogeneic and type of cells transplanted) was also tailored to the diagnosis, protocol and patient health. Furthermore, there were also marked differences in practices between the participating institutions for busulfan dosing and therapeutic drug monitoring. The possibility of any one or more of the aforementioned factors contributing to the large pharmacokinetic variability observed in busulfan could not be ruled out. Population pharmacokinetic (pop-PK) analysis can be applied to quantify the contribution of each factor and to characterise the variability

observed following administration with the use following administration of high dose busulfan.

### 3.1.2 Population Pharmacokinetic Modeling

Pharmacokinetic and pharmacodynamic (PK/PD) models describe the relationship between drug-concentration and drug effect. Given a population of patients, pop-PK models can be used to estimate PK parameters such as clearance (CL) and volume of distribution (V) in both the population, and also the individual patient. .[142] Population pharmacokinetic models are now routinely implemented in the design and monitoring of dosage regimens in drugs with variable pharmacokinetics or for drugs with a narrow therapeutic range. Pop-PK models can also be extended to simulate special patient groups where clinical trials are difficult or ethically challenging, namely paediatric and geriatric patients, or patients with renal or hepatic impairment. In recent years PK/PD models have become a regulatory requirement in the drug approval process. As models are generalized mathematical concepts, they are useful only if they appropriately fit the data.

### 3.1.3 Population Pharmacokinetic Analysis of the Busulfan Study Population

Busulfan pharmacokinetic models have been discussed extensively in the scientific literature, using either one- or two-compartment structural models.[61-74] [143] There is a continued theme of incorporating IIV and IOV to describe the PK variability, and also the inclusion of covariates relating to body-size and maturation on key PK parameters. The aim of building a pop-PK model for the current busulfan study population was to best describe the pharmacokinetic data on hand and to quantify the contribution of various

demographic and transplant-related factors to the observed variability in busulfan pharmacokinetics.

Given the variability in practice across the institutions, there were limited numbers of patients treated with medications of interest (eg. paracetamol and metronidazole) that are thought to affect PK variability in busulfan. While understanding the contribution of drugs to PK variability is of great interest in busulfan pharmacology, the incorporation of such low-powered covariates (small number of patients) would result in selection bias and harm the predictive performance of the model,[144] and therefore the impact of concomitant medications was tested *post hoc* by using robust parameter estimates from the model.

## 3.2 METHODS

### 3.2.1 Model Development

A pop-PK model was developed using nonlinear mixed effects (NLME) modeling. First-order conditional estimation method with interaction was used throughout the modeling process. Both, one- and two-compartmental structural models were analysed for best fit to the data. Model selection was based on diagnostic plots, decreases in the objective function value (OFV) of more than 3.84 ($P< 0.05$) for every covariate added and residual standard errors. The model was evaluated for precision using a bootstrap of 1000 simulated datasets and the $5^{th}$ and $95^{th}$ percentile confidence intervals were compared with the final model.[145] A visual predictive check (VPC) was performed using 1000 simulated datasets.[146] The area under the curve for every dosing occasion was calculated as dose divided by estimated individual clearance. Cumulative AUC (cAUC) was calculated as the sum of AUCs from every dose of busulfan for a patient for the conditioning episode.

#### *3.2.1.1 Pharmacokinetic Tools and Software*

Population pharmacokinetic analysis was performed using the nonlinear mixed-effects modeling program NONMEM version 7.3 (ICON developmental Solutions, Ellicott City, MD) with Perl-Speaks-NONMEM modules (version 2.9.2)[145] on a Pirana workbench (version 2.9.2).[147] Diagnostic plots and *post hoc* statistical analysis were performed on the statistical software R (version 3.5.0) on the R Studio platform (version 1.0.136) and GraphPad Prism (version 7.02).

#### *3.2.1.2 Base Model Development*

Plasma concentrations were log-transformed to allow greater weighting on values close to the lower limit of quantitation, thereby reducing the variance otherwise observed in untransformed data.[148] A one-compartment base model where zero-order absorption was used to describe the continuous intravenous infusion, was applied to the pharmacokinetic data.

Non-linear mixed-effects (NLME) models allow for the quantitation of variability using a combination of fixed-effects (denoted as θ, measurable sources of variability identified through a covariate analysis) and random-effects for unmeasurable sources of variability. An individual prediction of pharmacokinetic parameters therefore is a combination of fixed and random effects, where the random effect (denoted as η) is normally distributed over the population and the distribution is noted as ω.[142]

An additional component of random effects is a model to describe the residual unexplained variability (RUV), beyond the scope of the measurable and unmeasurable known sources of variability. The RUV, defined by ε quantifies the difference between what the model predicts in an individual compared to what is observed. The RUV in NONMEM is also assumed to be normally distributed and the variance is denoted as σ.[142]

For the purpose of this analysis, unexplained variability was tested for differences in CL and V between patients as inter-individual variability (IIV), amongst the various occasions of busulfan dosing in a single individual (inter-occasion variability or IOV). Inter-individual variability (IIV) was applied to the two pharmacokinetic parameters, clearance (CL) and volume of distribution (V), and a block matrix was introduced between the IIV distributions. Inter-occasion variability was also tested on both parameters. A proportional model to describe RUV was selected for the log transformed data.

### 3.2.2 Covariate Analysis

Demographic and transplant-related information collected during the PK BMT study formed the basis of covariate analysis in the study population. The first step in including covariates on the base model was a thorough exploration of the covariates identified, their relationship with each other, and the parameters CL and V. The continuous covariates assessed were weight (WGT, kg), adjusted ideal body weight (AIBW, kg), DuBois-calculated body surface area (BSA, $m^2$), height (HGT, cm), age from date of birth to date of transplant (AGE, years (y)) and pre-transplant albumin (ALB, g/L). Categorical covariates included sex (SEX), institution of transplant (HOSP), transplant type, whether autologous or allogeneic (TXTYPE), and HLA matched and mismatched transplantations (HLAMATCH). Covariate values were plotted against pharmacokinetic parameters of the

model to identify covariate relationships. Step-wise covariate modeling (SCM) analysis was used to identify potential covariate relationships where selection criterion for the inclusion of a covariate was $P< 0.05$ (dOFV -3.84) and the backward elimination step required $P < 0.01$ (dOFV 6.6) for the retention of the covariate. Other covariates and functions for maturation were also tested separate to the SCM process.

#### *3.2.2.1 Maturation Function*

Two maturation models have been described for busulfan clearance in neonates and very young children. The first is a sigmoidal function that factors in post-menstrual age (PMA) and has been validated for use over the human life-span.[62] The second model used age as an exponential function to calculate a maturation factor specifically in neonates and children under 12 kg,[69] who accounted for 12% (n = 40) of the study population. The maturation functions of both models were tested on the study population. Post-menstrual age in the sigmoidal maturation function was calculated as 40 weeks of gestation added to the age of all patients. As PMA was not recorded for patients in the study, both PMA (calculated by adding 40 weeks) and post-natal age were tested on the model to avoid the assumption of a consistent 40 week gestation on all patients.

### 3.2.3 Determination of AUC

The development of a population pharmacokinetic model allowed for the estimation of CL and V for all days of busulfan dosing. High-dose busulfan in myeloablative conditioning is given as a once-daily intravenous infusion for four full doses on consecutive days in all adult patients. Non-myeloablative and reduced intensity conditioning protocols used two days of busulfan dosing. To allow for timely TDM and to overcome logistical challenges of sending plasma samples to the Children's Hospital at Westmead, most patients were

administered the first dose of busulfan in two half doses where samples after the first half dose would be sent for TDM and subsequent full doses could be adjusted based on the first day half dose, to achieve the target full daily dose AUC of less than 24 mg.h/L or a cumulative AUC (cAUC), in myeloablative transplants between 56 mg.h/L and 86 mg.h/L. Therefore, prior to any *post hoc* analysis, the AUCs for all patients were standardised to a full day 1 dose of busulfan, according to the following equation. The cAUC was calculated for the analysis as the sum of all estimated AUCs for every dose administered to a patient.

*Equation 3-1*

$$Day\ 1\ AUC = AUC_{TDM} \times n$$

where $AUC_{TDM}$ is the AUC of the therapeutically-monitored dose and n is the number of doses into which the day 1 dose was fractionated, to account for the patients receiving the Day 1 dose either over two days, or as Q6H over four doses.

### 3.2.4 *Post Hoc* Analysis

The influence of other transplant-related factors such as concomitant medications, conditioning regimen, and inter-institutional differences on CL and V were analysed *post hoc*. To allow for comparison over all ages, busulfan normalized clearances ($CL_{NORM}$, L/h/kg) were calculated as busulfan CL divided by patient bodyweight, and average $CL_{NORM}$ was computed as the average of all the normalized clearances of an individual in one conditioning episode when a drug was either administered or not administered concomitantly with busulfan. Comparisons were made between $CL_{NORM}$ of patients using an unpaired Mann Whitney *U*-test when concomitant medications were used for all occasions of busulfan dosing. Average $CL_{NORM}$ was paired per patient based on concomitant use of a medication with busulfan dosing and assessed using a paired

Wilcoxon signed ranks W test. Net dose adjustments were calculated as a mg/kg change in doses on days 3, 4, or 5 based on TDM, compared to the first full dose AUC (either day 1 AUC or cAUC of days 1 and 2 in patients administered two-half doses), which were dosed according to body size.

## 3.3 RESULTS

A total of 3241 observations from 344 individuals were used to develop the population pharmacokinetic model. Another 210 observations were omitted from the analysis due to either being under the LLOQ or else contamination from busulfan from administration or flush solution was suspected.

### 3.3.1 The Base Model

The two-compartment model initially produced a lower OFV compared to the one-compartment model. A comparison of conditional weighted residuals for both one- and two- compartment base models over time after dose revealed an even distribution above and below the x-axis for the one compartment model. Systematically negative CWRES at initial concentrations, immediately followed by a higher density of positive CWRES in the two-compartment model also indicated bias in fitting the data. Prediction of inter-compartmental clearance and peripheral volume were accompanied with high relative standard errors (RSE > 50%). A comparison of the goodness of fit plots (supplementary) showed no major differences in predicted concentrations of individual or population values, and hence parsimony dictated the use of a one- compartment model.

a b

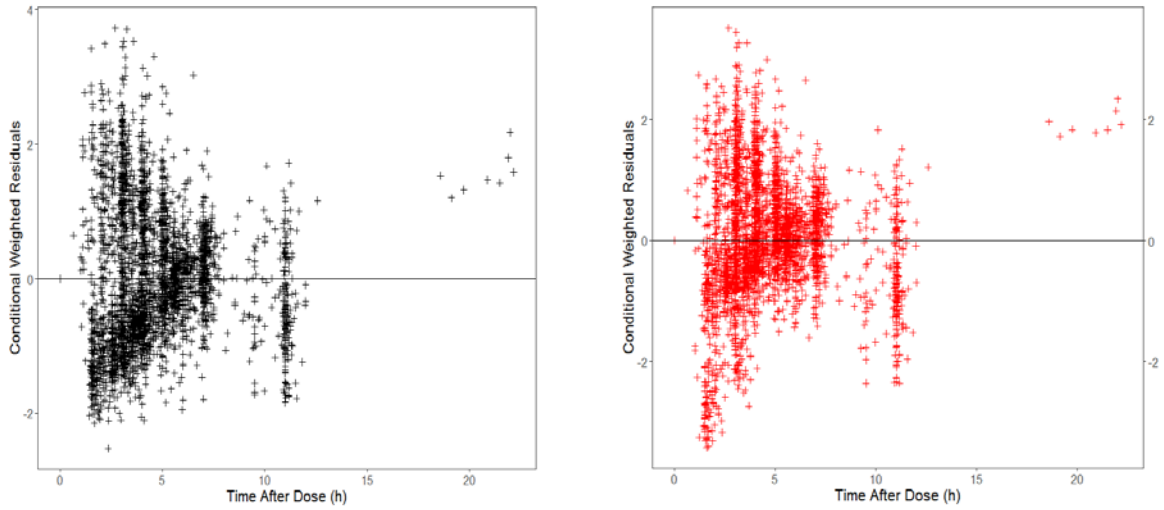


***Figure 3-1*** *A comparison between a) one-compartment and b) two- compartment model conditional weighted residuals (CWRES) plotted over time after busulfan administration (h).*

### 3.3.2 Stochastic Model for Random Effects

Inter-individual variability (IIV) on both CL and V, resulted in large decrease in OFV (dOFV -7335). A block matrix was introduced between the IIV distributions of CL and V, which further lowered the OFV by -885. The introduction of IOV on CL lowered the objective function by 1208 and IOV on V by a further 205 units. The variability between and within subjects and between population values for clearance and volume are described in equation 3-2 and 3-3 for Cl and V, respectively.

Equation 3-2 $$\mathbf{CL_i} = \mathrm{CL_{pop}} \times \mathrm{e}^{(\mathrm{IOV_{CL}} + \mathrm{IIV_{CL}})}$$

*Equation 3-3* $$\boldsymbol{V_i} = V_{pop} \times e^{(IOV_V + IIV_V)}$$

The model to describe RUV was chosen to complement log-transformed data as described in equation 3-4 where the observed concentration (Y) is the sum of the log transformed predicted value (f) and the parameter $\varepsilon_1$.

*Equation 3-4*

$$Y = Log(f(\theta, Time) + \varepsilon_1$$

The base model was used to assess the relationship between the parameters and potential covariates. The improvements in model OFV are documented in Table 3-1.

*Table 3-1 Development of a structural model with Objective Function Values and the difference between models where inter-individual and inter-occasion variability (IIV and IOV, respectively) )were incorporated log-normally on CL and V.*

| **Model No.** | **Model Description** | **OFV** | **dOFV** |
|---|---|---|---|
| Run101 | One Compartment with log transformed Plasma Concentrations | 1302 | |
| Run102 | Run101 + IIV-CL | -814 | -2116 |
| Run103 | Run102 + IIV-V | -6033 | -5219 |
| Run104 | Run103 + OMEGA BLOCK | -6960 | -927 |
| Run107 | Run104 + IOV-CL | -8127 | -1166 |
| Run108 | Run107 + IOV-V | -8332 | -205 |

### 3.3.3 Covariate Analysis

#### *3.3.3.1 Scatterplot Matrix of Continuous Covariates*

There was a high level of correlation between the measures of body-size, due to AIBW and BSA being functions of height and weight as illustrated in Figure 3-2. A moving average line of best fit identified these relationships between covariates. Age was mostly correlated with other covariates until early adolescence (14 years). There was no obvious relationship between ALB levels and other continuous covariates.

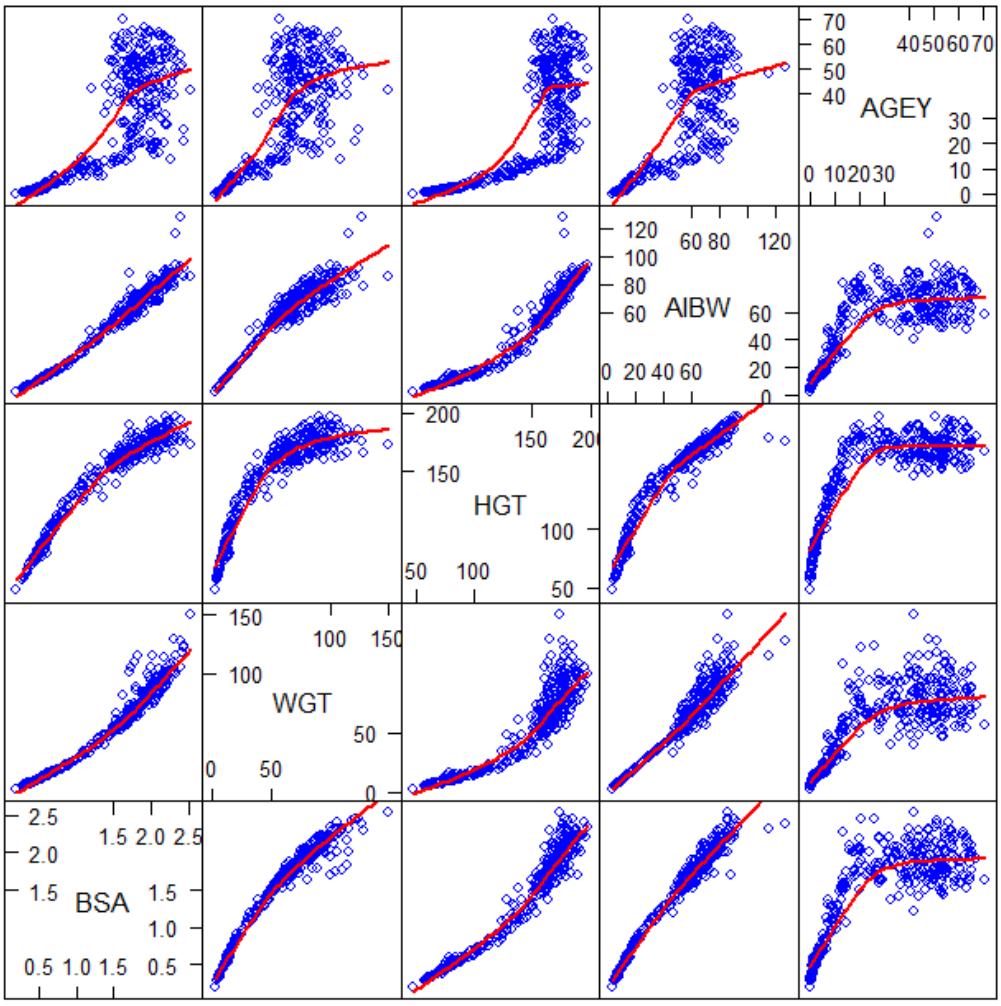


***Figure 3-2*** *Scatterplot matrix of continuous covariates showing relationships between weight, adjusted ideal bodyweight, body surface area, height and age (in years). A moving average line of best fit (red) describes any observable trends in the covariate relationships.*

### *3.3.3.2 Relationship Between Continuous Covariates and Pharmacokinetic Parameters*

Clearance and volume estimated from the base model were plotted against categorical and continuous covariates. A preliminary analysis of covariates found a high correlation between pharmacokinetic parameters and each measure of body-size. There was no observable relationship between pre-transplant albumin levels, and CL or V were highly variable over age beyond the adolescent years. Figure 3-3 illustrates the relationship between the PK parameters of CL and V and AIBW, BSA, height and weight.

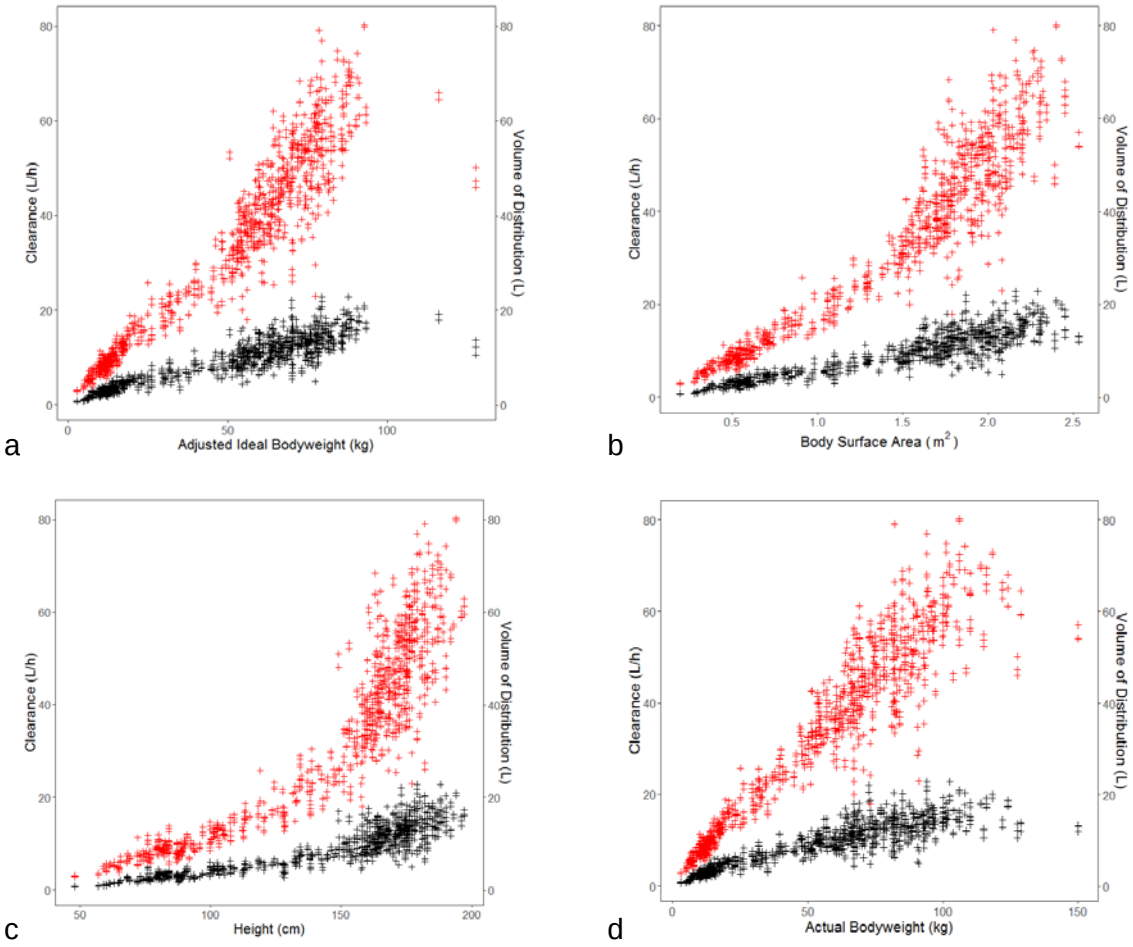


***Figure 3-3*** *Relationship between parameters, clearance (L/h, black) and volume of distribution (L, red), and covariates* ***a.*** *adjusted ideal bodyweight,* ***b.*** *BSA ($m^2$),* ***c.*** *height (cm) and* ***d.*** *Actual bodyweight (kg)*

### *3.3.3.3 Relationship Between Categorical Covariates and PK Parameters*

An analysis of categorical covariates identified several contributors to PK variability. Sex, hospital and conditioning regimen had one or more categories with significantly different CL or V compared to the other categories. While the difference initially seemed significant, the main contributing factor was body size, which varied vastly between paediatric and adult institutions, and also in conditioning regimens which were more frequently used in paediatric transplants. Figure 3-4 illustrates box and whisker plots highlighting differences in CL and V amongst the categorical covariates.

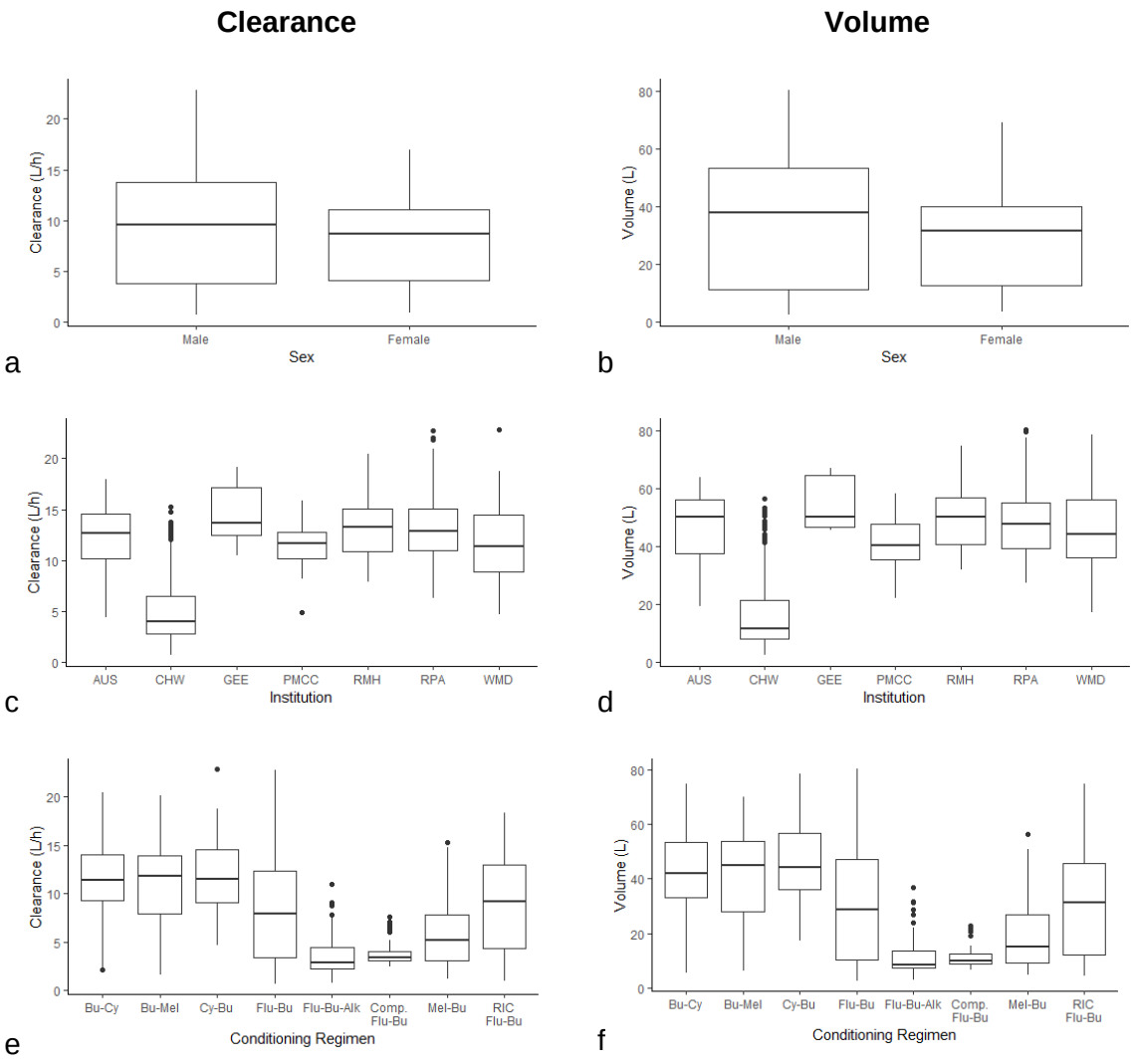


***Figure 3-4*** *Clearance and Volume of distribution for categories of the categorical covariates: Sex, Institution and Conditioning regimen, illustrated using box and whisker*

*plots. The box is comprised of the first and third quartiles separated by the median, while whiskers extend to 1.5 times above and below the interquartile range from the hinges of the box. All points beyond the aforementioned parameters of the box and whiskers are plotted individually as outliers.*

### 3.3.4 Step-wise Covariate Modeling

The step-wise covariate modeling analysis evaluated an additional 33 parameters to characterise the variability in CL and V. The lack of a physiological basis to covariate selection resulted in duplication of covariates accounting for body-size, such as weight and AIBW, both being incorporated on V. Hockey-stick relationships, where two linear functions have a point of intersection, were described between weight and V, and age and V in the analysis. For categorical covariates, parameters were incorporated as factors of the most common category, which was denoted with a value of 1. This resulted in factors being calculated for every category of the covariate, regardless of their effect on CL or V. Parameter coefficients (θ) for these categories were miniscule, with overly large residual standard errors as highlighted with (*) in Table 3-2.

*Table 3-2 Parameter estimates, residual standard errors and, and 5% and 95% confidence intervals for all covariates included from the SCM. Parameters with RSE greater than 50% are marked (*).*

| θ | Description | Estimate | Standard Error | Residual Standard Error | 5% – 95% CI |
|---|---|---|---|---|---|
| 1 | CL | 10.5 | 0.267 | 2.5% | 9.977 – 11.023 |
| 2 | V | 37.1 | 1.09 | 2.9% | 34.964 – 39.236 |
| 3 | CLBSA1 | 0.615 | 0.0054 | 0.9% | 0.604 – 0.626 |
| 4* | CLCONDITIONING1 | -0.0476 | 0.0402 | 84.5% | -0.126 – 0.031 |
| 5* | CLCONDITIONING2 | -0.0695 | 0.0403 | 58% | -0.148 – 0.009 |
| 6 | CLCONDITIONING3 | 0.961 | 0.0793 | 8.3% | 0.806 – 1.116 |
| 7* | CLCONDITIONING4 | -0.0138 | 0.0464 | 336.2% | -0.105 – 0.077 |
| 8 | CLCONDITIONING5 | -0.124 | 0.0581 | 46.9% | -0.238 – -0.01 |
| 9* | CLCONDITIONING6 | 0.0973 | 0.0537 | 55.2% | -0.008 – 0.203 |
| 10* | CLCONDITIONING7 | 0.302 | 0.193 | 63.9% | -0.076 – 0.68 |
| 11 | CLHOSP1 | 0.179 | 0.0397 | 22.2% | 0.101 – 0.257 |
| 12 | CLHOSP2 | 0.222 | 0.0551 | 24.8% | 0.114 – 0.33 |
| 13 | CLHOSP3 | -0.492 | 0.0122 | 2.5% | -0.516 – -0.468 |
| 14* | CLHOSP4 | 0.0438 | 0.0595 | 135.8% | -0.073 – 0.16 |
| 15* | CLHOSP5 | 0.00877 | 0.0667 | 760.5% | -0.122 – 0.14 |
| 16 | CLHOSP6 | -0.471 | 0.0867 | 18.4% | -0.641 – -0.301 |
| 17 | CLSEX1 | -0.0618 | 0.0208 | 33.7% | -0.103 – -0.021 |
| 18 | VAIBW1 | 0.0162 | 0.0002 | 1.3% | 0.016 – 0.017 |
| 19 | VAIBW2 | 0.00936 | 0.0019 | 20.7% | 0.006 – 0.013 |
| 20 | VHOSP1 | 0.124 | 0.0326 | 26.3% | 0.06 – 0.188 |
| 21 | VHOSP2 | 0.193 | 0.0398 | 20.6% | 0.115 – 0.271 |
| 22 | VHOSP3 | -0.118 | 0.0237 | 20.1% | -0.164 – -0.072 |
| 23* | VHOSP4 | 0.0846 | 0.0463 | 54.7% | -0.006 – 0.175 |
| 24* | VHOSP5 | -0.0765 | 0.0517 | 67.6% | -0.178 – 0.025 |
| 25 | VHOSP6 | -0.445 | 0.0714 | 16% | -0.585 – -0.305 |
| 26 | VSEX1 | -0.0591 | 0.0172 | 29.1% | -0.093 – -0.025 |
| 27* | VWGT1 | 0.00036 | 0.0011 | 303.4% | -0.002 – 0.002 |
| 28 | VWGT2 | 0.00623 | 0.001 | 15.4% | 0.004 – 0.008 |
| 29* | VCONDITIONING1 | -0.0231 | 0.0277 | 119.9% | -0.077 – 0.031 |
| 30 | VCONDITIONING2 | -0.0557 | 0.0247 | 44.3% | -0.104 – -0.007 |
| 31 | VCONDITIONING3 | 0.265 | 0.032 | 12.1% | 0.202 – 0.328 |
| 32* | VCONDITIONING4 | -0.00437 | 0.0247 | 565.2% | -0.053 – 0.044 |
| 33* | VCONDITIONING5 | -0.0634 | 0.0373 | 58.8% | -0.137 – 0.01 |
| 34 | VCONDITIONING6 | 0.0605 | 0.0299 | 49.4% | 0.002 – 0.119 |
| 35* | VCONDITIONING7 | -0.017 | 0.079 | 464.7% | -0.172 – 0.138 |

### 3.3.5 Other Covariates

#### *3.3.5.1 Accounting for Body-size*

Weight, AIBW and BSA were all independently tested on the base model, incorporated on both CL and V. Adjusted ideal-bodyweight resulted in the biggest improvement in OFV (-9318) compared to WGT (dOFV -40) and BSA (dOFV -197), and was retained for the rest of the analysis. An exponent of allometric scaling of ¾ was added to AIBW for CL and 1 to AIBW for V to account for the large range of body size in the study population. Inter-individual variability decreased for both CL and V from 69.4% and 76.9% to 21.3% and 13.5%, respectively. Equation 3-5 and 3-6 describes the coefficients for size incorporated on CL and V in the final model, respectively.

*Equation 3-5*

$$F_{sizeCL} = \left(\frac{AIBW}{70\ kg}\right)^{\frac{3}{4}}$$

*Equation 3-6*

$$F_{sizeV} = \left(\frac{AIBW}{70\ kg}\right)^{1}$$

#### *3.3.5.2 Maturation Function*

The sigmoid $E_{max}$ maturation function as described by McCune *et al*. yielded a lower OFV compared to the exponential maturation function developed by Savic *et al*. (dOFV -6.46). Although the sigmoid maturation function was developed using post-menstrual age of patients (PMA, calculated by adding 40 weeks to patient post-natal age),[62] the use of post-natal age resulted in no significant difference compared to post-natal age (dOFV 0.023). As the assumption of a full-term pregnancy (40 weeks) did not improve the fit of the model, post-natal age was retained for subsequent analysis.

Equation 3-7 describes the sigmoid function as the maturation factor ($F_{mat}$) that asymptotically approaches 1 (100% maturity) to explain the difference in CL over post-natal age (labeled AGE). Steepness of the change is governed by the exponent labeled "Hill" in Equation 3-7 and $TM_{50}$ is the age at which maturation is 50% of the adult value.

*Equation 3-7*

$$F_{mat} = \frac{1}{1+\left(\frac{AGE}{TM_{50}}\right)^{-Hill}}$$

### 3.3.6 The Final Model

The final model produced after the covariate analysis incorporated two important aspects of development over the human lifespan, which could contribute to the pharmacokinetic variability in the population: increasing body-size and enzymatic maturation. Individual CL ($CL_i$) and V ($V_i$) were described using the following equations.

*Equation 3-8*

$$CL_i = CL_{pop} \times F_{mat} \times F_{SizeCL} \times e^{(IOV_{CL} + IIV_{CL})}$$

*Equation 3-9*

$$V_i = V_{pop} \times F_{SizeV} \times e^{(IOV_V + IIV_V)}$$

#### *3.3.6.1 Goodness of Fit*

Figure 3-5a shows vast improvement in the population predicted concentrations compared to observed concentrations. The base model over-predicted for the paediatric population and under-predicted for the adult population, resulting in two separate clusters as observed in black. The incorporation of covariates resulted in a dramatic improvement in the predictive capacity of the model with predictive and observed concentrations aligning towards the line of unity.

Unexplained residual variability beyond the incorporation of covariates, IIV and IOV was assessed over time to see any trend in deviation over time or predictions. Trends beyond

y=0 are an indication of model misfit or poor prediction of high or low concentrations. The conditional weighted residuals, as illustrated in figure 3-5c and 3-5d, were dispersed evenly along the x-axis showing no obvious trends of model misfit or poor predictive capacity of the model for plasma concentrations.

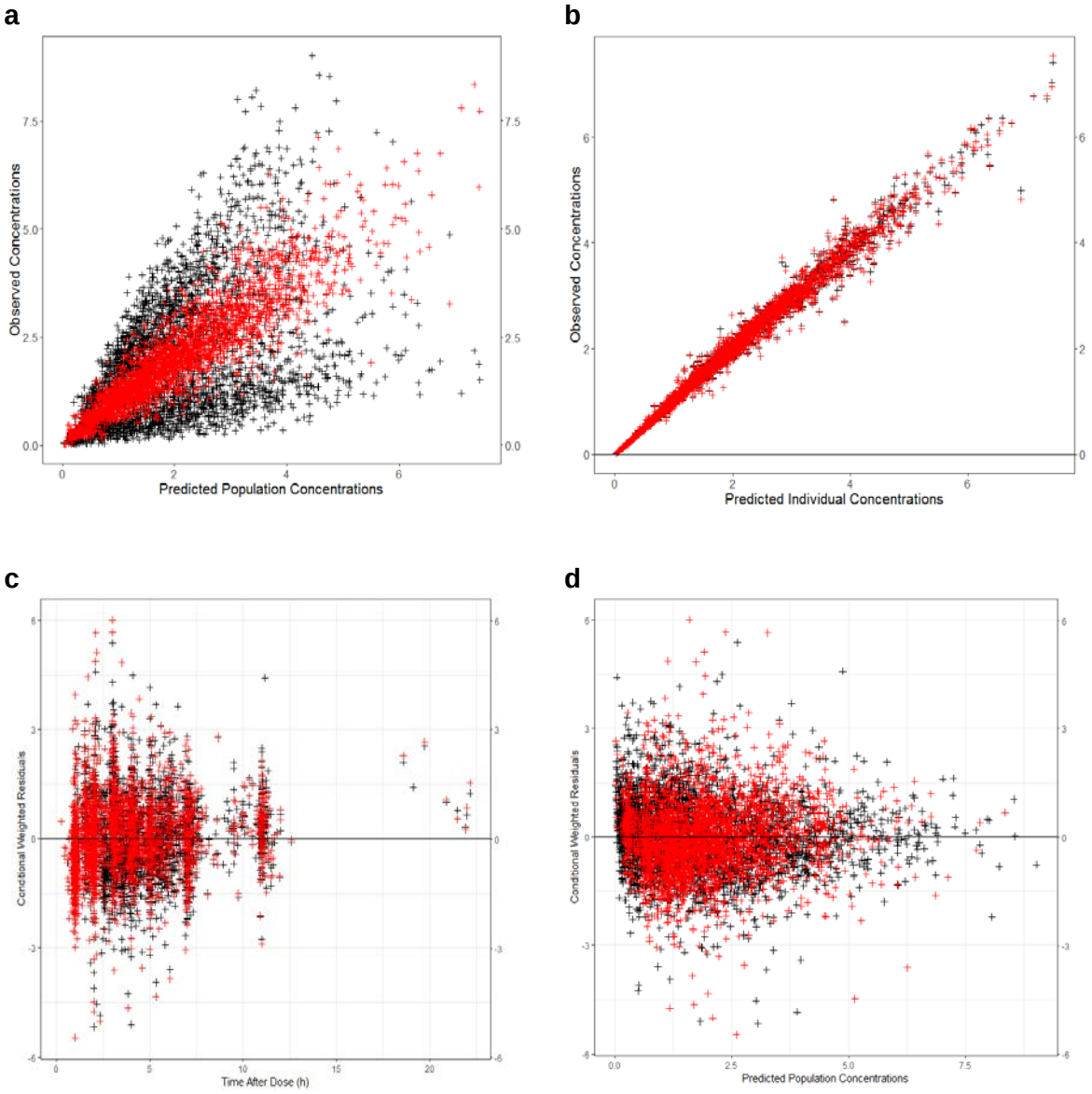


***Figure 3-5*** *Busulfan base and final model goodness of fit plots in black and red respectively, where observations are plotted against a) population predicted concentrations and b) individual predicted concentrations. Conditional weighted residuals (CWRES) are plotted against c) time after dose, and d) against population predicted values.*

#### 3.3.6.1.1 Bootstrap

The final parameter estimates for the model are described below in Table 3-3. A bootstrap of 1000 simulations was performed on the model and the results of the bootstrap median with $5^{th}$ and $95^{th}$ percentile confidence intervals are also provided. The bootstrap simulations confirmed an inter-individual variability of 20% and 13% for CL and V respectively. There was a 14% and 11% inter-occasional variability in CL and V, respectively for each individual. The residual unexplained variability (RUV) was 8%. The OFV for the final model was -9452 which was an improvement of 1120 compared to the base model.

*Table 3-3 Final Estimates of pharmacokinetic model with residual standard errors for the busulfan study population. The median bootstrap estimates of n=1000 simulations with $5^{th}$ and $95^{th}$ percentile confidence intervals are also stated.*

| ***Pharmacokinetic Parameter*** | **Population Estimate (%RSE)** | **Bootstrap Median (5% - 95% CI)** |
|---|---|---|
| *CL (L/h)* | 12.9 (2) | 12.8 (12.4 - 13.3) |
| *V (L)* | 48.1 (1) | 48 (47.3 - 48.7) |
| $IIV_{CL}$*(%)* | 20 (5) | 20 (18 – 22) |
| $IIV_V$ *(%)* | 13 (6) | 13 (12 – 15) |
| $IOV_{CL}$ *(%)* | 14 (7) | 13.5(12 – 15) |
| $IOV_V$ *(%)* | 11 (9) | 11 (9 – 12) |
| $TM_{50}$ *(y)* | 0.29 (17) | 0.30 (0.22 – 0.41) |
| *HILL* | 0.74 (13) | 0.75 (0.61 – 1.03) |
| ***Residual Variability*** | | |
| Proportional Error (%) | 8 (7) | 8 (7.6 - 8.5) |
| *ID/Obs* | 344/3241 | 344/3241 |

#### 3.3.6.1.2 Visual Predictive Check

A visual predictive check was constructed to observe the final model fit over 8 frequency bins (marked as orange scores on the x-axis) for the observed concentrations from the study population. One thousand simulations of concentrations were produced based on model parameters, where predictions were normalised for dose. Observed data from the study population (black scatter plot) fell well within the 5 – 95% confidence intervals of the simulated dataset (upper and lower orange bands) with good agreement between the median observed and simulated concentrations (solid red line and grey band, respectively).

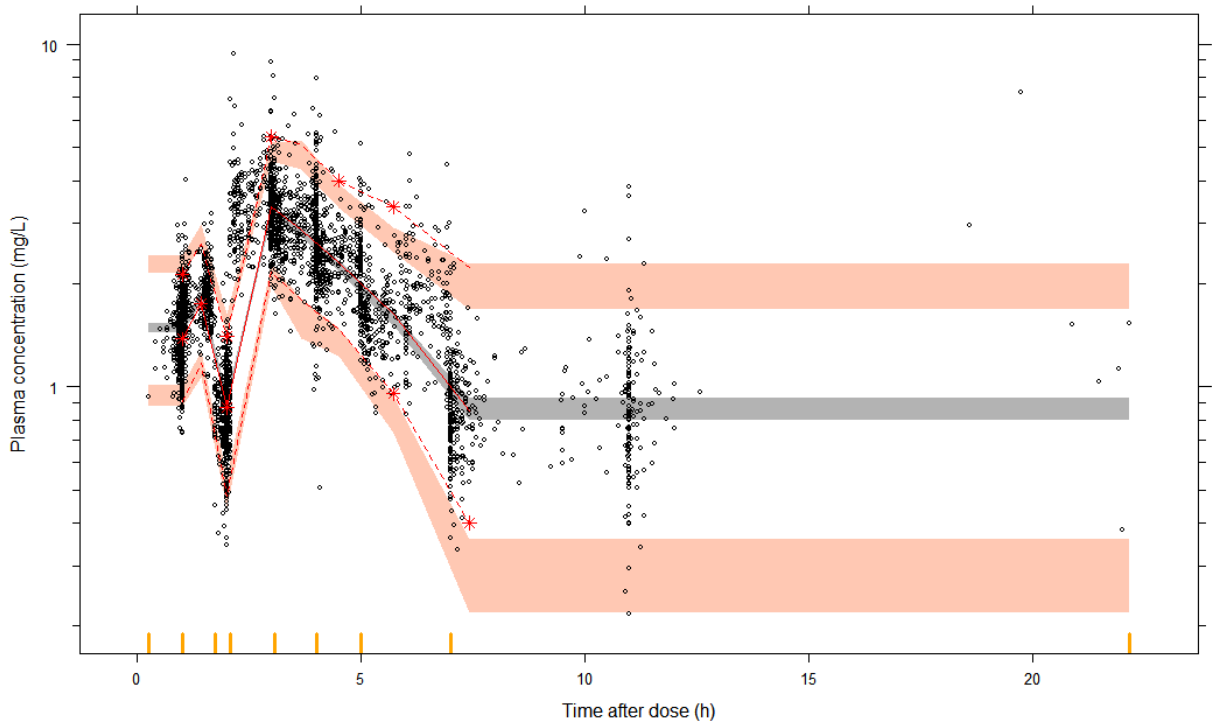


***Figure 3-6*** *Visual predictive check of one thousand simulations with log-transformed, observed plasma concentrations plotted as a scatter plot over time after dose in 8 bins. Median, and 5$^{th}$ and 95$^{th}$ percentile confidence intervals of the simulations are highlighted in bands of grey and orange, respectively, and as solid and dashed red lines, respectively for observed concentrations.*

### 3.3.7 Post Hoc Analyses

#### *3.3.7.1 Variability in AUC*

The *post hoc* calculation of AUC confirmed a high degree of variability in Day 1 AUC (9 to 40 mg.h/L). Dose adjustments based on day 1 AUC were implemented in 40% of the patients, to ensure a cumulative AUC (cAUC) target was reached. Twenty-nine percent of the patients were still out of range for the target AUC despite making dose adjustments. Figure 3-7 highlights dose changes in 142 patients based on the first day of busulfan, with 67 dose reductions and 75 dose elevations.

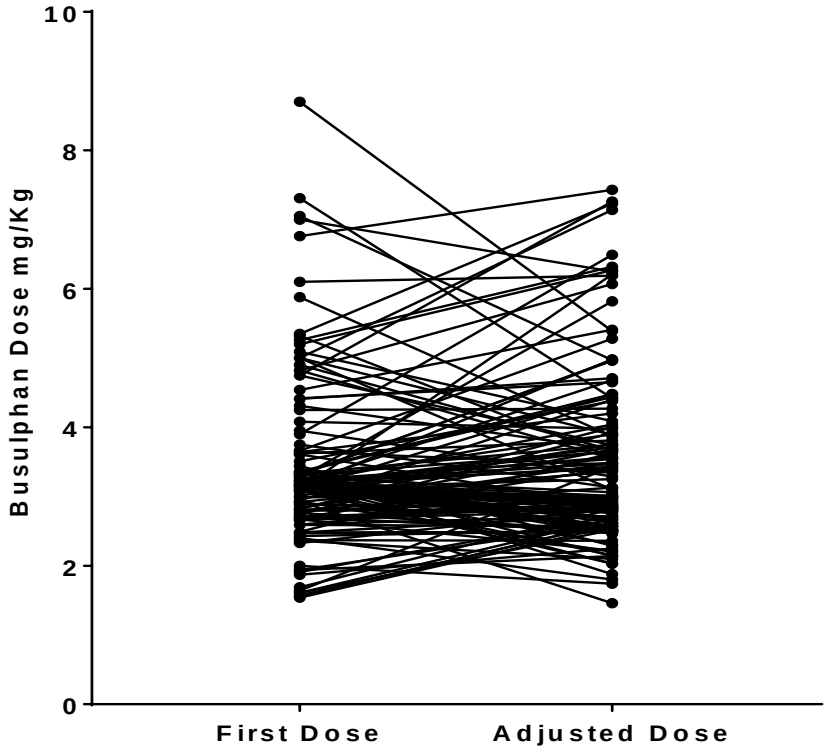


**Figure 3-7 a)** Busulfan dosing (mg/kg) on day one, calculated by body size compared to doses adjusted post TDM in 142 patients

The overall impact of dose adjustment on the cAUC is illustrated in Figure 3-8. Cumulative AUCs predicted from the first dose of busulfan were compared with the observed cAUC at

the end of therapy. Based purely on body-size, predicted cAUCs ranged from 35 mg.h/L to 167 mg.h/L whereas, dose adjustments narrowed the observed range from 48 mg.h/L to 123 mg.h/L. A two-tailed paired t-test of predicted vs. observed cAUCs identified a significant difference in mean cAUC (75 mg.h/L and 79 mg.h/L, respectively) amongst the 142 paired observations (t = 3.2 (df = 141), *P* = 0.0019). Figure 3-8 illustrates the change in cAUCs as a result of dose adjustments on the background of the target 56 – 86 mg.h/L target range. Overall, the dosage adjustments post pharmacokinetic analysis were able to bring cAUCs closer towards the target ranges.

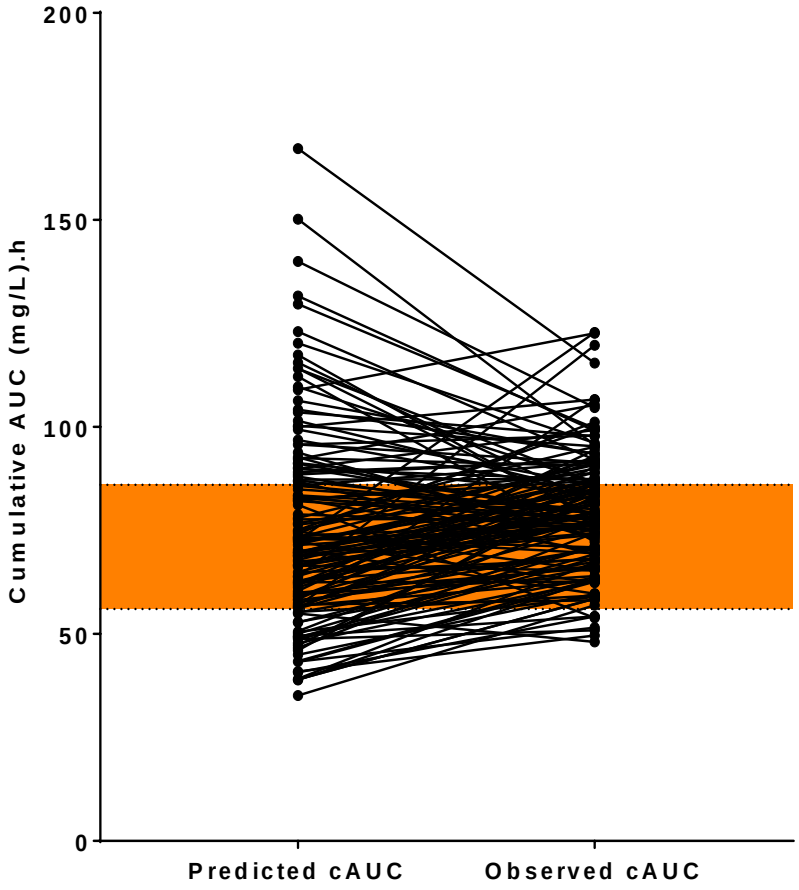


***Figure 3-8*** *The difference in predicted cAUC calculated for a total unadjusted dose from day 1 AUC, to observed cAUC post dosage adjustment in the 142 patients. The target range for cAUC (58 – 86 mg.h/L) is highlighted in orange.*

#### 3.3.7.1.1 Conditioning Regimen

Day 1 AUCs were compared between three groups of patients: those where busulfan was the first administered cytotoxic (n=132), busulfan was concomitantly administered with fludarabine (n=132) and busulfan was administered after a cytotoxic agent (n=80). There were no significant differences in median Day 1 AUCs between the three groups that could suggest prior or concomitant cytotoxic therapy affects busulfan AUC (Kruskal-Wallis H test H = 2.02, n = 344, *P* = 0.36). A similar analysis performed on cAUCs in the three groups also found no significant differences in the median and range (H = 0.73, n= 344, *P* = 0.69). Table 3-4 summarises the median and ranges of Day 1 AUCs and cAUCs of the three groups.

*Table 3-4 Median and ranges for Day 1 AUCs and cAUCs in patients who were administered Bu first, with or after a cytotoxic agent.*

| | n | Median Day 1 AUC (Range) | Median cAUC (Range) |
|---|---|---|---|
| Bu followed by Cytotoxic | 132 | 18.1 (12.4 – 31.2) | 76.4 (37.4 – 131 ) |
| Concomitant Bu-Flu | 132 | 18.3 (9.1 – 40.5) | 76.1 (28.2 – 131) |
| Cytotoxic followed by Bu | 80 | 19.3 (10.8 – 41.3) | 77.4 (19.5 – 120) |

#### 3.3.7.1.2 Changes in Clinical Practice

A change in practice occurred around the year 2012. After this date, TDM in children was performed on every day of busulfan dosing occasion at The Children's Hospital at Westmead. This resulted in a marked improvement in achieving target cAUCs in the paediatric cohort. Figure 3-9a shows the cAUCs of the adult transplant patients over date of transplant during the course of the study, where cAUCs in the adult patients were relatively well distributed around the target range. Figure 3-9b on the other hand illustrates the cAUCs of paediatric transplant patients where two occasion TDM was performed prior to 2012 (before the arrow) and daily therapeutic drug monitoring after the arrow. The

change in practice evidently allowed for better control of busulfan cAUC within the given target range. A chi-squared analysis on 136 patients achieving target cAUCs with or without daily TDM (71%(n = 66) vs 40% (n = 70)) identified significant differences, $X^2$(df = 1, N = 136) = 12.15, $P$ = 0.0005. Two patients who did not make it to transplant were not included in this analysis.

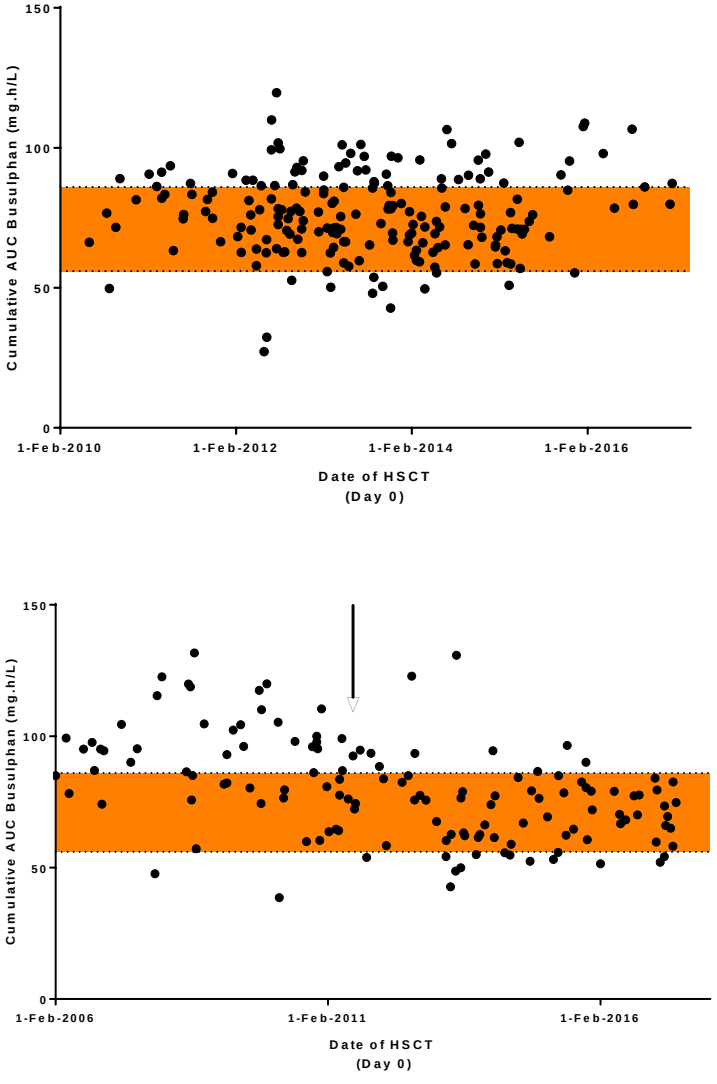


**Figure 3-9** The cumulative AUC of a) adults and b) children displayed chronologically by the date of transplant. A change in practice is observable for children, when two-day TDM was changed to daily TDM (indicated by an arrow in the children's HSCT plotted chronologically), thereby achieving better control on target cAUCs as highlighted in orange for both groups.

The improvement in achieving target cAUCs from daily TDM in the children's cohort was a key finding in this pharmacokinetic analysis of busulfan. Most population pharmacokinetic models of busulfan describe highly variable pharmacokinetics in the paediatric population, but seldom specify techniques to precisely achieve target cAUCs. This study identifies one such technique, which, although labour intensive and expensive due to intensive sampling over multiple days, is justified in achieving target cAUCs in the children's cohort.

#### 3.3.7.1.3 Institutional Differences

There was significant variability in patients achieving target cAUCs across the different institutions (47-91%). These differences could be attributed to a number of factors such as the number of diagnoses, conditioning regimens, concomitant medications and other aspects of the transplant procedure, beyond the scope of this study. Hospitals such as AUS and PMCC treated primarily non-Hodgkin's lymphomas using a single conditioning regimen for autologous transplants, while hospitals such as WMD, RPA, RMH and CHW used a variety of conditioning regimens.

#### 3.3.7.1.4 Concomitant Medications

Haematopoietic stem cell transplants are long and complicated procedures involving a range of medications, such as antibacterials, antifungals, antivirals, prophylactic medications, and immune-suppressants. The possibility of drug-drug interactions in contributing to the PK variability in busulfan cannot be ruled out. Many drugs have been speculated to affect busulfan clearance on theoretical or anecdotal evidence. The following analysis shows the impact of some commonly co-administered drugs on busulfan pharmacokinetics.

*3.3.7.1.4.1 Paracetamol*

Paracetamol is an analgesic that is usually avoided during busulfan dosing due to a drug-drug interaction involving the glutathione-related metabolic pathways of both drugs. The result of this drug interaction would be a potential increase in the serum levels of busulfan. Despite the precaution mentioned in the busulfan product information, there were still limited incidences of concomitant paracetamol use in the medical records of the patients in the study population. An unpaired, Mann-Whitney U test, used to compare the average CL normalized to bodyweight ($CL_{NORM}$, L/h/kg) for each patient for when paracetamol was administered compared to the rest of the population, showed a higher $CL_{NORM}$ (0.199 L/h/kg (n = 69) compared to 0.17 L/h/kg (n = 318), U = 9188, $P$ = 0.0340). In a smaller cohort of 45 patients where paracetamol was administered on some occasions of TDM, a paired, Wilcoxon signed-rank test was used to compare the average $CL_{NORM}$ on days with and without paracetamol administration. No significant differences were observed in the average $CL_{NORM}$ for these individuals (Median of differences = 0.003 L/h/kg, W = 99, $P$ = 0.59).

*3.3.7.1.4.2 Metronidazole*

Metronidazole is a broad-spectrum imidazole antibiotic commonly used to treat gastro-intestinal infections, such as *helicobacter pylori* and *clostridium difficile*. One institution in the study routinely administered metronidazole in transplant patients during myeloablative conditioning at the start of the recruitment period (2010 – 2012). Although the practice has ceased following case reports of a potential drug-interaction between metronidazole and busulfan, the retrospective analysis allowed for a comparison of busulfan AUCs in patients who were administered metronidazole. An unpaired Mann-Whitney U-analysis revealed a lower median busulfan $CL_{NORM}$ in patients receiving metronidazole (0.13L/h/kg (n = 17) compared to 0.18 L/h/kg (n = 327), U = 1203, $P < 0.0001$). To ensure the result was not an

artifact of the paediatric population having a higher $CL_{NORM}$, the same comparison was made in the adult cohort and still found to be significant (0.13 L/h/kg (n = 16)/ 0.17 L/h/kg (n = 191), U = 868, *P* = 0.0035).

*3.3.7.1.4.3 Corticosteroids*

Steroidal agents are potent anti-inflammatory, anti-nausea and appetite-stimulating drugs that can be beneficial in improving the quality of life of an individual during the conditioning regimen. Corticosteroid use was documented in the study population (dexamethasone (n = 58), methylprednisolone (n = 12), hydrocortisone (n = 12) prednisolone (n = 5). An unpaired Mann-Whitney analysis in children showed markedly lower average $CL_{NORM}$ of busulfan in patients with concomitant corticosteroid administration (median 0.2 L/h/kg (n = 34) compared to 0.23 L/h/kg (n = 119), U = 1543, *P* = 0.03). Conversely, a similar test found a slightly higher median $CL_{NORM}$ in adults taking corticosteroids (0.17 L/h/kg (n = 55) compared to 0.16 L/h/kg (n = 154), U = 3187, *P* = 0.006). The majority of adult patients received dexamethasone (n = 49) which resulted in a slightly higher $CL_{NORM}$ in the cohort (median 0.17 L/h/kg (n = 49) compared to 0.16 L/h/kg (n = 159), U = 2861, *P* = 0.005).

*3.3.7.1.4.4 Antifungals*

Triazole antifungals are routinely used as prophylaxis during HSCT. Interactions between some triazole antifungals, such as itraconazole, have been documented with oral busulfan, but not the intravenous formulations. Triazole antifungals were used in 112 patients on at least one occasion of Bu TDM; fluconazole (n = 86), itraconazole (n = 5), posaconazole (n= 11) or voriconazole (n=9). An unpaired Mann-Whitney analysis of triazole antifungal use in patients showed an unexpected higher median average $CL_{NORM}$ (0.19 L/h/kg (n = 112) compared to 0.18 L/h/kg (n = 234), U = 10843, *P* = 0.0094). A similar observation

was made in an unpaired comparison considering only fluconazole administration (0.2 L/kg/h (n = 86) compared to 0.18 L/h/kg (n = 259), U = 8807, *P* = 0.0035). Liposomal amphotericin was also used in 28 patients on at least one occasion of busulfan TDM. An unpaired Mann-Whitney analysis also showed a higher average $CL_{NORM}$ with concomitant amphotericin administration (0.2 L/h/kg (n = 28) compared to 0.18 L/h/kg (n = 316), U = 3413, *P* = 0.04). However, as busulfan $CL_{NORM}$ is known to change over age, the effect of antifungal medications on busulfan CL was reassessed in paediatric and adult populations for triazole antifungals and amphotericin. A repeated unpaired Mann-Whitney U test indeed found an artefact of age confounding results. No significant differences were found for busulfan $CL_{NORM}$ in adults administered or not administered antifungals (0.16 L/h/kg (n = 50) compared to 0.17 L/h/kg (n = 158), U = 3530, *P* = 0.26) or children (0.22 L/kg/h for both groups (n = 62 and n = 76, respectively), U = 2251, *P* = 0.66). Therefore, the use of neither triazole antifungals nor amphotericin affected busulfan $CL_{NORM}$ .

*3.3.7.1.4.5 Antivirals*

Antivirals were commonly administered in patients undergoing a HSCT who had tested serologically-positive for viruses such as CMV, EBV, RSV or hepatitis viruses. Transplantation is usually avoided in acute infections and prophylactic antiviral medications are also administered during the conditioning regimen to avoid opportunistic viral infections during myelosuppression from the transplant procedure. Antivirals were administered to 137 patients in the study cohort on at least one occasion when busulfan TDM was performed (aciclovir (n=63), valciclovir (n =21), valganciclovir (n = 36), ganciclovir (n=19), ribavirin (n=1). An unpaired Mann-Whitney analysis indicated significantly lower median $CL_{NORM}$ of busulfan in patients when administered an antiviral medication (0.17 L/h/kg (n = 137) compared to 0.19 L/h/kg (n= 216), U 11501, *P* = 0.004). However, the difference was confirmed to be an artifact due to changing $CL_{NORM}$ over age.

Separate analyses found no significant differences in the $CL_{NORM}$ for concomitant administration of antiviral medications or not, in children (0.21 L/h/kg (n = 26) vs 0.22 (n = 120), U = 1394, *P* =0.40) or adults (0.16 L/h/kg (n = 96) vs 0.17 L/h/kg (n = 111), U = 5085, *P* = 0.57).

## 3.4 DISCUSSION

In this analysis the pharmacokinetic variability of busulfan was explored in a diverse study population of paediatric and adult transplant patients from multiple institutions. While busulfan pharmacokinetics have been thoroughly explored in adults and children separately, there is limited literature on the evolution of pharmacokinetic variability over the human lifespan. Using data from 337 individuals, this study confirms a high degree of pharmacokinetic variability at a younger age, which requires greater intervention to maintain target exposures (cAUCs).

Exploration using population pharmacokinetic modeling allowed for quantification of the variability between and within individuals, over the various dose events of busulfan administration. The pop-PK analysis successfully produced a robust description of busulfan pharmacokinetics and allowed the estimation of parameters from each individual for further post hoc analyses.

### 3.4.1 The Model

A one-compartment structural model best described the data in the study population. The literature is divided on the structural models used to describe busulfan pharmacokinetics. Dosing guidelines for busulfan based on pop-PK analyses for the FDA[70] and EMA[55], both

use one-compartment models, while more recent descriptions from other larger studies have employed two-compartment models.[61 62] While the reasons for selecting the one-compartment model over the two-compartment alternative have been discussed previously in this chapter, use of the two-compartment model would not have greatly affected the substantive conclusions, which were based on CL and AUC (dose divided CL) in our analysis. Model misspecifications can impact on precision and bias of pharmacokinetic estimates,[149] which were both taken into consideration when selecting the structural base model. A two-compartment model, with similar covariates has been developed for the calculation of initial and Bayesian-adjusted doses by McCune et al.[62]

#### *3.4.1.1 Covariate Analysis*

As part of the population pharmacokinetic analysis, potential contributors to the variability in busulfan pharmacokinetic parameters were tested and identified. Different techniques of model building were employed to reduce selection bias for the inclusion of covariates. Step-wise covariate modeling provides an unbiased method to identify the best fitting covariate relationships on the data based on statistical power, rather than on prior assumptions. Based on the statistical measures for adding and removing covariates, the SCM incorporates as many covariates as needed to characterise the pharmacokinetic variability. This process of adding covariates can result in over-parameterisation, which was observed in the busulfan study population. Thirty-three covariates were estimated to characterise the pharmacokinetic variability in CL and V as stated in Table 3-2. The inherent problems in the methodology of the step-wise covariate modeling analysis were apparent from the large relative standard errors observed for several covariates in the analysis. Evaluation of categorical covariates saw the inclusion of every single category as a covariate, which may or may not have contributed to variability. A large number of parameters can also affect model stability and impact on the robustness of the parameters

for predictions.[142] One way to overcome the issues of overparameterisation are by setting more stringent statistical cut-offs for the inclusion and removal of covariates. Instead of entering an iterative cycle of improving covariate selection in the SCM, the pop-PK model was developed using the most relevant and robust covariates selected by the SCM, such as body-size.

#### *3.4.1.2 Shrinkage*

The pop-PK model produced after an extensive analysis of the study data confirms large variability among individuals and between the various occasions of dosing. Estimates of IIV of 20% and 13% for CL and V respectively are consistent with literature values.[150] Inter-occasion variability in the study population was 13.6% and 10.7% for the two parameters, which is low compared to older studies of busulfan, albeit using different structural models.[150] Given the extensive sampling of busulfan concentrations over time for each occasion of TDM, there was relatively low shrinkage on IIV-CL and V (9% and 12%, respectively). Variability between the occasions over a four to five-day course of dosing, on the other hand, was mostly informed by the cohort of children, in whom TDM was performed daily. The two-occasion TDM for busulfan dosing in adults, and children before 2012, left large gaps in data on occasions when TDM was not performed, and this in turn, affected the precision of parameter estimates for those occasions, resulting in high shrinkage on the estimates of IOV. Shrinkage ranged from 27% to 68% for $IOV_{CL}$ and 35% to 66% for $IOV_{V}$ on occasion 2-5.

#### *3.4.1.3 Allometric Exponent*

Differences in body size explained much of the pharmacokinetic variability present in the busulfan study population. Various measures of body size, such as weight, body surface

area and fat-free mass have also been proposed to effectively explain pharmacokinetic variability in busulfan pop-PK models.[70 75 151 152] While the incorporation of AIBW improved model fit, the addition of the allometric exponent of ¾ actually increased the OFV (dOFV 17). Estimating the allometric exponent on clearance in the model resulted in values (0.69) inconsistent with the literature. One of the reasons for this discrepancy was the discrete distribution of ages in the study population. The allometric exponent on body size is known to be different in children under two and alternative exponents have been recommended for this younger age group.[80] Other studies have used dynamic allometric exponents, that evolve over the human lifespan.[81] The population in the study was not large enough, nor did it have a balanced representation of patients over the entire human lifespan to accurately estimate the allometric exponent for scaling clearance according to body-size and hence the literature value of ¾ was retained for further analysis despite the resultant increase in OFV.

#### *3.4.1.4 Maturation Models*

Maturation functions were tested on the model to account for the significant number of infants and very young children in the study, who are reported to have immature metabolic pathways for busulfan metabolism.[153] There have been two maturation models described for busulfan PK in very young children.[62 69] While both models resulted in an improved OFV, indicating altered busulfan pharmacokinetics in the youngest participants of this study, the sigmoid maturation function was a better option, given the adaptability of the function over the entire human lifespan. As this maturation function significantly improved the model, there was no need for the development of a new function.

Information was not recorded on post- menstrual age for any participants in this study. Given the high number of children under two in the study population, the assumption of a full-term pregnancy by adding forty weeks may have impacted on the maturation profile of children who were indeed premature and would be expected to have a lower GSTA1 activity. Therefore, post-natal age was implemented in the model. As the function is intended to describe differences in enzymatic maturation in the youngest of patients, the degree of change is highest in this population after which, allometric exponent on body-size takes over in explaining the variability in CL. Physiologically however, enzymatic maturation commences during gestation and continues throughout childhood.[78] Therefore, any prospective validation of the model should be conducted using post-menstrual age and parameters should be reassessed.

### 3.4.2 *Post Hoc* Analysis

A range of *post hoc* analyses were conducted to characterise the contribution of various transplant-related factors to pharmacokinetic variability in the study population. While this investigation was by no means exhaustive on all transplant-related factors, a significant focus on concomitant medications and TDM practices allowed us to investigate potential contributions to PK variability. Lower $CL_{NORM}$ was confirmed in patients administered metronidazole in this study, while dexamethasone was found to result in a slightly higher $CL_{NORM}$. Although differences in $CL_{NORM}$ were opposite for adults and children concomitantly administered corticosteroids, the small magnitude of the difference may not result in a significantly different clinical outcome. A significant problem in teasing out differences in CL from the use of concomitant medications was the varying $CL_{NORM}$ profile of busulfan over age. Clearance normalised per kilogram of bodyweight decreases with age[58] which consistently contributed to artefactual differences in analyses of the effect of concomitant medication for antifungals and antivirals. An alternative to overcome the age-

relevant problems in future would be to incorporate concomitant medications into the pop-PK model, however such an analysis would require a larger, more balanced sample-size of patients administered concomitant medications.[144]

The most significant aspect of the *post hoc* analysis arose from the change in TDM practices for the children's cohort, where daily TDM resulted in dramatically better control of cAUC compared to two-occasion TDM, which is still routinely performed in adults. Studies have reported greater unpredictability in busulfan PK of children[58], with only 60% of patients achieving target exposure based on dosing guidelines from pack inserts.[129] However, daily TDM could be explored as a viable alternative, given the established pharmacodynamic relationship between busulfan exposure and clinical outcomes of transplant.[107 116]

A high variability in cAUCs could be attributed to a variety of reasons, such as the heterogenous population, various diagnoses and different conditioning regimens used within the same institution. The Children's Hospital at Westmead treated patients with the largest variety of conditions, ranging from immune-deficiencies to haematological and other malignancies. Most immune-deficiencies surface early in a patient's life and treatment is essential to ensure survival. Transplantation protocols developed for these conditions can also vary significantly in the intensity of the conditioning regimen, and associated surgeries or debridement as performed in conditions such as neuroblastoma. Austin Health and PMCC focused only on autologous transplantations for non-Hodgkin's lymphomas during the time of the study and hence their cohort was homogeneous, with similar conditioning regimens and treating physicians, reducing in part the high variability observed in other institutions.

While this investigation into the pharmacokinetic variability in the study population resulted in the analysis of several aspects of the transplant procedures, there were still several limitations as part of the analysis. Six institutions conducted transplants in adults and only one performed transplants in children. Therefore, there was no absolute way of deducing if the highly variable cAUCs in the children's cohort were a result of demographic factors, or institutional practices. Post hoc analysis in the small number of patients identified differences in CL through univariate comparisons. However, to accurately identify differences in clearance would require a multivariate analysis that includes the fixed and random effect parameters observed in the population.

In all, this chapter identifies several sources of pharmacokinetic variability in adults and children. However, the utility calculating pharmacokinetic parameters lies in the translation of the data into pharmacodynamic effects. Chapter four investigates the relationship between busulfan clearance and the primary endpoint Sinusoidal Obstruction Syndrome (SOS).

# Chapter 4.

## ASSESSING THE INCIDENCE OF SINUSOIDAL OBSTRUCTION SYNDROME AFTER BUSULFAN THERAPY

## 4.1 INTRODUCTION

Sinusoidal Obstruction Syndrome (SOS), previously known as veno-occlusive disease is a known complication of haematopoietic stem cell transplants (HSCT). Associations between busulfan use and an increased incidence of SOS have been frequently documented in the literature and several strategies have been discussed to reduce the incidence and/or severity of SOS. Despite these strategies, SOS is still a persistent side-effect of Bu therapy prior to HSCT. This chapter builds on the pharmacokinetic findings from Chapter 3 to analyse in depth the relationship between busulfan use and the incidence of SOS.

### 4.1.1 Sinusoidal Obstruction Syndrome

Sinusoidal Obstruction Syndrome is a multimodal complication of transplantation where inflammatory responses are followed by increased coagulation and obstruction of hepatic sinusoids, which lead to hepatic dysfunction and, if left untreated, cause liver and multi-organ failure and death. The incidence of SOS can vary from 8% - 30% depending on institution, age of patients, the conditioning regimen used and other transplant-related factors.

The presentation of SOS can range from early (<21 days post transplantation) to later onset (> 21 days). Symptoms of sudden weight-gain from fluid retention, ascites and upper-right quadrant pain and tender hepatomegaly, are usually followed by elevated liver enzymes and bilirubin and a rapid consumption of platelets. Histological changes in the liver and elevated blood pressure in the portal arteries can be identified using doppler ultrasounds. Notable multi-organ dysfunction (MOD) or even -failure (MOF) can be observed in very severe cases of SOS, both associated with poor prognosis.

Much of the treatment for SOS revolves around controlling presentations of the syndrome such as fluid retention and hyperbilirubinaemia. There are currently no therapeutic measures with complete efficacy against SOS and hence, early intervention and treatment are essential to maximise chances of survival in patients. Early detection of SOS requires daily monitoring for weight gain, fluid retention, oedema and ascites, tenderness in the upper right quadrant, hepatomegaly and early signs of jaundice.

Resolution of SOS depends on severity and control over symptoms, as there are still no curative treatments available. Supportive treatment is provided for fluid retention using diuretics such as frusemide or spironolactone, and peritoneocentesis is also used in severe cases of ascites. Beyond the scope of symptomatic treatment of fluid overload, the only proven pharmacological treatment for SOS is defibrotide.[111 154]

### 4.1.2 The Relationship Between Busulfan and SOS

The use of busulfan in HSCT has long been associated with SOS. The earliest descriptions of hepatic injury from busulfan use were recorded in Phase 1 trials for transplant in the 1980's when crystalline deposits were found in liver post autopsy in patients exposed to cumulative doses higher than 16 mg/kg.[35 155] Subsequently SOS was characterised as a potential complication of oral busulfan therapy in patients with higher than expected AUCs from the standard 16 mg/kg doses.[13] As pharmacokinetic variability in patients receiving busulfan became more apparent from PK analyses, an association was made between patients with AUC > 6.2 mg.h/L after a single dose of oral busulfan (given Q6H for sixteen doses) and increased incidence of SOS.[156]

Incomplete and variable absorption of oral busulfan was a contributing factor to busulfan pharmacokinetic inter-individual variability (IIV). Furthermore, possible upregulation of busulfan metabolism pathways in the enterocytes was also thought to increase busulfan clearance (CL/F) in young children, resulting in lower clearances at adult equivalent doses.[84] The replacement of oral busulfan with an intravenous formulation overcomes some of the IIV through bypassing enteric absorption and metabolism, although the impact on the incidence of SOS is conflicting.[157]

As SOS became better characterised, several prophylactic measures, such as the use of ursodeoxycholic acid and therapeutic drug monitoring (TDM), were implemented. Despite all measures, SOS persists in patients receiving busulfan prior to transplantations.

#### *4.1.2.1 Therapeutic Drug Monitoring and SOS*

As TDM of busulfan became common practice during HSCTs, more refined therapeutic windows were identified for optimal efficacy of transplant vs. toxicity of the conditioning regimen and many studies confirmed the association between busulfan overexposure and incidence of SOS. The use of TDM allowed for dose adjustments, which resulted in lower incidences of SOS as reported by Grochow.[109] There was however no association to suggest the severity of SOS was proportional to the exposure to busulfan.

As discussed at length in chapter 1, an exposure-outcome relationship has been documented in several studies exploring SOS incidence in patients receiving busulfan prior to transplant. Regimens where busulfan is administered in sixteen doses (1 mg/kg administered Q6H over 16 doses) with AUC > 6.2 mg.h/L (after a single dose of busulfan given every six hours) have been commonly associated with an increased incidence of

SOS.[13 156 158] A large multi-institutional retrospective analysis of busulfan use in paediatric transplant centres suggested a higher incidence after cumulative AUCs (cAUCs) of all dosing occasions of busulfan of 101 mg.h/L while other studies have found no association between SOS incidence and busulfan exposure[141].

Busulfan TDM has been implemented in adults and children at several institutions in an attempt to overcome interpatient variability in achieving target exposures (cAUC 58 – 86 mg.h/L).[141] The efficacy of TDM in reducing the incidence of SOS is unclear. Studies that do show an exposure outcome relationship recommend the use of TDM for dosing busulfan. However, one large retrospective analysis (n = 13,097) from patients registered in the centre for international blood and marrow transplant research (CIBMTR) registry found a higher incidence of SOS following busulfan TDM, compared to patients with no dose-adjustment.[138] The authors theorised that dose elevations made to achieve target exposures (AUC or $C_{ss}$) were the main reason for the increased incidence of SOS in these patients, although no thorough assessment was made on TDM or dose adjustment practices. This paper contradicts the perceived overall benefit of TDM for busulfan, given the improvement in overall survival and reduced non-relapse related mortality.

### 4.1.3 The Study

The relationship between busulfan exposure and the incidence and time of onset of SOS in transplant patients was assessed in this part of the study. While overexposure of busulfan has been assumed to be associated with an increased incidence, and TDM is suggested to keep exposures within a target window, a recent investigation found a greater rate of SOS in patients receiving TDM as part of their busulfan therapy.[138]

## 4.2 METHODS

### 4.2.1 Data Collection and Analysis

Information was collected from medical records of all patients on symptoms of SOS leading up to diagnosis, as specified in the modified Seattle and Baltimore criteria.[112] Additional information was also collected on rapid consumption of platelets and pulmonary involvement, which were not originally part of the criteria. Time to SOS was calculated from first dose of busulfan to the first mention of SOS (suspected or diagnosed). Patient cAUC and CL as calculated from the pop-PK analysis on the study population in chapter three were used for the investigation. Statistical analysis was performed on the software R (version 3.5.0) on the R Studio platform (version 1.0.136).

### 4.2.2 Exploring the Association Between Busulfan Use and SOS Incidence

Busulfan exposure calculated as cAUC was compared between patients with and without SOS using unpaired Mann-Whitney tests. The association was stratified by post-natal age categories of infant (0 – 2 years), children (2- 10 years), adolescent and young adult (AYA, 10- 25 years) and adults (over 25 years). Differences in cAUC for patients of different conditioning regimens and diagnoses were also compared using Mann- Whitney tests.

The role of TDM in reducing the incidence of SOS was also assessed in comparative Mann-Whitney analyses, where patients with and without dose adjustments and patients with dose adjustments were further divided into patients with dose escalations and dose reductions. Chi-squared tests were used to compare the number of SOS cases in patients over and under the 86 mg.h/L cAUC upper limit, above which incidence of SOS is expected to be greater. A recent recommendation to raise the upper limit was also

assessed using a Chi-squared test to explore the incidence of SOS in patients with a cAUC above or equal to 101 mg.h/L.

Maximum concentration on Day one of busulfan administration is also known to be higher in patients with SOS.[105] To test the association in the study population, day 1 $C_{max}$ was calculated for all patients in the pop-PK analysis using Equation 4-1 where "Dose" refers to the dose of busulfan administered, duration of infusion is identified as "Duration", "CL" the clearance of busulfan and "ke", the rate of elimination. Calculated day 1 $C_{max}$ were then analysed *post hoc* using unpaired Mann-Whitney analyses.

*Equation 4-1* $$C_{max} = \left(\frac{Dose}{CL * Duration}\right) \times (1 - e^{-k_e \times Duration})$$

### 4.3 RESULTS

A total of 66 patients were diagnosed with SOS post busulfan therapy in the study population. Of these, two patients did not have a recorded date of SOS onset and were excluded from the Cox regression analysis for time to SOS post busulfan therapy. The observations were still retained for all association studies.

Onset of SOS ranged from day 9 post first dose of busulfan to day 47, with two outliers who were diagnosed on days 110 and 161 after the commencement of busulfan. Median patient age was 7.5 years (3 months to 64.8 years). The incidence of SOS was 19% for both autologous and allogeneic transplantations (12/63 and 54/281, respectively), and also 19% between HLA matched and mismatched transplants (27/139 and 25/130, respectively). HLA-matching information was not available for 13 patients.

Ursodeoxycholic acid was used as SOS prophylaxis in 215 of the 344 transplant occasions. A further 14 paediatric patients were given defibrotide in addition to ursodeoxycholic acid. The proportion of patients with SOS was 22% (n =47), 50% (n = 7) and 10% (n = 12) in patients given ursodeoxycholic acid, defibrotide and ursodeoxycholic acid, and no SOS prophylaxis, respectively.

There was high variability in the proportion of SOS cases within the various conditioning regimens and diagnoses. The incidence of SOS in the two largest cohorts of diagnoses, AML and myelodysplastic syndrome (MDS) were 14% (n = 117) and 20% (n = 24), respectively. Comparatively, SOS was more prevalent in children with high-risk neuroblastoma (n = 19), acute lymphoblastic leukaemia (n = 21) and juvenile myelomonocytic leukaemia (n = 6), with significantly higher incidences of SOS (47%, 45% and 83%, respectively).

### 4.3.1 Association Between SOS and cAUC

The association between cAUC and the incidence of SOS was assessed in the study population using non-parametric analyses. Median cAUC for patients with and without SOS was 77.5 (42.7 – 122) vs 76.0 (19.5 – 131) mg.h/L, respectively. An unpaired Mann-Whitney U test showed no significant difference in the median cAUCs of patients with or without SOS (U = 9451, $P$ = 0.7).

A chi-squared analysis was performed on patients with or without SOS separated by the upper limit of the accepted therapeutic window for cAUC of busulfan, which was 86 mg.h/L. A total of 18/79 and 48/265 patients were diagnosed with SOS, who had cAUCs

above and below the upper limit of 86 mg.h/L, respectively. There was no significant increase in SOS cases over 86 mg.h/L, $X^2$ (df = 1, N = 344) = 0.86, *P* = 0.35.

#### *4.3.1.1 SOS Stratified by Age*

An initial exploration of the relationship between cAUC, SOS and age, conducted by plotting cAUCs of SOS cases and non-cases over patient age at transplant, found no observable trends that indicate an exposure-response profile for SOS. Figure 4-1 illustrates the cAUCs of patients diagnosed with SOS (blue), which are evenly spread between the highest and lowest cAUC values. As expected from the literature, the density of SOS cases was higher at a younger age.

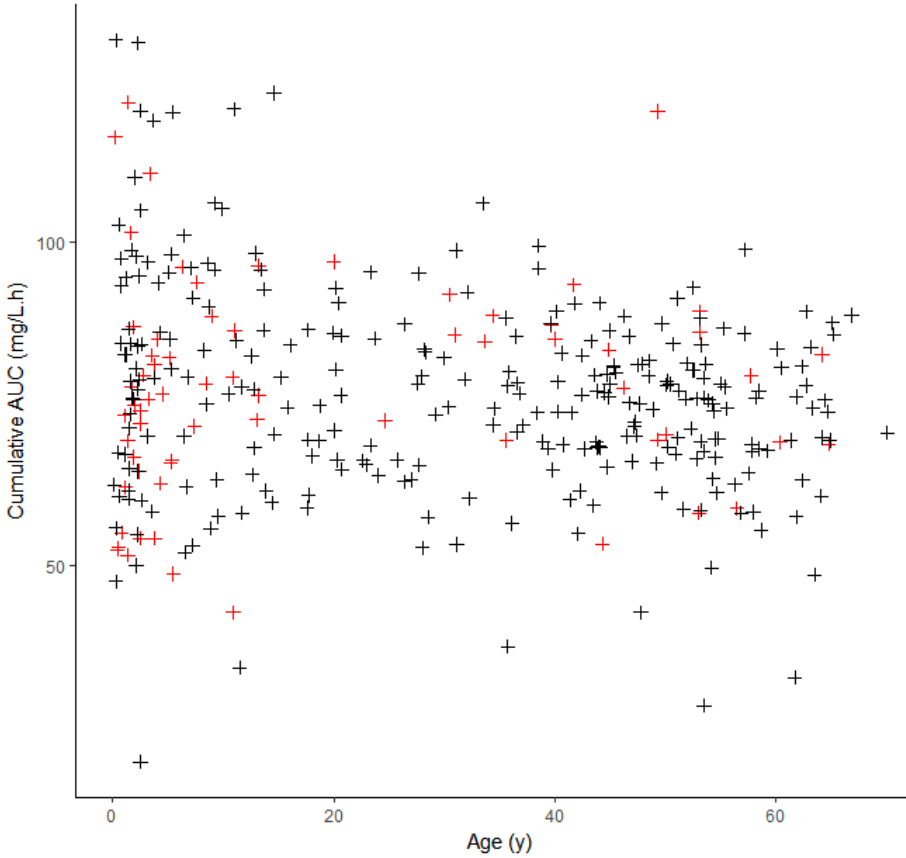


***Figure 4-1*** *Cumulative AUC (mg.h/L) of patients with or without SOS (blue and red, respectively) are plotted over age (years) at transplantation.*

The incidence of SOS was 36%, 32%, 16% and 13% over the infant, children, AYA and adult age groups respectively. There is a higher than average incidence of SOS in infant and children populations, but AYA and adults SOS incidences are consistent with the literature. A Mann-Whitney analysis of the cAUCs between patients with and without SOS in each age category identified no significant differences as summarised in Table 4-1.

A Fisher's exact test was used to analyse the incidence of SOS in patients with cAUCs above and below the 86 mg.h/L cut off. The odds of developing SOS did not increase with exposures higher than 86 mg.h/L for any age category, except in children where higher exposures were associated with a lower incidence of SOS. While the *P* value was significant for the odds ratio in children, the 5 – 95% CI included 1, indicating non-effect and hence could not be used to confidently describe the statistic. Table 4-2 summarises the number of patients with or without SOS, above and below the cAUC upper limit, with odds ratio and 5-95% confidence intervals. Significant results ($P < 0.05$) are marked with an asterisk.

*Table 4-1* Summary of Mann-Whitney U-tests exploring the differences in cAUCs of four age categories of patients with or without SOS

| Age Category | n = | Regimen | % Cases of SOS diagnosed (n) | Median cAUC mg.h/L | | *P* < 0.05 (*) | Median Cmax µg/mL | Median Cmax µg/mL | *P* < 0.05 (*) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | SOS (range) | No SOS (range) | | SOS (range) | No SOS (range) | |
| Infants (0-2 years) | 39 | Bu-Cy, Flu-Bu, Flu-Bu-Alkylating agent, consecutive Flu-Bu, Mel-Bu, RIC Flu-Bu | 36% (14) | 71.2 (51.5 – 122) | 75.99 (47.5 – 131) | 0.46 | 3.51 (2.07 - 4.31) | 3.01 (1.55 – 4.63) | 0.14 |
| Children (2-10 years) | 68 | Bu-Cy, Bu-Mel, Flu-Bu, Flu-Bu-Alkylating agent , consecutive Flu-Bu, Mel-Bu, RIC Flu-Bu | 32% (22) | 76 (48.6 – 111) | 84 (19.5 – 131) | 0.09 | 3.19 (0.93 – 6.15) | 3.12 (1.30 – 7.34) | 0.84 |
| AYA (10-25) | 51 | Bu-Cy, Bu-Mel, Cy-Bu, Flu-Bu, Flu-Bu-Alkylating agent, Mel-Bu, RIC Flu-Bu | 16% (8) | 77.7 (42.7 – 97) | 76.3 (34.1 – 123) | 0.44 | 3.16 (1.79 – 4.15) | 2.11 (0.95 – 3.64) | 0.02* |
| Adults (25+) | 186 | Bu-Cy, Bu-Mel, Cy-Bu, Flu-Bu, RIC Flu-Bu | 13% (22) | 83 (53.3 – 120) | 75.1 (28.2 – 106) | 0.07 | 2.12 (1.58 – 2.95) | 1.92 (1.32 – 5.81) | 0.06 |

Table 4-2 Fisher's exact test summarising the odds of developing SOS in patients with cAUC greater than 86 mg.h/L

| Age Category | cAUC < 86 mg.h/L | | cAUC > 86 mg.h/L | | Odds Ratio (5-95% CI) | *P* < 0.05 |
|---|---|---|---|---|---|---|
| | n SOS | n No SOS | n SOS | n No SOS | | |
| Infants (0-2 years) | 10 | 18 | 4 | 7 | 1.03 (0.176-5.34) | 1 |
| Children (2- 10 years) | 18 | 25 | 4 | 21 | 0.27 (0.06 – 1) | 0.03* |
| AYA (10-25) | 5 | 33 | 3 | 10 | 1.95 (0.26 – 12.3) | 0.40 |
| Adults (25+) | 15 | 141 | 7 | 23 | 2.84 0.88- 8.43 | 0.06 |

#### *4.3.1.2 SOS Stratified by Conditioning Regimen*

The incidence of SOS across various conditioning regimen was between 14% - 32%. There were no significant differences in the cAUCs of patients with or without SOS when grouped under the same conditioning regimens, with the exception of the Cy-Bu regimen.[159] The incidence of SOS amongst conditioning regimens was not significantly different, although certain regimens, such as Cy-Bu have been associated with a lower incidence of SOS compared to Bu-Cy.[159]. Table 4-3 summarises the cases of SOS per conditioning regimen and median cAUCs. Significance was tested once again using unpaired Mann-Whitney tests.

*Table 4-3 The incidence of SOS across the various conditioning regimen used and their corresponding median cAUCs. Significant differences in cAUC tested using unpaired Mann-Whitney tests are highlighted with (*)*

| Conditioning Regimen | n = | % Cases of SOS diagnosed (n) | Median cAUC SOS (range) | Median cAUC mg.h/L No SOS (range) | (P < 0.05) |
|---|---|---|---|---|---|
| Bu-Cy[•] | 58 | 17% (10) | 74.7 (53.3 – 102) | 69.5 (37.4 – 106) | 0.55 |
| Bu-Mel[†] | 74 | 14% (10) | 77.6 (62.7 – 111) | 79.5 (55.4 – 131) | 0.38 |
| Cy-Bu | 42 | 16% (7) | 92.0 (72.4 – 120.4) | 78.4 (53.2 – 106) | 0.04* |
| Flu-Bu[§] | 80 | 16% (13) | 85.0 (43.3 – 109) | 76.0 (34.1 – 123) | 0.20 |
| Concomitant Flu-Bu/ + Cy/Mel or Thiotepa[ɣ] | 43 | 30% (13) | 74.7 (54.2 – 96.46) | 77.3 (55.7 – 131) | 0.59 |
| Complete Flu followed by Bu | 17 | 24% (4) | 70.0 (52.7 – 116) | 94.8 (19.5 – 120) | 0.41 |
| Mel-Bu | 11 | 27% (3) | 82.5 (73.9 – 86.9) | 70.2 (58.3 – 93.7) | 0.50 |
| RIC Flu/Bŭ | 19 | 32% (6) | 53.7 (42.7 – 86.4) | 57.4 (28.2 – 75.2) | 1 |

[•]three patients had concomitant administration of etoposide, [†]one patient had concomitant administration of gemcitabine, [§]concomitant administration of thiotepa in five patients and clofarabine in two other patients, [ɣ]one patient was administered concomitant clofarabine, [ŭ]five patients received concomitant thiotepa out of which four were diagnosed with SOS.

#### 4.3.1.2.1 SOS Incidence Stratified by Number of Chemotherapy Agents per Conditioning Regimen

All 344 transplant occasions of busulfan were given as a combination therapy of either concomitantly administered fludarabine or consecutive administration of fludarabine or an alkylating agent. Patients were divided into three categories:

- Busulfan with fludarabine
- Busulfan with an alkylating agent
- Busulfan with fludarabine and an alkylating agent

Where, additional alkylating agents include: Cyclophosphamide, Melphalan or Thiotepa

The incidence of SOS was 13% (n = 12), 16% (n = 30) and 33% (n = 24) in patients administered busulfan with fludarabine, an alkylating agent, and fludarabine and alkylating agent, respectively. A significantly higher number of SOS cases were initially observed in patients administered busulfan with fludarabine and an alkylating agent, compared to the rest of the cohort $X^2$ (df = 2, N = 344) = 12.6, $P$ = 0.002. The high incidence of SOS could be explained by the fact the aforementioned conditioning regimen was only used in paediatric transplants. A focus on SOS incidence in only the paediatric cohort showed no significant differences in SOS cases over the three regimens, $X^2$ (df = 2, N = 136) = 3.87, $P$ = 0.14.

#### 4.3.1.2.2 SOS Stratified by Dose Adjustments

As described in Chapter 3, dosage adjustments were made as part of TDM to ensure patients received a daily AUC between 4000 and 5260 µMol.min (14.5 and 21.5 mg.h/L). A total of 67 net dose reductions and 75 net dose elevations were made in the study population. The incidence of SOS was 24% (n = 16), 20% (n = 14) and 17% (n = 36) in

patients with net dose reductions, net doses elevations and no dose adjustments, respectively. No significant difference was found in the number of SOS cases in patients with or without dose adjustments, $X^2$ (df = 2, N = 344) = 1.35, *P* = 0.51.

### 4.3.2 Busulfan $C_{max}$ and SOS

Busulfan $C_{max}$ calculated for the first day of administration was compared in adults and children with SOS. At first instance, model calculated $C_{max}$ were not significantly higher in adults or children, although as highlighted in Figure 4-2, there was a tendency for patients with SOS to have a higher $C_{max}$.

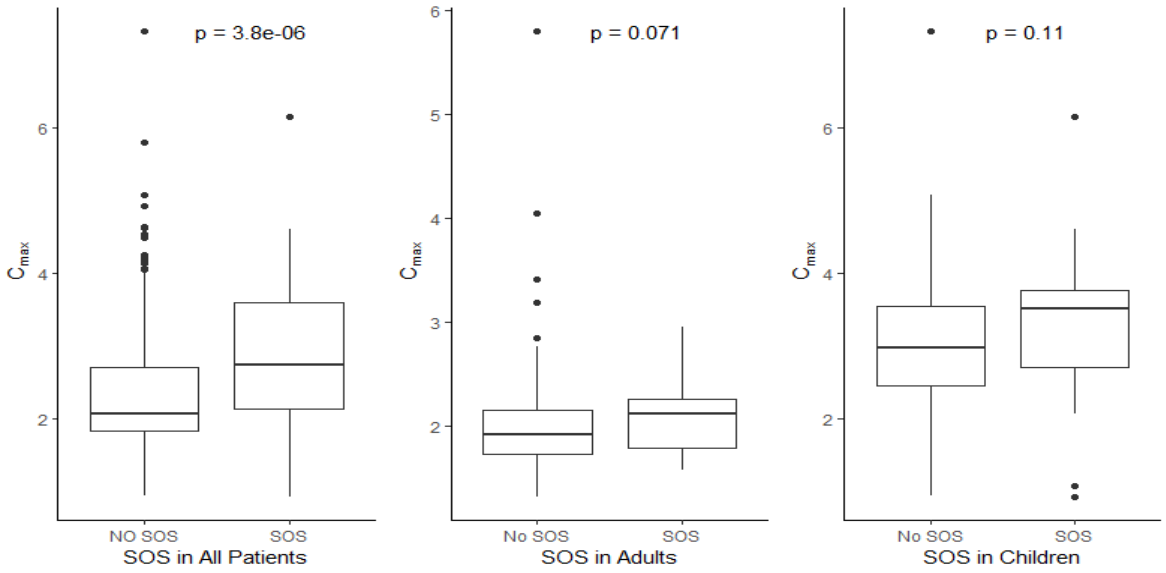


***Figure 4-2*** *Day 1 Boxplot figures illustrating differences in $C_{max}$ of All patients , Adult and children with or without SOS from the study population. Median $C_{max}$ is identified as a bold line dividing the box marking the 25% and 75% quartiles. The whiskers extend to the smallest observation and largest observations within 1.5 x IQR. Points beyond the parameters of the box and whiskers were plotted individually as outliers.*

Patients were divided by dosage regimen, the largest subset of whom (190 adults and 61 children) received the first dose in two halves were retained for this part of the analysis. Here a significant difference in Cmax was evident for the adult population, but no similar finding was observed in the children, as highlighted in Figure 4-3.

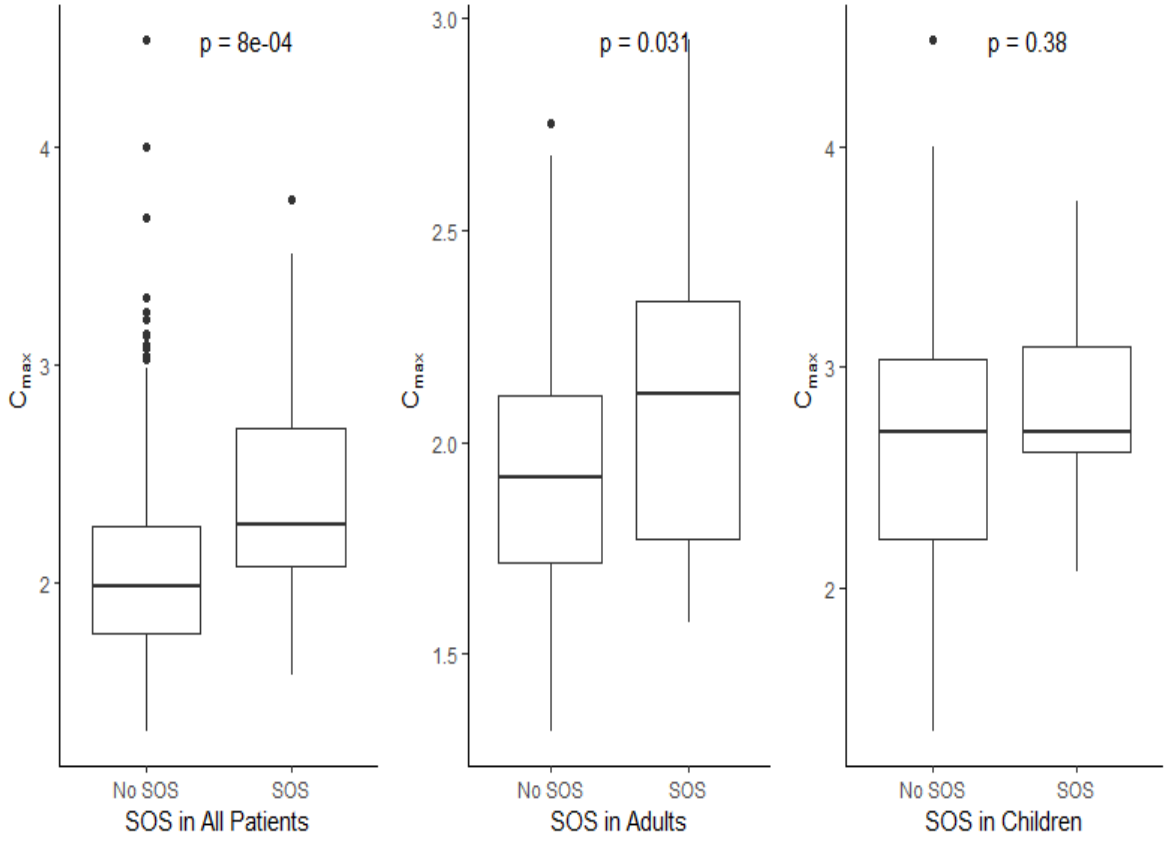


***Figure 4-3*** *Day 1 Boxplot figures illustrating differences in $C_{max}$ of All patients , adult and children with or without SOS receiving the first dose of busulfan in two halves. Median $C_{max}$ is identified as a bold line dividing the box marking the 25% and 75% quartiles. The whiskers extend to the smallest observation and largest observations within 1.5 x IQR. Points beyond the parameters of the box and whiskers were plotted individually as outliers.*

A re-analysis of measured $C_{max}$ concentrations, however unveiled significant differences in patients with or without SOS. Data from patients whose first sample was collected more than 5 minutes past the end of infusion was excluded from the analysis to minimise error for the assumption of true $C_{max}$ post-infusion. A total of 52 and 210 patients, with and without SOS were retained for comparison. Mann-Whitney analyses revealed a

significantly higher $C_{max}$ in patients with SOS (U = 7798, *P* = $1.8x10^{-6}$). As $C_{max}$ is dependent on the dose administered, dosage regimens were stratified into three groups of patients who received the first dose as a half dose (n = 199), as a full dose (n = 55) and others dose forms which included Q12H busulfan for 8 doses and and Q6H for 16 doses(n = 8).

Figure 4-4 illustrates the differences in $C_{max}$ between patients receiving a half or full dose of busulfan. A Mann-Whitley U test failed to identify a significant difference in median $C_{max}$ concentrations of patients with or without SOS receiving a full dose of busulfan on Day 1 (3.71 µg/mL (2.2 µg/mL – 6.11 µg/mL) and 3.5 µg/mL (2.14 µg/mL – 6.22 µg/mL), respectively (U = 411, *P* = 0.23). However, there was a strong effect of median $C_{max}$ for patients administered a half dose of busulfan on Day 1. Thirty-one of 199 were diagnosed with SOS and median $C_{max}$ between patients with and without SOS were 2.66 µg/mL (1.66 µg/mL – 3.78 µg/mL) and 2 µg/mL (1.22 µg/mL – 3.93 µg/mL), respectively (U = 3912, *P* = $9.1 \times 10^{-6}$).

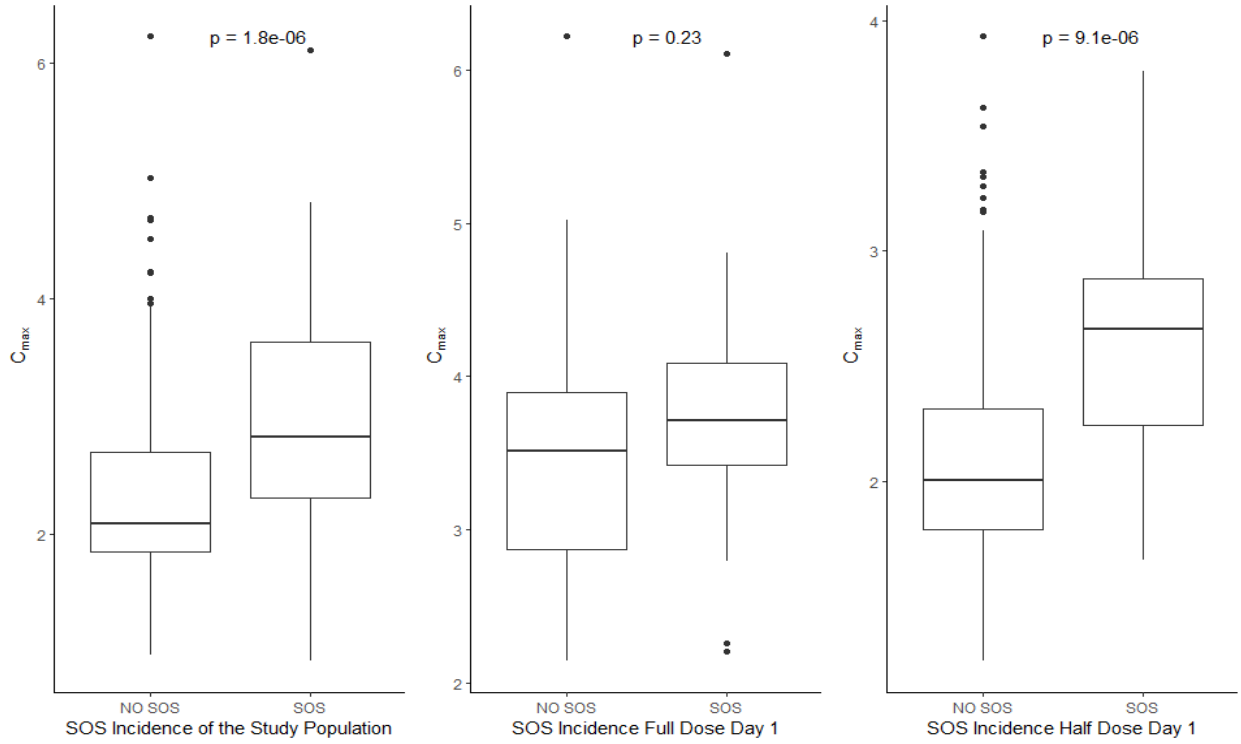


***Figure 4-4*** *The difference in $C_{max}$ of patients with or without SOS, for All patients with a measured $C_{max}$ within 5 minutes at the end of infusion, patients receiving a full dose of busulfan on Day 1 and patients receiving a half dose of busulfan on Day 1. Median $C_{max}$ is identified as a bold line dividing the box marking the 25% and 75% quartiles. The whiskers extend to the smallest observation and largest observations within 1.5 x IQR. Points beyond the parameters of the box and whiskers were plotted individually as outliers.*

The maximum concentration measured in patients receiving half a dose of busulfan on day one had a highly significant association with SOS. Patients transplanted using a myeloablative regimen were administered busulfan over five days, where a full dose of busulfan was divided and administered daily over the first two days. A total 191 patients received the total five day myeloablative therapy, of whom 143 were adults and 48 were children. The differences in $C_{max}$ were reassessed in these subsets of patients.

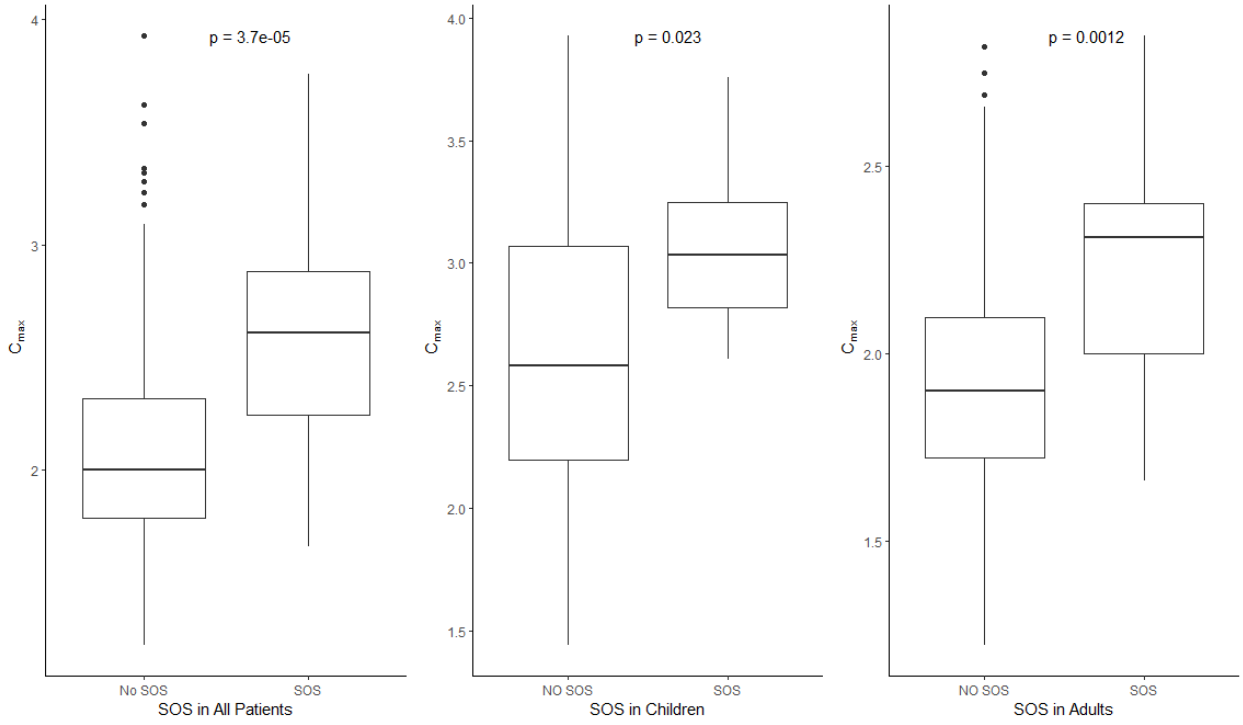


***Figure 4-5*** *Differences in $C_{max}$ of a) all, b) children and c) adult patients with or without SOS receiving the five day myeloablative conditioning regimen. Median $C_{max}$ is identified as a bold line dividing the box marking the 25% and 75% quartiles. The whiskers extend to the smallest observation and largest observations within 1.5 x IQR. Points beyond the parameters of the box and whiskers were plotted individually as outliers.*

Table 4-4 summarises the Day 1 $C_{max}$ observed in all patients, children and adults with or without SOS, receiving the five-day myeloablative conditioning protocol. The significance was maintained in both paediatric and adult patients as demonstrated by a Mann-Whitney analysis of medians, as summarised in Table 4-4.

*Table 4-4 Summary of Mann-Whitney analysis of median busulfan $C_{max}$ in patients with or without SOS receiving myeloablative conditioning over five days.*

| Patient Groups | n = SOS | $C_{max}$ in patients with SOS (µg/mL) | | n = No SOS | $C_{max}$ in patients without SOS (µg/mL) | | *P* - Value |
|---|---|---|---|---|---|---|---|
| | | Median | Range | | Med | Range | |
| All | 27 | 2.61 | 1.66 – 3.76 | 164 | 2 | 1.22 – 3.93 | $3.7 \times 10^{-5}$ |
| Children | 10 | 3.04 | 2.61 – 3.76 | 38 | 2.58 | 1.44 – 3.93 | 0.023 |
| Adults | 17 | 2.31 | 1.66 – 2.85 | 126 | 1.9 | 1.22 – 2.82 | 0.0012 |

## 4.4 DISCUSSION

In this chapter the incidence and time to SOS was examined in a large, heterogenous population of HSCT patients. Given the wide range in patient age, diagnoses, conditioning regimens and transplant-specific factors, an appropriate pairing of patients with and without SOS was not possible. Hence, the study population was divided and analysed in as many ways as statistically advisable.

### 4.4.1 Prophylaxis and SOS

The incidence for SOS was not seen to be higher in patients with allogeneic or HLA-mismatched transplants in the study population. Ursodeoxycholic acid is used as an SOS prophylactic in all paediatric transplants and also in some institutions for adult transplants. Defibrotide however, was only used in addition to ursodeoxycholic acid in a small proportion of paediatric patients (n = 14) at 25 mg/kg, for a separate study that recruited patients known to be at a higher risk of developing SOS. The use of defibrotide for SOS prophylaxis reduced the incidence of SOS in children as part of a Phase III clinical trial[160]

but this was not observed in this study population. Higher than expected SOS incidence was observed in the defibrotide and ursodeoxycholic acid group, possibly due to the patients already being at a higher risk of developing SOS. Also, further analysis was not pursued for this group given the small number of individuals.

### 4.4.2 Diagnosis and SOS

Comparisons between SOS incidence in various diagnoses were not performed as part of this analysis, given the diverse range of patients recruited in the study population. With over 50 different diagnoses, a comparison of statistically balanced numbers was not possible, other than incidences already discussed for AML and MDS. Patients with high-risk neuroblastoma are known to have a high incidence of SOS at around 24%,[161] although the incidence of SOS reported (47%) was almost twice as high in the study population. The proportion of SOS cases for each diagnosis was more influenced by patient age e.g. JMML and ALL are both predominantly paediatric conditions; as was the case for conditioning regimens.

### 4.4.3 Busulfan Exposure and SOS

The large variability in cAUC of individuals receiving busulfan has already been discussed in depth in Chapter 3 of this thesis, however the association between higher cAUCs and SOS, which has routinely been documented in literature, was largely missing in this analysis. One of the reasons for a lack of exposure-outcome relationship is the nature of the concentration-dependent dose modifications that were made during the TDM. Drugs with narrow therapeutic ranges, such as busulfan, were initially given to achieve target doses (16 mg/kg) rather than target concentration. Inter-individual variability resulted in concentrations far higher than expected, and positive associations were identified between

high exposure and SOS. The paradigm shift to concentration-controlled dosing limited exposure of busulfan to a smaller targeted window, with most patients falling within a narrow range not large enough to show a dose-response relationship. This phenomenon has been reported in phase one and dose escalation type studies and is one plausible reason for why an association was not observed.[162]

### 4.4.4 Busulfan $C_{max}$ and SOS

One of the most significant findings of this chapter was the higher observed $C_{max}$ in patients with SOS on Day 1 of busulfan therapy. A greater $C_{max}$ as calculated using pop-PK analysis has recently been associated with SOS in a paediatric population.[105] The analysis mentioned in this chapter, however, did not find a significant difference in calculated $C_{max}$ but in observed $C_{max}$ concentration (based on samples obtained within five minutes of the end of infusion) in patients with or without SOS. The observation was consistent in both adults and children. One of the reasons for this may be inherent bias in the model to fit observations to a one compartment model, and therefore under-predicting $C_{max}$ concentrations. However, a comparison of observed and model drived $C_{max}$ concentrations showed good agreement ($r^2$ = 0.89) and provides little reason to doubt the validity of the model.

A measured concentration $C_{max}$ taken at the end of infusion is a robust, cost-effective predictor which does not require the construction of complicated models. If prospectively validated, $C_{max}$ on day 1 may have a predictive capacity to determine patients at risk of SOS providing timely access to treatment, thereby preventing multi-organ failure.

### 4.4.5 Age and SOS

The incidence of SOS was consistent with the literature for three of the four age categories studied here. Infants and children have always reported higher frequency of cases of SOS[111], albeit not as high as 36%. Out of the seven institutions, only one was involved in paediatric HSCTs and hence cross-institutional comparisons could not be made to thoroughly identify the source of the higher than average incidence. The Fisher's exact test was used in lieu of the chi-squared test to compare the number of SOS cases, due to small numbers of patients diagnosed with SOS in each of the sub-categories. The lower number of children (2 – 10 y) having SOS upon over-exposure of busulfan (>86 mg.h/L) was a surprising outcome for patients with malignancies and immune conditions. Thus far, lower AUC has only been associated with SOS in *β*-Thalassaemia patients. A higher clearance is postulated to either deplete glutathione or produce a toxic metabolite that leads to SOS.[163]

### 4.4.6 Emendation Conditioning and SOS

Over the eight conditioning regimens investigated in the study population, the incidences varied according to the age of patients in which the regimen was used. The incidence of SOS was not found to be different between regimens, although studies have shown lower incidence in patients receiving the Cy-Bu regimen compared to the Bu-Cy.[159] The reasons for this finding remain unclear as SOS cases in both populations (Cy-Bu and Bu-Cy) were similar in their diagnoses (AML, MDS or myelofibrosis), demographic and measures of busulfan exposures. The only major difference in patients receiving the Cy-Bu regimen was the concomitant use of metronidazole which may not have affected SOS incidence, even though cAUCand number of dose changes in both regimens were not different.

The influence of age on SOS incidence became more apparent when conditioning regimens were grouped into three larger categories. Conditioning regimens such as Mel-Bu, Flu-Bu with an additional alkylating agent and consecutively administered fludarabine and busulfan were primarily used in paediatric transplants. These were also regimens with some of the highest incidences of SOS (24-30%). Surprisingly, reduced intensity conditioning with Flu-Bu with or without an additional alkylating agent had the highest proportion of SOS cases (32%). One possible explanation for this finding is pre-existing liver dysfunction in patients prompting the use of RIC regimens over myeloablation. Also, the concomitant use of thiotepa in the children population receiving the RIC regimen may have also confounded the results of the analysis.

### 4.4.7 Dose Adjustments and Physician Discretion

Choice of conditioning regimen or use of prophylaxis were both at physician's discretion and therefore, the possibility of using a more conservative regimen, narrower targets or dose increases of prophylaxis cannot be ruled out. In our study population, no significant differences in terms of SOS incidence were observed between patients with or without dose adjustments. While a recent investigation suggested increased incidence of SOS in patients with doses adjusted using TDM, the underlying causes of the increase were not explored. We compared both dose increases and decreases compared to no adjustment, and found neither to make a significant impact on SOS incidence.[138]

Physician rationale for using a particular conditioning regimen with regards to risk of developing SOS was not studied in this analysis. The onset of SOS is multifactorial and has been shown to depend on several other transplant-related factors, as recently outlined in the revised European guidelines for the current situation of SOS in HSCTs.[111] Several of

these risk factors are unavoidable prior to or during the transplant procedure, which could impact on the number of SOS incidences reported in the study population.[162] Although this is a perceived limitation of this study, the aim was to assess the exposure-response relationship of busulfan and SOS incidence, and hence not all risk factors as highlighted by the EBMT were exhaustively analysed.

In all, this chapter confirms a higher incidence of SOS in younger patients compared to adults. In the study population, the number of cases diagnosed with SOS was not greater in patients receiving busulfan cAUCs higher than the upper limit of 86 mg.h/L. There were also no significant differences in the cAUCs of patients with or without SOS, that could suggest a relationship between busulfan exposure and SOS incidence. However, a higher $C_{max}$ on the first dose was predictive of SOS in patients receiving the same busulfan dosing regimen. This finding likely suggests a predisposition to SOS which, can be identified through a higher $C_{max}$, but cannot be controlled by adjusting total busulfan exposure (cAUC) to a set target. Although this analysis fails to demonstrate a relationship between busulfan exposure and SOS, there may still be a possibility of busulfan use accelerating the incidence of SOS post-transplant which will be evaluated in Chapter 5.

# Chapter 5.

## TIME TO EVENT ANALYSIS OF SINUSOIDAL OBSTRUCTION SYNDROME AFTER BUSULFAN USE

## 5.1 INTRODUCTION

Chapter Four provided insights into sinusoidal obstruction syndrome (SOS) with a detailed analysis of incidence and time to event in the study population. Consistent with the latest EBMT review of SOS, the incidence was higher in children compared to adults. From various analyses we found no association between busulfan cAUC and incidence of SOS. There was however, an association between high busulfan $C_{max}$ (measured within five minutes after the end of infusion) and incidence of SOS.

The complicated course of diagnosis and the retrospective nature of gradation make SOS a complicated toxicity to treat. Furthermore, timely treatment is essential for optimal outcome, due to the possibility of multi-organ failure (MOF) and death if left untreated. Understanding an individual patient's hazard of developing SOS post transplantation can flag patients at most risk to improve treatment and outcome. This chapter aims to implement non-parametric and semi-parametric survival analysis techniques to model time to the incidence of SOS post busulfan administration in the study population.

### 5.1.1 Survival and Hazard Modeling

For the purposes of this chapter, the hazard h(t) was defined as the probability of an individual having SOS at any given time (t) after the first busulfan administration. Survival probability S(t), strictly analysed the cumulative probability of not developing SOS in the study population, from the first busulfan administration to the end of the study. The interplay between the hazard and survival can be modelled using several methods to reflect the time course of an event in a population.

### 5.1.2 Non-Parametric Survival

Survival probability can be analysed using non-parametric methods of analysis such as Kaplan-Meier estimation. Here, events (in this case SOS) are assumed to occur independently of one another and therefore survival probability can be calculated from one interval to the next, and cumulative survival probability is calculated as a multiple of the survival probability between intervals. Equation 5-1 describes the probability of survival (not developing SOS) at time $t_i$ from time $t_{i-1}$ where number of events are described as $n_{SOS}$ and number of patients without SOS at time $t_i$ are described as $n_i$.

*Equation 5-1*

$$S(t_i) = S(t_{i-1}) \times (1 - \frac{n_{SOS}}{n_i})$$

Survival is assumed constant between times of events, resulting in a step function. Kaplan-Meier analyses are particularly effective in comparing survival between two or more groups of patients, through non-parametric statistical tests. The Log rank test is a chi-squared analysis that calculates the number of events expected since the last event between the groups.

The use of Kaplan-Meier analyses is a simple and effective way of determining the difference in survival between two or more groups of patients. However, the analysis is limited by nature to categorical data. Also, for events as complicated as SOS post HSCT, there may be more than one confounding factor, which may not be recognised by a Kaplan Meier analysis alone. Lastly, while Kaplan-Meier analyses are effective at calculating survival, the hazard of developing SOS cannot be easily calculated and hence other means of analysis must also be employed.

### 5.1.3 Cox Proportional-Hazards Modeling of Busulfan

Cox proportional hazards models overcome the issue of confounding factors by offering a method of multivariate analysis, where more than one covariate can contribute to the hazard of developing an event. A Cox proportional hazards model is often referred to as a semi-parametric survival analysis that describes the relationship between the incidence of an event, the time over which the events occurred and the covariates that impacted on the hazard. Equation 5-2 describes the hazard at time t (h(t)) as an exponential function where the baseline hazard at time 0 ($h_0$ (t)) is multiplied by the exponential increase in hazard by p number of covariates ($x_p$), each with their individual weighting or coefficient ($b_p$).

*Equation 5-2*

$$h(t) = h_0(t) \times e^{(b_1x_1 + b_2x_2 + b_3x_3 + \ldots + b_px_p)}$$

The multivariate aspect of the Cox proportional hazards analysis can be described as a multiple linear regression of a logarithm of hazard. Much like linear regression, an assumption of proportionality is made, where a proportional increase or decrease in hazard is observed per unit or category of covariate.

## 5.2 METHODS

### 5.2.1 Software

All statistical analyses were conducted on the software R (version 3.5.0) through the graphical interface R Studio (version 1.0.136). Busulfan pharmacokinetic information is as derived from the population pharmacokinetic model described in Chapter 3. Information on SOS incidence was described in Chapter 5. Time to SOS was calculated in days from first dose of busulfan administration to first mention of potential SOS in medical records.

### 5.2.2 Analysing Hazard for Time to SOS in the Study Population

Kaplan Meier curves were constructed to assess the hazard of developing SOS after commencement of busulfan therapy for conditioning regimens and age categories. Significance was assessed upon both, visual analysis of curves, plotted with 5 – 95% confidence intervals, hazard ratios and log-rank scores between various categories and SOS incidence.

Univariate Cox proportional hazards assessments of various transplant-related factors were undertaken on the time to SOS onset from commencement of busulfan therapy. Continuous covariates such as institution of transplantation, weight, adjusted ideal bodyweight, body surface area, busulfan clearance (L/h/Kg), cAUC, age at transplantation and pre-transplant albumin levels (g/mL) were assessed as causing a proportional increase or decrease in the hazard of SOS per unit increase of covariate at a significance of $P < 0.05$. Dichotomous categorical covariates, such as sex (male/female), transplant type (autologous/allogeneic) and concomitant use of serotherapy, paracetamol, metronidazole or fluconazole with busulfan were assessed as a hazard ratio with 5-95% confidence intervals and significance of $P < 0.05$. A multivariate Cox proportional hazards model was then constructed by incorporating all continuous and categorical covariates into a single analysis to identify the most significant covariates for time to SOS incidence in the study population.

## 5.3 RESULTS

### 5.3.1 Non-Parametric Time to Event Analysis

A total of 341 transplant occasions (64 SOS cases) had incidence or censoring information available for time to event analysis. For the time to event analysis, survival probability is the probability of not having SOS and will be referred to as survival probability hereon. The results of non-parametric and semi-parametric analyses are as reported below:

#### *5.3.1.1 Time to SOS and Conditioning Regimen*

The onset of SOS was analysed in the eight conditioning regimens for busulfan as described in Chapter 2. Median survival probability was plotted from first dose of busulfan to 160 days after, which was the last case reported for SOS. Large overlapping confidence intervals for each conditioning regimen were not plotted for clarity. A log-rank test identified no significant difference in hazards of SOS incidence for the various conditioning regimens ($P$ = 0.19). Survival probability appeared lower for conditioning regimens predominantly used in children, such as consecutively or concomitantly administered fludarabine and busulfan followed by an alkylating agent, or melphalan followed by busulfan.

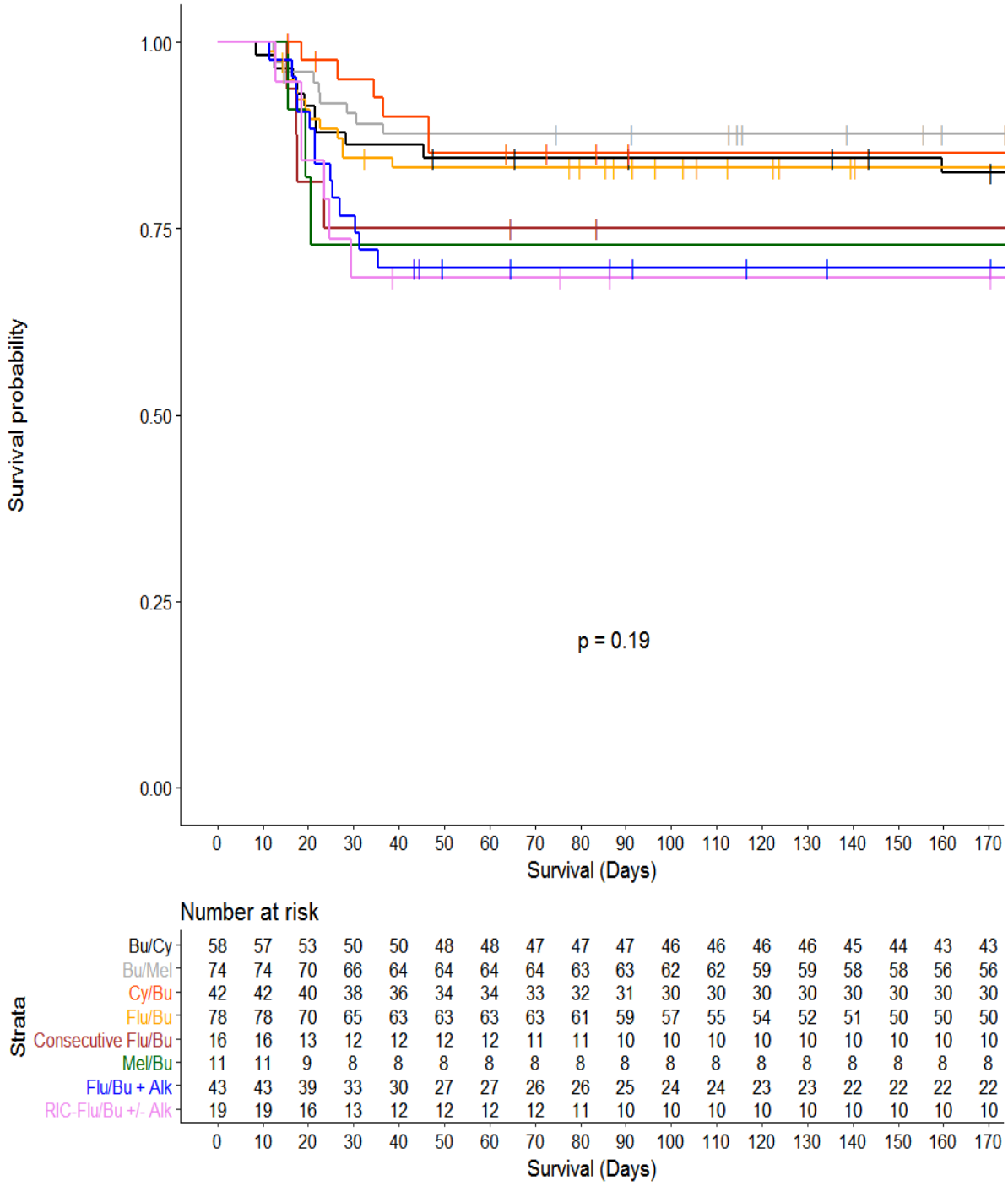


***Figure 5-1*** *Survival probability of developing SOS over Time observed by conditioning regimen: Bu-Cy (black), Bu-Mel (grey), Cy-Bu (orange), concomitant Flu-Bu (gold), consecutive Flu-Bu (brown), Mel-Bu (dark green), concomitant Flu-Bu followed by Cy/Mel or thiotepa (purple) and RIC- concomitant Flu-Bu (grey).*

#### *5.3.1.2 Time to SOS and Autologous vs. Allogeneic Transplantations*

Kaplan Meier curves of time to SOS onset in autologous and allogeneic transplantations revealed no significant differences in survival probabilities as indicated by overlapping confidence intervals and log rank test ($P$ = 0.21) in Figure 5-2.

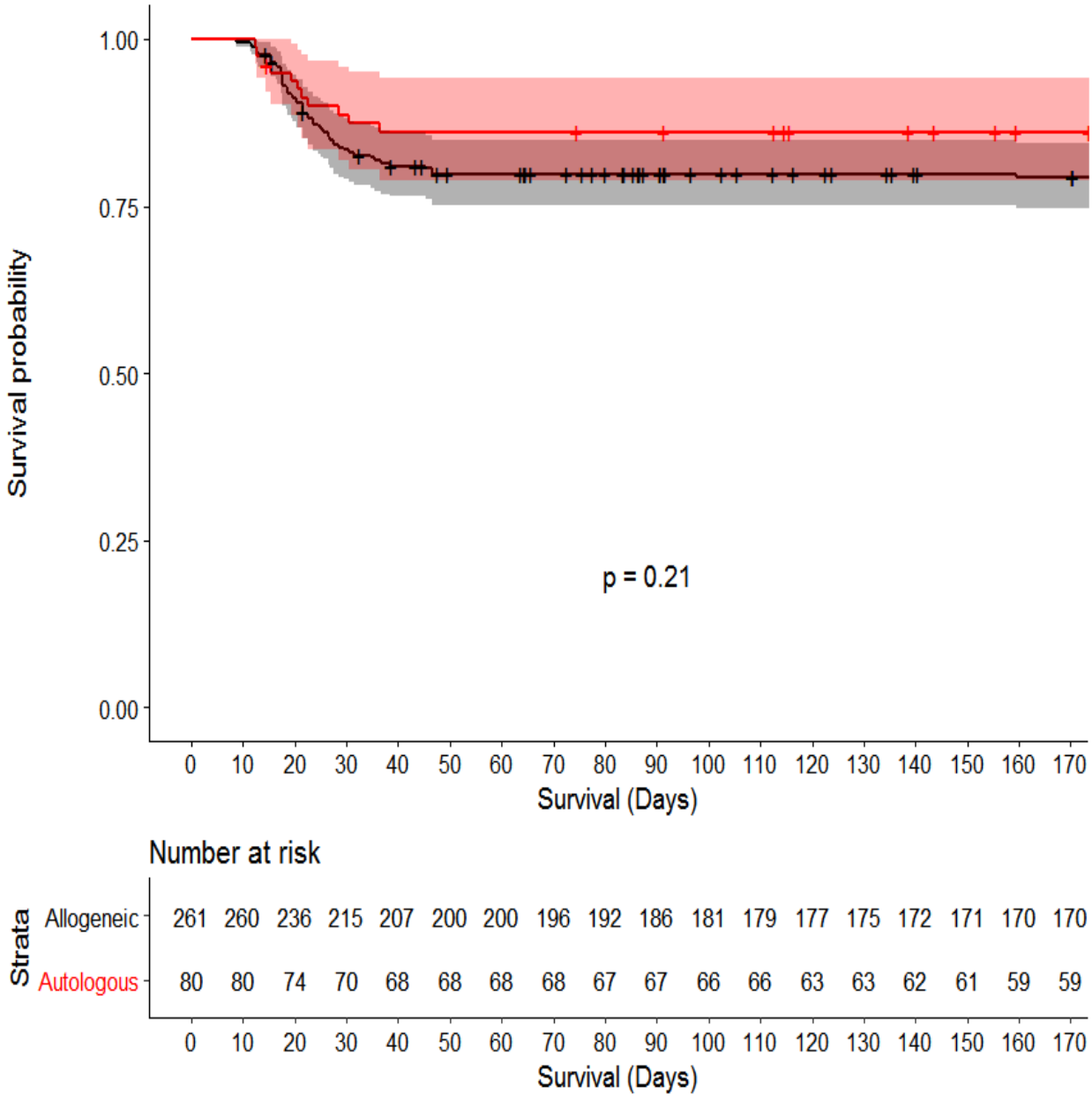


***Figure 5-2*** *Survival probability of developing SOS over Time observed by type of transplant: Allogeneic transplants with 5 – 95% confidence intervals (black), Autologous transplantations with 5 – 95% confidence intervals (red).*

### *5.3.1.3 Time to SOS and Age*

Survival probabilities were significantly different in the adult population compared to the infant and children population as indicated in the Kaplan Meier figure 5-3. A log-rank test for trends analysed age as an ordered categorical covariate and indicated a significant improvement in survival over the categories ($P < 1x10^{-4}$). This was an expected result from association studies in 5.3.1.1 given the marked difference in SOS incidence from infants to adults.

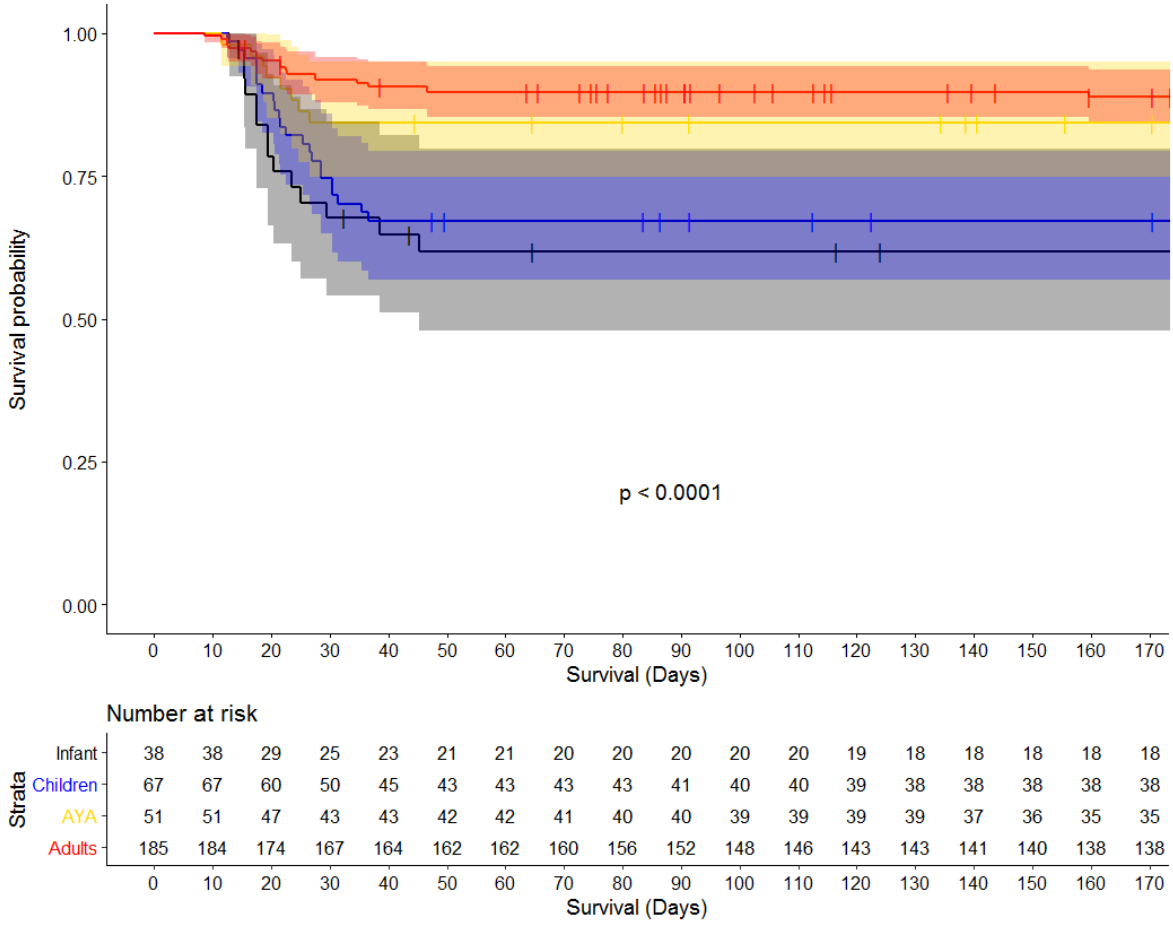


***Figure 5-3*** *Survival probability of developing SOS over Time observed by the following age categories with 5 – 95% confidence intervals: infants (black), children (grey), adolescents and young adults (orange) and adults (red).*

### *5.3.1.4 Time to SOS, and SOS Prophylaxis*

The Kaplan Meier curves were analysed in patients with or without prophylaxis in the study population. A log-rank test identified significant differences ($P$ = 1x10$^{-4}$) over three groups of patients receiving ursodeoxycholic acid, defibrotide with ursodeoxycholic acid and no SOS prophylaxis during transplantation. Figure 5-4 highlighted lower survival probability in patients with SOS prophylaxis, particularly in high risk patients receiving defibrotide with ursodeoxycholic acid.

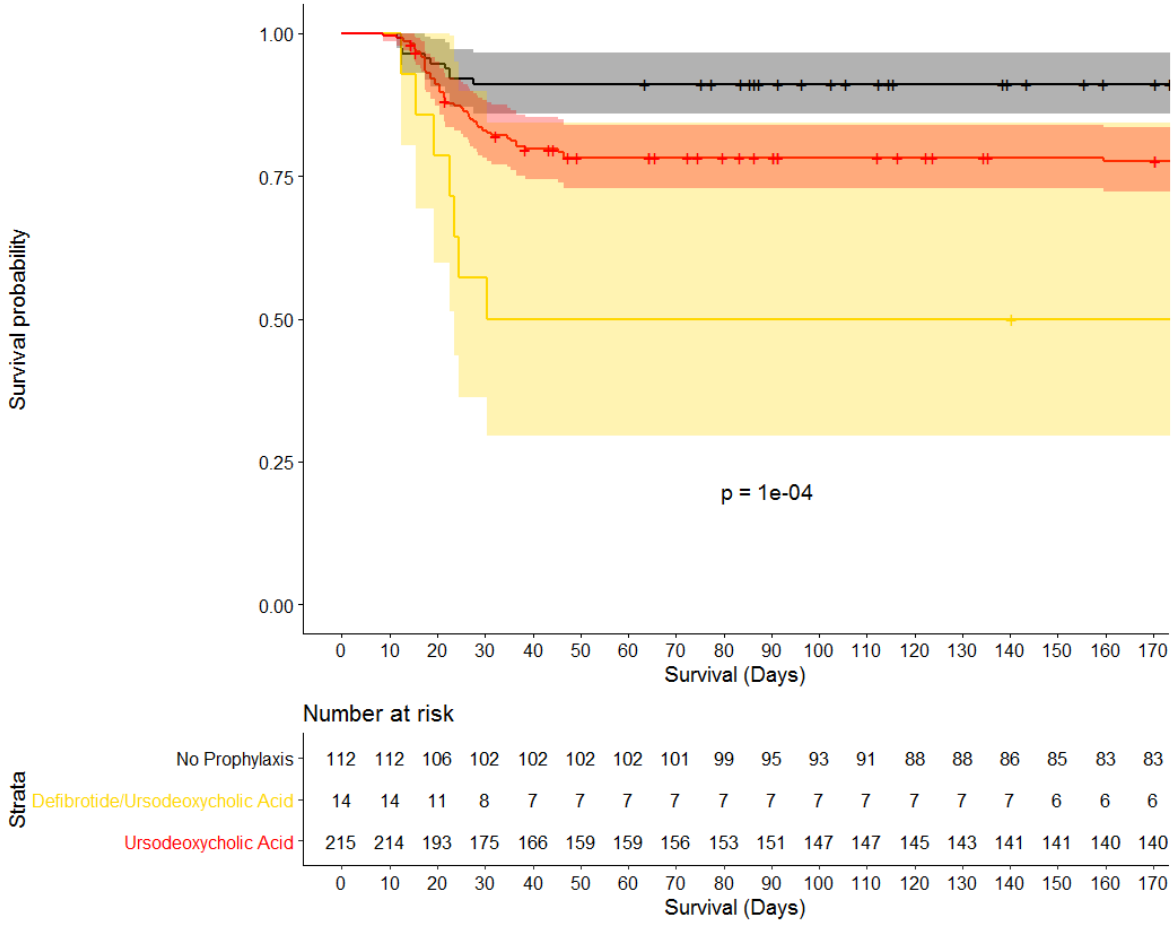


***Figure 5-4*** *Probability of developing SOS over Time observed in patients receiving SOS prophylaxis with 5 – 95% confidence intervals: no prophylaxis (black), ursodeoxycholic acid with defibrotide (orange) and ursodeoxycholic acid (red).*

### 5.3.2 Proportional Hazards Model of SOS incidence

#### *5.3.2.1 Univariate Cox Proportional Hazards Model*

Time to SOS was recorded in 64/66 incidences in the study population. A Cox proportional hazards univariate analysis identified several continuous and categorical covariates that were significantly associated with SOS.

5.3.2.1.1 Continuous Covariates

Analysis of continuous covariates revealed a correlation between body size and SOS, such that adjusted- ideal bodyweight (kg), weight (kg) and age (y) were all significant covariates in the univariate analysis. Also, pharmacokinetic parameters such as busulfan volume of distribution (L), clearance (L/h) (both retaining an element of body-size) and observed $C_{max}$ (µg/mL) were also found to be significant. Pre-transplant albumin (g/L), a marker for liver heath was also identified as significantly associated with the hazard of developing SOS. The magnitude of hazard identified, as the beta coefficient, was highest for $C_{max}$ where a positive correlation of 0.43 was calculated per µg/mL increase and development of SOS, implying higher $C_{max}$ increased the hazard of developing SOS. There was no significant association between cAUC and hazard to SOS in the univariate analysis. The beta coefficient for cAUC also had one of the lowest Wald chi-squared test statistics, indicating the least influence as a covariate on the model. Adjusted-ideal bodyweight scored the highest Wald chi-squared statistic, indicating high significance, confirmed by a highly significant *P* value of $8.7 \times 10^{-8}$. Table 5-1 summarises all continuous covariates tested on time to SOS from day 1 of busulfan dosing. Covariates are sorted in order of the highest Wald chi-squared test score.

*Table 5-1 Continuous covariates for a univariate Cox- proportional hazard model of time to SOS from first dose of busulfan.*

| | $\beta$-coefficient | Hazard Ratio (5%-95% CI for HR) | Wald chi-squared test | *P* value |
|---|---|---|---|---|
| Adjusted Ideal Bodyweight (kg) | -0.026 | 0.97 (0.97-0.98) | 29 | $8.70 \times 10^{-8}$ |
| Volume of Distribution (L) | -0.037 | 0.96 (0.95-0.98) | 27 | $1.70 \times 10^{-7}$ |
| Body Surface Area (m2) | -1 | 0.36 (0.25-0.54) | 26 | $3.10 \times 10^{-7}$ |
| Clearance (L/h) | -0.15 | 0.86 (0.82-0.91) | 26 | $3.50 \times 10^{-7}$ |
| Bodyweight (kg) | -0.02 | 0.98 (0.97-0.99) | 23 | $1.70 \times 10^{-6}$ |
| Age (y) | -7.80E-05 | 1 (1-1) | 18 | $1.80 \times 10^{-5}$ |
| $C_{max}$ (µg/mL) | 0.4315 | 1.54 (1.26 – 1.28) | 4.35 | $1.3 \times 10^{-5}$ |
| Pre-transplant Albumin (g/L) | -0.035 | 0.97 (0.95-0.99) | 12 | 0.00068 |
| Cumulative AUC | 0.00013 | 1 (0.98-1) | 0 | 0.99 |

5.3.2.1.2 Categorical Covariates

A range of categorical dichotomous covariates such as concomitantly administered metronidazole, fluconazole or paracetamol, which are believed to increase SOS incidence through various mechanisms, were not identified as significant in the univariate cox-proportional hazards analysis. Other covariates such as sex, allogeneic vs. autologous transplants and concomitant T-cell depleting serotherapy were also not significant. Surprisingly, concomitant defibrotide for SOS prophylaxis was a significant covariate for the development of SOS with a hazard ratio of 3.5 (1.6 – 7.8), *P* = 0.0016. Fludarabine administration was identified as a weak contributor to the development of SOS with a hazard ratio of 1.7 (1 - 2.7), *P* = 0.044. Table 5-2 identifies all categorical dichotomous

covariates tested on the Cox-proportional hazards model, sorted by highest Wald chi-squared test. .

*Table 5-2 Categorical covariates for a univariate Cox- proportional hazard model of time to SOS from first dose of busulfan.*

| | *β*-coefficient | Hazard Ratio (5%-95% CI for HR) | Wald chi-squared test | *P* value |
|---|---|---|---|---|
| Defibrotide | 1.3 | 3.5 (1.6-7.8) | 10 | 0.0016 |
| Fludarabine | 0.5 | 1.7 (1-2.7) | 4.1 | 0.044 |
| Sex | 0.43 | 1.5 (0.94-2.5) | 2.9 | 0.087 |
| Paracetamol | 0.47 | 1.6 (0.93-2.8) | 2.9 | 0.09 |
| Type of Transplant (Autologous vs Allogeneic) | -0.31 | 0.73 (0.4-1.3) | 1 | 0.32 |
| Antivirals | -0.2 | 0.82 (0.49-1.4) | 0.61 | 0.44 |
| Thymoglobulin | -0.15 | 0.86 (0.5-1.5) | 0.31 | 0.58 |
| Cyclophosphamide | -0.13 | 0.88 (0.53-1.5) | 0.24 | 0.62 |
| Metronidazole | -0.26 | 0.77 (0.24-2.5) | 0.19 | 0.67 |
| Melphalan | 0.086 | 1.1 (0.66-1.8) | 0.11 | 0.74 |
| Cancer diagnosis | -0.1 | 0.9 (0.47-1.7) | 0.1 | 0.75 |
| Fluconazole | 0.014 | 1 (0.58-1.8) | 0 | 0.96 |

#### *5.3.2.2 Multivariate Analysis*

A multivariate Cox proportional hazards model of all significant continuous and categorical covariates from the univariate analyses is described in Table 5-3.. No covariates by themselves were found to be significant in the multivariate analysis confirming the multifactorial nature of SOS onset. The *P*-values for the likelihood ratio test, the Wald test and the Score (logrank) test for the overall models were $4.75 \times 10^{-6}$, $5.26 \times 10^{-6}$ and $2.75 \times 10^{-7}$, respectively, indicating the effect is significant.

*Table 5-3 Multivariate Cox- proportional hazard model of time to SOS from first dose of busulfan.*

| | β-coefficient | HR (5 – 95% CI) | Wald chi-squared test | *P* value |
|---|---|---|---|---|
| **Defibrotide** | -0.63 | 0.53 (0.23 – 1.22) | -1.5 | 0.14 |
| **Pre-transplant Albumin (g/L)** | -0.015 | 0.98 (0.96 – 1) | -1.4 | 0.18 |
| **$C_{max}$ (µg/mL)** | 0.18 | 1.2 (0.90 – 1.6) | 1.25 | 0.21 |
| **Age (y)** | $1.8 \times 10^{-5}$ | 1 (1 – 1) | 0.52 | 0.60 |
| **Adjusted Ideal bodyweight (kg)** | -0.018 | 0.98 (0.91 – 1.06) | -0.45 | 0.65 |
| **Volume (L)** | -0.014 | 0.98 (0.79 – 1.2) | -0.13 | 0.90 |
| **Clearance (L/h)** | *0.021* | 1.02 (0.59 – 1.7) | 0.08 | 0.94 |

## 5.4 DISCUSSION

### 5.4.1 Time to SOS- Analysis by Non- and Semi-parametric Investigations.

An attempt to study time to SOS was made using non-parametric and semi-parametric time to event analyses. Kaplan Meier curves produced for conditioning regimens failed to show significant differences in survival probabilities of the various regimens. Survival probabilities were significantly different in the age categories from infants to adults. This was expected from the aforementioned association studies and is a phenomenon well-supported by the literature.[111 164] While it is unclear as to why there is a higher incidence of SOS in children, there is a trend in recent guidelines towards treating SOS in both patient groups as separate diseases with their own presentations, risk factors, diagnoses and gradations [113 114]. Age was also an underlying risk factor in almost all significant findings of the non-parametric analyses. All paediatric patients received ursodeoxycholic acid as

prophylaxis and some received defibrotide with ursodeoxycholic acid. Therefore, the largest contribution to SOS incidence in the SOS prophylaxis groups was made by the entire paediatric cohort. The marked improvement in survival over the age categories prompted an investigation into how the hazard of SOS would change over age as a continuum. Therefore, a Cox proportional hazards model was employed where age was incorporated as a continuous covariate.

### 5.4.2 Uni- and Multi-variate Cox Proportional Hazards Models

Univariate Cox regression analysis allowed for the incorporation of transplant-related factors as covariates that could influence the hazard of SOS in the study population. The analysis identified several covariates in relation to body-size that affected time to SOS from first day of busulfan dosing. Most of the investigations conducted in this study until this point identified age as the underlying factor in determining SOS incidence, however the results of the univariate analysis identified AIBW as the strongest covariate for characterising SOS hazard. This was attributed to a more consistent distribution of weight over the entire range of the study population compared to age. The association between AIBW and SOS was not maintained in the multivariate analysis, rather a multitude of covariates were found to affect the hazard of SOS in a transplant patient. Of course, the dramatic fall in the rate of SOS incidence between children and young adults as observed in the association studies implies a non-linear relationship between SOS hazard and age, which cannot be tested in a Cox proportional hazard model and is a limitation of the analysis.

Although several confounding factors relevant to busulfan use in transplantation were investigated in this analysis, there were still many aspects missing. Sinusoidal obstruction

syndrome is a complication of transplantation, partly attributed to busulfan use. Other chemotherapeutics such as gemtuzumab and ozogamicin and prior radiation have also been associated with SOS, as have concomitant norethisterone and thalassaemia.[111] The presentation of SOS can be ambiguous in its early stages and differential diagnosis between acute graft vs. host disease of the gut amongst other complications can be difficult. Therefore, inaccurate determination of time of SOS diagnosis, in addition to the potential for under- or over-diagnosis may have influenced the results. Lastly, a time-course of SOS development in patients according to the most recent guidelines requires sophisticated modeling of multiple events such as thrombocytopaenia, hyperbilirubinaemia and also involvement of other organs such as the kidneys and lungs. All of these were beyond the scope of this analysis and are a possible extension for future research.

In all, this chapter aimed to analyse time to SOS after the administration of busulfan. While busulfan cAUC did not impact on the hazard of developing SOS, Day 1 $C_{max}$ as observed at the end of infusion was observed to be a significant covariate. Chapter 6 proceeds to explore predisposition to SOS through an exploratory pharmacogenetic analysis of the study population as a final attempt to understand the development of SOS after busulfan administration.

# Chapter 6.

## PHARMACOGENETIC VARIABILITY OF GLUTATHIONE-S-TRANSFERASES AND OTHER ADME ENZYMES IN PATIENTS RECEIVING BUSULFAN

## 6.1 INTRODUCTION

Chapter three characterised variability in busulfan clearance across the study population using a population pharmacokinetic approach. The effect of concomitant medications on busulfan clearance was also noted, as were the effects of conditioning regimens. Chapter 4 explored the pharmacodynamic relationship between busulfan and the toxicity of Sinusoidal Obstruction Syndrome (SOS). While targeted busulfan exposure, estimated as a cumulative area under the curve (cAUC), was not significantly associated with SOS, a higher $C_{max}$ on the first dose was observed in patients with SOS compared to those unaffected and receiving similar dosage regimens.

### 6.1.1 Genetic Influences on pharmacokinetic variability

A large proportion of busulfan pharmacokinetic (PK) variability has been attributed to differences in metabolism between individuals. Section 1.4.2.3 outlined the metabolism pathway for busulfan through various enzymes. The impact of one or more polymorphisms of the listed enzymes on busulfan clearance have been previously studied. Busulfan is predominantly metabolised by the glutathione *S* transferase (GST) family of enzymes, and various polymorphisms of GST enzymes, particularly GST-A1, -M1 and -T1, have been associated with a lower busulfan clearance.[165] A recent analysis incorporated GSTA1 polymorphisms into a population pharmacokinetic model based on enzymatic activity, to further characterise variability in busulfan clearance.[92] While that study quantified the genetic contribution to busulfan pharmacokinetic variability in a paediatric population, there is no similar study in an adult cohort.

Apart from the GST family of enzymes, there is little information on other enzymes and metabolic pathways that are associated with busulfan PK variability. An exploratory

analysis in a panel of 1936 SNPs in ADME genes, as described by Ten Brink *et al*. in an adult transplant population, did not find any influence of genetic variants on busulfan clearance, with the exception of GSTA1.[166] Enzymatic maturation has been discussed in previous chapters as having an effect on busulfan clearance in young children, and this may also be subject to genetic variation. This level of complexity may be beyond the scope of this thesis. Nevertheless, the impact of genetic variation in a panel of ADME genes could shed further light on sources of pharmacokinetic variability.

#### *6.1.1.1 Linear Regression Analysis of the Influence of Genotype on Busulfan Clearance*

Associations with busulfan clearance are commonly studied using linear regression analysis. Similar to the models constructed in Chapters 3 and 5, linear regression allows for the prediction of a dependent variable (in this case clearance) in an individual. Assuming a linear relationship, clearance can be predicted as the sum of all significant covariates that are present in the individual. A weighting or coefficient describes the magnitude of the effect of each covariate on the parameter. Equation 6-1 describes the mathematical relationship for linear regression where busulfan clearance (CL) is affected by an $n$ number of covariates, each with their own coefficient ($\beta$). Covariates are retained in the analysis based on the *P*-value calculated from the Wald *Z*-score (calculated as the coefficient divided by standard error).

*Equation 6-1* $$CL = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \ldots + \beta_n x_n$$

### 6.1.2 GST and SOS

Beyond the influence of metabolizing enzymes on busulfan pharmacokinetics, genetic variants of GSTA1 and GSTM1 have also been linked to pharmacodynamic outcomes such as a higher incidence of SOS. Although the association is not observed consistently,[167] recent analysis of GSTA1 polymorphisms have found a sex-linked predisposition to developing SOS in patients with the GSTA1*B/*B diplotype.[92] Furthermore, patients with genetic variants of cystathionase (CTH), an enzyme involved in glutathione synthesis, had a greater risk of developing SOS when these variants occurred in conjunction with the GSTA1*B/*B genotype[168]. Patients null for the GSTM1 enzyme are also at increased risk of SOS[169]. Other genetic polymorphisms in enzymes such as methylene-tetrahydrofolate-reductase (MTFR-A1298C) have been shown to independently affect peak levels of bilirubin and duration of hyperbilirubinaemia in patients with SOS after busulfan therapy.[170]

#### *6.1.2.1 Logistic Regression Analysis of Genotypes affecting SOS*

Logistic regression can describe the relationship between a binary variable such as the presence or absence of SOS with one or more covariates. Like linear regression, covariates are incorporated in an additive process using Equation 6-2. Significant covariates are retained based on the p-value calculated from the Wald *Z*- score.

*Equation 6-2*

$$ln\left(\frac{p}{1-p}\right) = \beta_o + \beta_1 x_1 + \beta_2 x_2 \ldots + \beta_n x_n$$

This chapter describes an exploratory analysis on a panel of drug metabolising enzymes and their relationship with busulfan clearance and SOS. Furthermore, this chapter aims to

characterise the contribution of GSTA1 polymorphisms to variability in busulfan clearance and also their association with SOS.

## 6.2 METHODS

### *6.2.1.1 Materials*

Tris EDTA (TE) buffer was sourced from invitrogen by Thermo Fisher Scientific (LOT 00369409) for the dilution of patient DNA samples. DNA concentration was measured using a NanoDrop Microvolume Spectrophotometer® and confirmed using a Qubit® 2.0 fluorescence detector. Working solution and standard solutions (Qubit® dsDNA HS Assay Kit) were supplied by the Bosch Institute at the University of Sydney.

### *6.2.1.2 DNA Extraction*

Patient EDTA blood samples were collected at the time of busulfan therapy. DNA was extracted using Roche MagNA from the fresh blood samples following the manufacturer's instructions and stored as two separate aliquots. The DNA samples and any remaining whole blood were then stored at -80 ºC until further use. Frozen DNA samples were thawed immediately before vortex mixing and centrifuged at 14,000 rpm for 2 minutes prior to use.

### *6.2.1.3 DNA Quantitation*

6.2.1.3.1 Calculating DNA concentration using UV-spectrophotometry

DNA concentrations were measured using UV-spectrophotometry. The Nanodrop™ is a UV-spectrophotometer that can analyse microliter volumes of solutions of DNA, RNA or protein for purity and concentration. The Nanodrop™ was initialised using 1 µL of nuclease free water and TE buffer. One microlitre samples were then placed on the nanodrop

detector to quantify the concentration of DNA. DNA purity was also determined using the 260/280nm ratio as calculated by the Nanodrop. Calculated volumes of DNA samples were aliquoted into Eppendorf tubes to produce 100 µL samples normalized to 5 ng/µL diluting in TE buffer.

6.2.1.3.2 Calculating DNA concentration by fluorescence

Prior to shipping for analysis, DNA sample concentrations were reconfirmed using fluorescence at the Bosch Institute at University of Sydney. The Qubit® working solution was prepared by diluting Qubit® dsDNA HS Reagent at a ratio of 1 µL of reagent to 199 µL of TE buffer. Standards were prepared by diluting 10 µL of standard solution in 190 µL of working solution in an Ultra-clear Qubit™ assay tube.

All normalized samples were tested by adding 5 µL of sample to 195 µL working solution in Ultra-clear Qubit™ assay tubes. Concentration of the normalized samples were back-calculated to the original concentration of the stock and samples were remade to 5 ng/µL concentrations prior to plating on a 96-well plate.

### *6.2.1.4 Exploratory analysis of ADME enzymes and association with busulfan clearance and Sinusoidal Obstruction syndrome*

6.2.1.4.1 The Patient Population

DNA samples were available in a subset of 217 patients (141 adults and 76 children) transplanted between 2010 and 2016 from six out of seven institutions (all except PMCC). Median age was 37 years (4 months – 67 years) and median weight was 66 kg (5.2 kg – 122 kg). One hundred and sixty-three patients received allogeneic transplants and 54

patients received autologous transplants. The largest groups of patients transplanted were treated for AML (n = 70), NHL (n = 32) and MDS (n = 16).

#### 6.2.1.4.2 Analysis

Samples were sent to the Australian Genomics Research Foundation (AGRF) for analysis of common genetic variant in a panel of absorption, distribution, metabolism and elimination (ADME) enzymes using iPLEX PRO chemistry on an ADME PGx Pro panel.

*"AGRF is accredited by the National Association of Testing Authorities, Australia to ISO/IEC 17025:2005 in 8.81.02 Genotyping (Accreditation No. 14332). The test(s) reported have been performed (and this document is issued) in accordance with NATA's requirements. AGRF is also a registered Agena Certified Service Provider (CSP). Agena works closely with CSP providers to ensure technical staff, equipment and workflows meet the standards needed for the highest quality MassARRAY System® research services."*

#### 6.2.1.4.3 Quality Control

Patients with a call rate of less than 90% were excluded from the analysis. All SNPs were tested for deviation from Hardy-Weinberg equilibrium for a *P* – value cut-off of 0.05 with a Bonferroni correction (*P* – value <0.05 divided by the number of SNPs tested). SNPs with a minor allele frequency of less than 1% were excluded from analysis

#### 6.2.1.4.4 Association with normalised CL and SOS

Individual clearance normalised to bodyweight ($CL_{NORM}$) was calculated from the population pharmacokinetic analysis as described in Chapter 3. Age was incorporated as a

covariate for all patients to account for age-related maturation processes. Patients with SOS were identified as described in Chapter 4. A linear regression analysis was constructed for each SNP significantly associated with $CL_{NORM}$ ($P < 0.01$).

Logistic-regression analysis was used to determine associations between SNPs and SOS ($P < 0.01$). Age was incorporated as a covariate on both models to account for changes in clearance over age. Linear regression analysis and additive model development on PLINK was conducted by Alexis Liu (PhD Candidate in Pharmacogenomics) and her contribution to this aspect of the thesis chapter is duly acknowledged.

Copy number variations (CNVs) are repeating numbers of genetic regions, which have been associated with variability in drug responses[171] and were therefore of interest in this study. Copy number variations can occur in individuals due to gene deletions, insertions, inversions, duplications or complex recombinations.[172] Gene deletions are common in the GST family of enzymes, occurring in roughly 50% and 30% of the population for GSTM1 and GSTT1.[173] As the GSTM1 and GSTT1 enzymes are involved in the metabolism pathways for several drugs (including busulfan), patients homozygous for the gene deletions are reported to have greater toxicities, such as leukocytopaenia and thrombocytopaenia from R-CHOP (rituximab with cyclophosphamide, vincristine, doxorubicin and prednisolone) therapy in patients with diffuse large B-cell lymphomas.[174] Similar toxicities have also been reported for GSTM1 and GSTT1 gene deletions in patients administered busulfan prior to HSCTs.[165] Therefore in addition to the SNPs investigated, the influence of CNVs in GSTM1, GSTT1 and also GSTT2 were also studied as part of the exploratory analysis.

#### *6.2.1.5 Assessment of GSTA1 polymorphisms in busulfan clearance and SOS.*

DNA samples collected for 184 patients (117 adults and 67 children) were sent for analysis to the CanSearch group (Université de Genève, Switzerland) under the guidance of Professor Marc Ansari for determination of GSTA1 using a previously reported algorithm.[92] Single nucleotide polymorphisms in the promoter region of GSTA1 at positions T-513-C(rs119649968), A-567-C (rs4715332), A-631-C (rs4715333) and C-1142-G (rs58912740) were analysed and patients categorised into six different haplotypes as summarised in Table 4-1.[175]

*Table 6-1 Summarising the corresponding SNP combinations to denote the haplotype of GSTA1*

| T-513-C | A-567-C | A-631-C | C-1142-G | Haplotype |
|---|---|---|---|---|
| T | A | C | G | *A2 |
| T | A | A | C | *A3 |
| T | A | A | G | *A1 |
| T | C | C | C | *B1a |
| T | C | C | G | *B2 |
| C | C | C | C | *B1b |

Patient diplotypes were consolidated into four ordinal categories separated by activity,[92] as shown in Table 6-2.[176] The impact of diplotype on busulfan $CL_{NORM}$ was assessed between the four categories using a post-hoc analysis of non-parametric Mann-Whitney U-tests.

*Table 6-2 GSTA1 diplotypes divided into four activity groups where group I) is extensive metabolisers, II is normal metabolizers, III normal/slow metabolisers and IV are slow metabolisers.*

| GSTA1 | *A2 | *A3 | *A1 | *B1a | *B2 | *B1b |
|---|---|---|---|---|---|---|
| *A2 | I | I | II | II | II | IV |
| *A3 | I | I | II | II | II | IV |
| *A1 | II | II | III | III | III | IV |
| *B1a | II | II | III | IV | IV | IV |
| *B2 | II | II | III | IV | IV | IV |
| *B1b | IV | IV | IV | IV | IV | IV |

#### *6.2.1.6 Data Analysis*

Association studies and quality control were performed using PLINK v 1.9, GraphPad Prism 7. Haplotype analysis was performed on PHASE 2.1. Analysis of GSTA1 polymorphisms was conducted on R (version 3.5.0) on the R Studio platform (version 1.0.136).

## 6.3 RESULTS

### 6.3.1 Linear and And Logistic Regression Analysis For ADME Enzymes Associated With Busulfan Clearance And SOS

A total of 217 individual DNA samples were available for the exploratory analysis. As problems of power with a smaller sample size were reported in a previous exploratory study where patients were divided into training and validation cohorts, this analysis was conducted using all available samples. Six SNPs were excluded from the analysis due to

a missing rate of >10%. A further 102 SNPs were removed due to minor allele frequencies of <1%. Eleven SNPs were excluded for deviation from the Hardy-Weinberg equilibrium, leaving a total of 67 SNPs for analysis. A list of excluded SNPs is provided in Appendix 5 of this thesis. Given the vast age range, both linear and logistic regression analyses were conducted with age incorporated as a prior covariate.

Linear regression analysis as summarized in Appendix 3 identified the weighting (B coefficient) for each of the genes as a covariate. The significance of each covariate was assessed using the Wald Z-score (B coefficient divided by standard error) and the corresponding *P*-value. No SNPs from the ADME panel were identified to be of significance with regards to busulfan clearance or incidence of SOS by the linear regression analysis.

### 6.3.2 The Influence of GSTM1, GSTT1 and GSTT2 Variants on Busulfan $CL_{NORM}$ and SOS Incidence

#### *6.3.2.1 GSTM1*

A total of 217 patients were included for the analysis of GSTM1, GSTT1 and GSTT2 activity on busulfan $CL_{NORM}$ and SOS. In GSTM1, 125 patients were observed to have a gene deletion (CNV = 0). A further 76 patients had a single copy of the GSTM1 gene and the remaining 22 patients had both copies present. A Mann Whitney comparison of $CL_{NORM}$ in the three CNV groups showed no significant differences in the median $CL_{NORM}$ ( 0.18 L/h/kg (0.09 L/h/kg – 0.33 L/h/kg), 0.18 L/h/kg (0.12 L/h/kg – 0.38 L/h/kg) and 0.16 L/h/kg (0.13 L/h/kg – 0.27 L/h/kg) for CNV = 0,1, and 2 respectively) of the three groups (*P* = 0.47). Figure 6-1 illustrates the difference in $CL_{NORM}$ for the three activity groups of GSTM1.

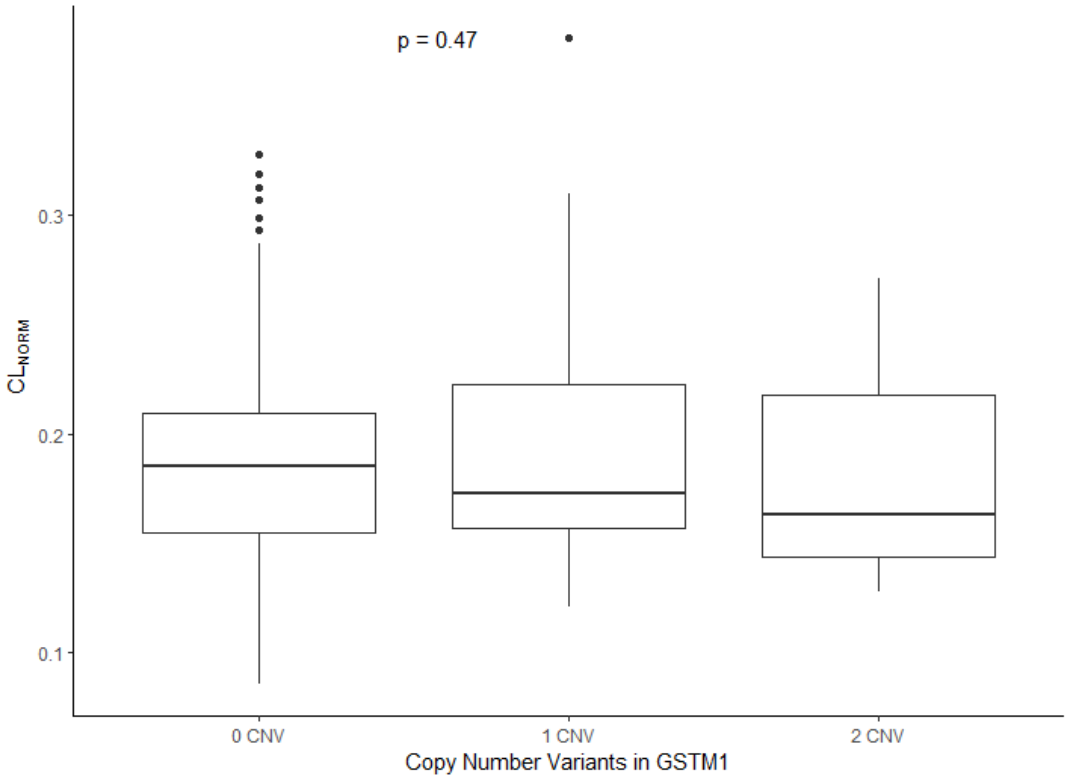


***Figure 6-1*** *$CL_{NORM}$ in patients with a gene deletion, one or two copy number variants for GSTM1. Median $CL_{NORM}$ is identified as a bold line dividing the box marking the 25% and 75% quartiles. The whiskers extend to the smallest observation and largest observations within 1.5 x IQR. Points beyond the parameters of the box and whiskers were plotted individually as outliers.*

For the 92 patients with at least one copy of the gene (CNV > 0), patient alleles could be divided into homozygous *A, homozygous *B or heterozygous *A*B alleles. However, both alleles of GSTM1 have been shown to have comparable activity and therefore were treated as a single group of patients active for GSTM1. A Mann-Whitney analysis failed to reveal any significant differences in median busulfan $CL_{NORM}$ of patients with (0.17 L/h/kg, 0.12 L/h/kg – 0.92 L/h/kg) or without (0.18 L/h/kg, 0.09 L/h/kg – 0.48 L/h/kg) a functional GSTM1 genotype ($U$ = 5388, N = 217, $P$ = 0.429.)

The incidences of SOS in patients with or without functional GSTM1, were 30% and 23%, respectively. No significant differences were observed in SOS incidence of the two groups $X^2$ (d.f .= 1,N = 217,*P* = 0.5) = 0.45. Furthermore, a comparison of SOS incidence between patients with gene deletion, one or two alleles for GSTM1 (23%, 17% and 18%, respectively) did not reveal any significant influence of GSTM1 genotype on SOS incidence $X^2$ (d.f. = 2, N = 217, *P* = 0.69) = 0.74.

### 6.3.3 GSTT1

Gene deletion (CNV = 0) was observed in 49 of 217 patients. A Mann Whitney analysis of busulfan median busulfan $CL_{NORM}$ found no significant differences in patients with (0.18 L/h/kg ,0.09 L/h/kg – 0.92 L/h/kg) or without (0.18 L/h/kg, 0.09 L/h/kg – 0.81 L/h/kg) GSTT1 gene deletion (*U* = 4289, *N* = 217, *P* = 0.66). Furthermore, a $X^2$ analysis of SOS incidence in patients with or without GSTT1 gene deletion (24% and 20%, respectively) failed to find any significant differences $X^2$ (d.f .= 1,N = 217,*P* = 0.66) = 0.20.

### 6.3.4 GSTT2

A total of 56 patients had a gene deletion for GSTT2. A Mann Whitney analysis of median busulfan $CL_{NORM}$ found no significant differences in patients with (0.18 L/h/kg ,0.10 L/h/kg – 0.30 L/h/kg) or without (0.18 L/h/kg, 0.09 L/h/kg – 0.92 L/h/kg) GSTT2 gene deletion (*U* = 4491, *N* = 217, *P* = 0.97). A $X^2$ analysis of SOS incidence in patients with or without GSTT2 gene deletion (18% and 22%, respectively) did not find any significant differences $X^2$ (df = 1, N = 217, *P* = 0.60) = 0.27.

## 6.4 CHARACTERISATION OF GSTA1 POLYMORPHISMS IN THE STUDY POPULATION

Glutathione-S-transferase- α1 polymorphisms were characterised in a separate analysis of 216 patients in the study population. Of these, 23 samples were not usable for analysis and eight samples were uncharacterized beyond determination of the C-69-T SNP on the promoter region. They were included in analysis where only one SNP was used to categorise patients. The analysis reported in this thesis was based on genotyping of either A-52-G or A-567-C, which are in complete linkage disequilibrium with C-69-T. Patients were divided into three groups based on their genotype for -52 or -567, based on GSTA1*A, GSTA1*A/*B and GSTA1*B genotypes. The influence of GSTA1 diplotypes on busulfan clearance normalised to bodyweight ($CL_{NORM}$ L/h/kg) reported purely on the basis of the one SNP are summarised in a boxplot (Figure 6-2).

*Table 6-3 GSTA1 diplotype frequencies in the patient population with mean $CL_{NORM}$ and standard deviation for all patients genotyped for GSTA1 in the study population.*

| Diplotype | Number of patients (%) | Mean $CL_{NORM}$ (range) | SD |
|---|---|---|---|
| GSTA1*A | 86 (45%) | 0.22 (0.07 – 0.37) | 0.056 |
| GSTA1*A/*B | 76 (39%) | 0.19 (0.09 – 0.35) | 0.057 |
| GSTA1*B | 31 (16%) | 0.18 (0.10 – 0.31) | 0.051 |

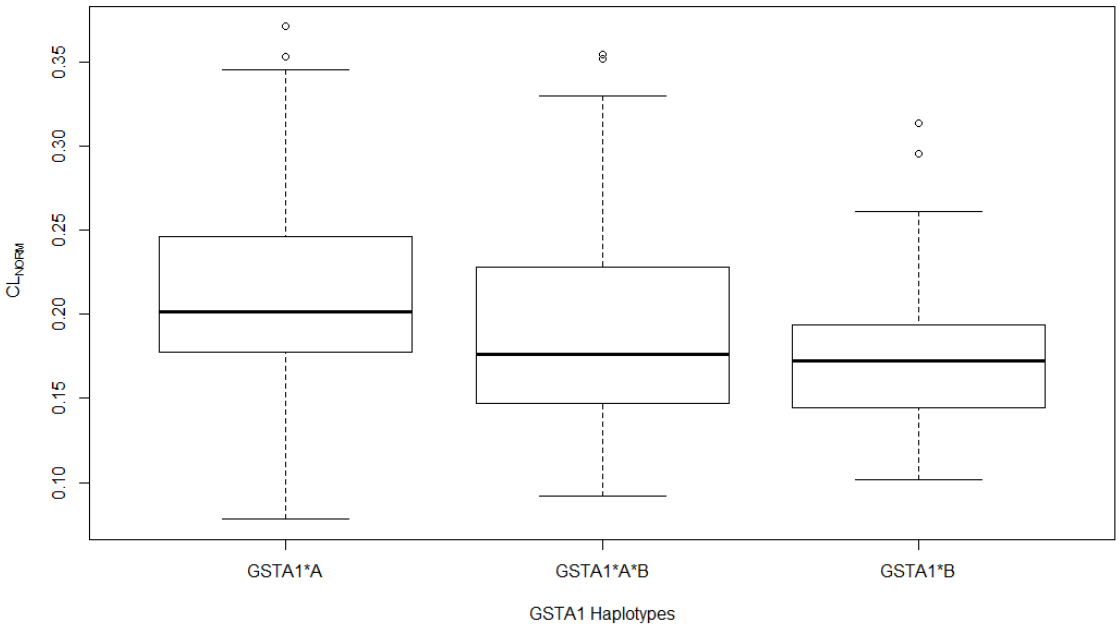


***Figure 6-2*** *Boxplots comparing median $CL_{NORM}$ (L/h/kg) in three haplotypes of GSTA1 determined by the A-52-G or A-567-C SNPs. Median $CL_{NORM}$ is identified as a bold line dividing the box marking the 25% and 75% quartiles. The whiskers extend to the smallest observation and largest observations within 1.5 x IQR. Points beyond the parameters of the box and whiskers were plotted individually as outliers, as present in the intermediate, normal and slow metabolisers.*

An unpaired t-test between all groups found $CL_{NORM}$ in patients with the GSTA1*A genotype (mean $CL_{NORM}$ = 0.22, SD = 0.056) to be significantly higher than that in patients with either the GSTA1*A*B genotype (mean $CL_{NORM}$ = 0.19, SD = 0.057) or GSTA1*B genotype (mean $CL_{NORM}$ = 0.18, SD = 0.051). The t-statistic, degrees of freedom and significance are summarised in Table 6-4.

*Table 6-4 Patient GSTA1 polymorphisms on C-69-T using an unpaired t-test of mean $CL_{NORM}$. Significance is reported with 5$^{th}$ and 95$^{th}$ percentile confidence intervals of the difference*

| Group 1 (n = ) | Group 2 (n = ) | *T (df)* | *P* – Value | Mean difference in $CL_{NORM}$ | 5$^{th}$ – 95$^{th}$ percentile confidence intervals of the difference in mean $CL_{NORM}$ |
|---|---|---|---|---|---|
| GSTA1*A (86) | GSTA1*A/*B (76) | 2.89 (156) | 0.004 | 0.03 | 0.008 – 0.04 |
| GSTA1*A/*B (76) | GSTA1*B (31) | -1.43 (63) | 0.15 | 0.02 | -0.039 – 0.006 |
| GSTA1*A (86) | GSTA1*B (31) | 3.84 (58) | 0.0003 | 0.04 | 0.02 – 0.06 |

#### *6.4.1.1 The Association Between Busulfan Clearance and GSTA1 Activity*

Beyond the distribution of patients into GSTA1*A,*B or *A*B haplotypes, GSTA1 polymorphisms were categorised according to the recently-described functional groups developed from the combinations of four SNPs. Table 6-5 characterises the patient diplotypes and summarises their frequency. The table divides the various diplotypes into the four functional groups of rapid, intermediate, normal and slow metabolisers as described by Ansari *et al*.[92]

*Table 6-5 Number of patients per diplotype of GSTA1, separated into the four categories of groups I -Rapid, II - Intermediate, III- Normal and IV- Slow metabolisers of busulfan.*

| | GSTA1 Diplotype | Number of Patients (%) | Number of patients per functional group |
|---|---|---|---|
| *Group I (Fast)* | GSTA1*A2/*A2 | 14 (7%) | 16 (8%) |
| | GSTA1*A3/*A3 | 2 (1%) | |
| *Group II (Fast-Normal)* | GSTA1*A1/*A2 | 22 (11%) | 59 (31%) |
| | GSTA1*A1/*A3 | 2 (1%) | |
| | GSTA1*A2/*B1a | 20 (10%) | |
| | GSTA1*A2/*B2 | 1 (1%) | |
| | GSTA1*A3/*B1a | 1 (1%) | |
| | GSTA1*A3/*B2 | 13 (7%) | |
| *Group III (Normal)* | GSTA1*A1/*A1 | 41 (21%) | 63 (33%) |
| | GSTA1*A1/*B1a | 18 (9%) | |
| | GSTA1*A1/*B2 | 4 (2%) | |
| *Group IV (Slow)* | GSTA1*A1/*B1b | 9 (5%) | 44 (23%) |
| | GSTA1*A2/*B1b | 3 (2%) | |
| | GSTA1*A3/*B1b | 1 (1%) | |
| | GSTA1*B1a/*B1a | 23 (12%) | |
| | GSTA1*B1a/*B1b | 5 (3%) | |
| | GSTA1*B1b/*B1b | 2 (1%) | |
| | GSTA1*B2/*B2 | 1 (1%) | |
| *Undetermined* | GSTA1*A/*A | 3 (2%) | 9 (5%) |
| | GSTA1*A/*B | 6 (3%) | |
| Total | | 193 (100%) | |

Distribution of patients across the four categories of predicted GSTA1 activity in adults was consistent with literature available on paediatric data, with the exception of Group 4, which was higher (23% vs 14.5% in literature).[92] Categorising patients by activity allowed for the inclusion of patients with diplotypes of lower frequency in the analysis. Busulfan clearances calculated using the population pharmacokinetic model were normalised to bodyweight to account for size-related differences. Table 6-6 highlights the median $CL_{NORM}$ (L/kg/h) for patients across the four activity groups with median ages. Difference in median busulfan clearance between two groups were assessed using a one-way analysis of variance, which found no significant difference in $CL_{NORM}$ of patients grouped in either activity group ($F(3,180) = 2.17$, $P = 0.09$).

*Table 6-6 The patients as divided in each category of GSTA1 metabolisers.*

| GSTA1 category | Median Age (y, range) | Median $CL_{NORM}$ (L/kg/h, range) |
|---|---|---|
| I | 19 (0.6 – 58) | 0.20 (0.15 – 0.37) |
| II | 40 (1.4 – 65) | 0.18 (0.11 – 0.35) |
| III | 41 (0.12 – 65) | 0.19 (0.07 – 0.35) |
| IV | 25 (0.3 – 65) | 0.17 (0.10 – 0.31) |

Figure 6-3 summarises the differences in distribution of $CL_{NORM}$ across the four functional groups through a boxplot. A marked difference in the $CL_{NORM}$ of slow metabolisers can be observed compared to the fast, intermediate and normal metabolising functional groups.

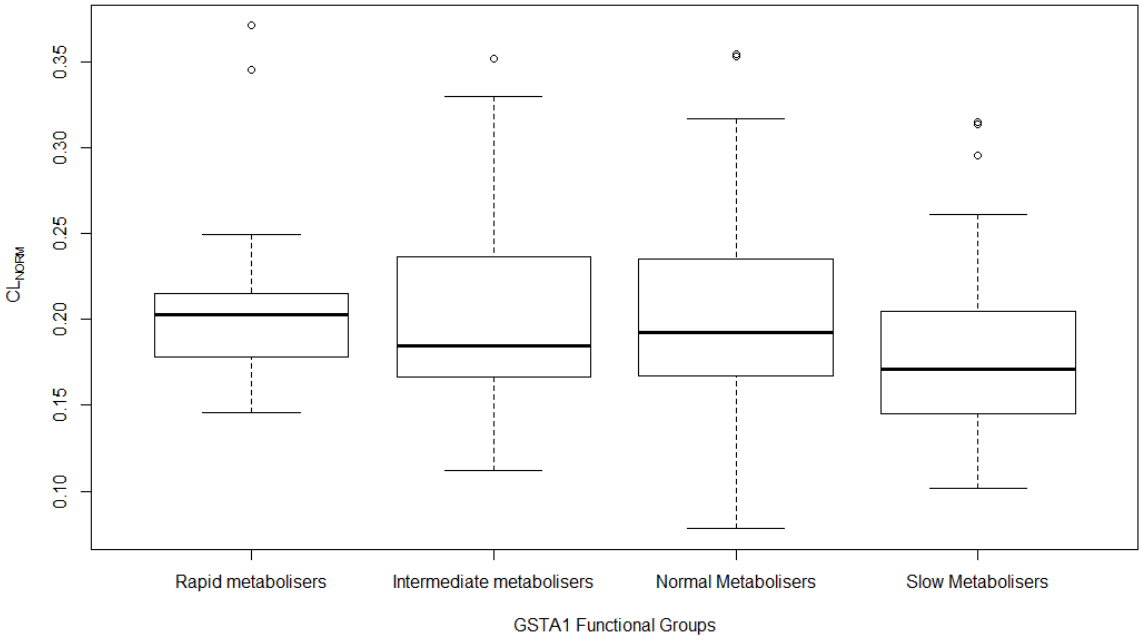


***Figure 6-3*** *Boxplots comparing median $CL_{NORM}$ (L/h/kg) in the four categories of patient GSTA1 activity. Median $CL_{NORM}$ is identified as a bold line dividing the box marking the 25% and 75% quartiles. The whiskers extend to the smallest observation and largest observations within 1.5 x IQR. Points beyond the parameters of the box and whiskers were plotted individually as outliers, as present in the intermediate, normal and slow metabolisers.*

#### *6.4.1.2 Relationship between GSTA1 activity and SOS*

Sinusoidal obstruction syndrome was diagnosed in 16 (14%) patients in the subpopulation characterised for GSTA1 polymorphisms. Based on the single SNP discrimination of GSTA1 subtypes, SOS was diagnosed in 6 (12%), 6 (13%) and 4 (20%) patients with GSTA1*A, *A*B and *B haplotypes, respectively. A $X^2$ analysis revealed no significant differences in SOS incidence between the three groups of GSTA1 $X^2$ (df = 2, N = 116) = 0.83, $P$ = 0.66.

The incidence of SOS in adult patients according to predicted phenotype was 21% (n =3), 10% (n =3), 13% (n = 6) and 15% (n = 4) for rapid, intermediate, normal and slow metabolisers, respectively. Given the small number of SOS incidences per functional group, a $X^2$ squared analysis could not be performed. Also, there were no significant differences in median $CL_{NORM}$ values of patients with or without SOS in any of the phenotypic enzyme function groups for GSTA 1 as illustrated in a box plot (Figure 6-4).

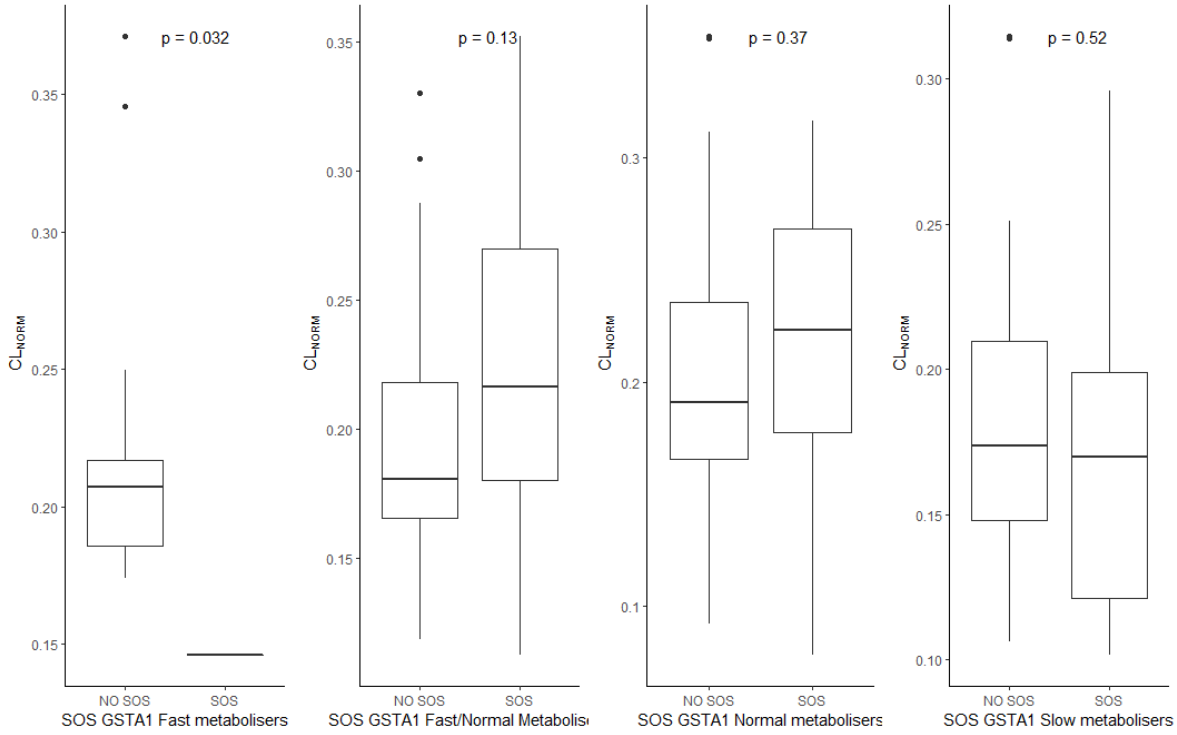


***Figure 6-4*** *Normalised clearances (L/h/kg) of patients with or without SOS for each category of GSTA1. Median $CL_{NORM}$ is identified as a bold line dividing the box marking the 25% and 75% quartiles. The whiskers extend to the smallest observation and largest observations within 1.5 x IQR. Points beyond the parameters of the box and whiskers were plotted individually as outliers, as present in the intermediate, normal and slow metabolisers.*

## 6.5 DISCUSSION

This chapter explored the genetic variability in the study population and attempted to explore the effect of genetic variants on busulfan clearance and SOS incidence. The analysis was divided into two segments; one an exploratory panel of ADME enzymes for a cohort of 217 individuals with available extracted DNA and secondly a detailed analysis of GSTA1 polymorphisms on a group of 184 patients from the study cohort. Beyond the glutathione-transferase superfamily of enzymes, there is a dearth of literature on busulfan metabolism. An exploratory analysis by Ten Brink *et al*. identified genes significantly associated with busulfan clearance using linear regression analysis in adult patients. However, there has been no similar exploratory analysis reported to date for paediatric patients. The current study population provided a unique opportunity to explore enzymatic associations in patients spanning the entire human lifespan.

One of the challenges associated with an exploratory analysis in the study population was the varying clearance of busulfan over age. The addition of age as a covariate prior to commencing the linear regression analysis for the various SNPs sought to overcome the differences in clearance. However, enzymatic immaturity of GST enzymes has been reported and characterised in populations previously, and also in the analysis described in Chapter 3.

Given the small number of patients available and to maximise the statistical power of the exploratory analysis of ADME enzymes, the study population was not divided further into training and validation cohorts. An initial exploration of the linear regression did find two SNPs significantly associated with busulfan clearance in the linear regression analysis,

however on further Despite the larger group of 217 patients, linear and logistic regression analyses failed to identify further effects of SNPs on either busulfan clearance or SOS.

Comparison of genotype groups for GSTM1, GSTT1 and GSTT2 also failed to show significant differences in busulfan clearance or SOS incidence in the study population. Given the modest differences in incidence of SOS (4 – 6% between patients with or without gene deletions for GSTT1, GSTT2 and GSTM1), the sample size was not large enough to yield significant results. In the literature, patients with a combination of gene deletions for both GSTM1 and GSTT1 have been reported to have a higher incidence of SOS.[177] However, only 14 patients in the study population had no activity for both GSTM1 and GSTT1, of whom three developed SOS, and this relationship was not assessed further due to lack of statistical power.

However, in the cohort of adult patients where GSTA1 polymorphisms were characterised, significant differences in normalised clearance were observed. The division of patients into *A and *B haplotypes alone was sufficient to highlight significant differences in busulfan $CL_{NORM}$ for patients with or without the *B allele. Lower GSTA1 activity in patients with the *B haplotype has been reported previously to result in a lower busulfan clearance.[178]

The further characterisation of GSTA1 haplotypes into the six polymorphisms was shown to refine the classification of enzymatic activity relative to the conventional single SNP genotyping.[175] Patient diplotypes could be divided into four phenotypic groups that reflect GSTA1 activity and may explain the differences in busulfan $CL_{NORM}$. These phenotypic groups have also been successfully incorporated into population pharmacokinetic models for paediatric populations. This was the first attempt at characterising GSTA1 haplotype

variability according to the most recent model and to identify the impact of functional GSTA1 diplotype groups on busulfan $CL_{NORM}$. In the adult population, no significant differences in $CL_{NORM}$ were observed for rapid, intermediate or normal metaboliser phenotypes. However, all three phenotypic groups (mean $CL_{NORM}$ = 0.20 L/h/kg) had significantly higher $CL_{NORM}$ values compared to the slow metabolisers (mean $CL_{NORM}$ = 0.18 L/h/kg) t = 2.62 (df 80.9) = *P* = 0.01. Based on the differences observed in both types of GSTA1 characterisations, a prospective validation of GSTA1 genotyping prior to busulfan administration may help target busulfan cAUCs more effectively.

To compare between the two methods of analysing GSTA1 polymorphisms, patients with a *B allele, either homo- or heterozygous, represented 56% of GSTA1 genotyped patients (65 patients). The slow metaboliser phenotypic groups however, represented 22% of the genotyped patients for GSTA1 (26 patients). The determination of *A and *B alleles is a simpler process that requires the genotyping of a single SNP, while characterising phenotypic groups requires the analysis of four SNPs, which then need to be sorted into four activity groups. The process of determining GSTA1 diplotype groups can be expensive and time consuming and, based on this analysis, did not provide additional benefit in adult patients to characterise pharmacogenomic variability. Given the small number of patients genotyped relative to the entire study population (337 individuals) and the smaller groups tested for lower clearance, such as *B or the slow metaboliser phenotypic group, incorporation into the population pharmacokinetic model was not attempted.

In all, the results of this chapter have identified a clear relationship between the *B polymorphism of GSTA1 and lower busulfan clearance. Furthermore, this analysis finds sufficient discrimination in busulfan clearance using determination of C-69-T genotype for

GSTA1 as either *A or *B, and no further benefit of the complicated phenotypic activity groups as described by Ansari et al. However, despite marked differences in busulfan clearance of the GSTA1 diplotypes, there was no identifiable relationship between GSTA1 polymorphisms and the development of SOS.

# Chapter 7.

## CONCLUSIONS AND FUTURE DIRECTIONS

This thesis has, through a number of experimental techniques, investigated key concepts of busulfan clinical pharmacology and has characterised contributing factors associated with clinical outcomes, such as SOS. Each chapter in this thesis has furthered the understanding of particular aspects of busulfan therapy using contemporary pharmacokinetic, pharmacodynamic and pharmacogenetic techniques, the results of which will be summarised in this chapter. As mentioned in the introduction, busulfan therapy is vastly complicated and one thesis cannot possibly address all of the associated issues. This thesis does however, assess current clinical practice and and individualisation of treatment in the context of avoiding toxicity.

## 7.1 RECAP

### 7.1.1 Chapter 1: Busulfan use in Haematopoietic Stem Cell Transplantation

Chapter 1 introduced the current applications of busulfan as a myeloablative conditioning agent prior to Haematopoietic Stem Cell Transplantation (HSCT). Issues pertinent to busulfan therapy, such as inter-individual pharmacokinetic variability and toxicities such as Sinusoidal Obstruction Syndrome (SOS) were discussed with a thorough exploration of previous studies. Following a brief history of transplantations and the introduction of busulfan as a conditioning agent in the 1970's, the review explored subsequent improvements in therapy, particularly after the introduction of the intravenous formulation of busulfan. The application of therapeutic drug monitoring (TDM) was discussed along with an assessment of recent arguments surrounding appropriate target windows of AUC for busulfan administration. The different approaches to TDM were also outlined and differences between TDM techniques highlighted. The development of busulfan dosing through the use of nomograms and Bayesian dose calculators in reducing inter-individual variability and the potential impact of genetic differences was discussed.

### 7.1.2 Chapter 2: The Patients- A Detailed Analysis of the Patient Population Recruited for Analysis

Chapter 2 introduced the retrospective analysis of HSCT patients at seven institutions across New South Wales and Victoria, Australia. The patient population of 337 transplant patients was described, along with an *a priori* exploration of interpatient differences such as the wide range of age, diagnoses and conditioning regimens used during transplantation. The chapter also provided a prelude on inter-institutional differences in addressing busulfan therapy - including institution-specific approaches to targeting busulfan and to SOS prophylaxis. The range of heterogeneity in the study population set the scene for the investigations performed in subsequent chapters.

### 7.1.3 Chapter 3: Characterising Pharmacokinetic Variability in the Busulfan Study Population Using Population-Pharmacokinetic Analysis

Chapter 3 aimed to characterise the degree of inter-individual and inter-occasion variability in busulfan pharmacokinetics in the study population. Through the use of population pharmacokinetic analysis, the chapter discussed the application of a one-compartment model that explained inter-patient variability through differences in body size and also enzymatic maturity from paediatric through to adult transplant patients. Following a thorough assessment of goodness of fit, and robustness of parameter estimates using techniques such as visual predictive checks and bootstrap simulations, the population pharmacokinetic model was used to estimate individual clearance, volume of distribution and area under the curve for every occasion of busulfan administration. These parameter estimates were then applied in a range of *post hoc* analyses, exploring the impact of conditioning regimens and concomitant medications on busulfan clearance. Patients coadministered metronidazole were found to have decreased clearance (0.13L/h/kg vs. 0.18 L/h/kg, $P < 0.0001$).

While the large inter-individual variability in paediatric patients was already known, the impact of daily TDM for busulfan in this patient group has not yet been published. The dramatic improvement in cumulative area under the curve (cAUC), as observed in Figure 3-9 of Chapter 3, clearly highlights improved control of cAUC in patients receiving daily TDM (71% with daily TDM vs 40% without daily TDM, $P$ = 0.0005

### 7.1.4 Chapter 4: Assessing the Incidence of Sinusoidal Obstruction Syndrome After Busulfan Therapy

Chapter 4 examined the incidence of sinusoidal obstruction syndrome (SOS) as an outcome of busulfan therapy in the study population. Using a variety of statistical techniques, the incidence of SOS was studied in patients receiving various conditioning regimens, in the different age groups. While no significant differences in busulfan cAUC were observed in patients with or without SOS, measured $C_{max}$, sampled within five minutes at the end of the first busulfan infusion, was higher in patients with SOS (2 µg/mL vs. 2.66 µg/mL, $P = 9.1 \times 10^{-6}$). This finding has important clinical implications for predicting SOS and providing earlier therapy with agents such as defibrotide to improve clinical outcome but raises questions as to why model based $C_{max}$ was not able to identify the relationship as strongly as measured $C_{max}$.

### 7.1.5 Chapter 5: A Parametric Time to Event Analysis of Sinusoidal Obstruction Syndrome After Busulfan Use

The development of SOS after transplant was studied using non- and semi- parametric analyses. Age was suspected to confound results in the non-parametric analysis, a finding confirmed when SOS incidence was compared between the four age categories of

patients. A proportional hazards analysis found a range of significant covariates such as lower age and pre-transplant albumin levels, and increased weight and day 1 $C_{max}$ concentrations as predictors of SOS in a univariate analysis. Multivariate Cox proportional-hazards analysis however could not determine a single covariate for predicting SOS, rather a combination of the aforementioned covariates.

### 7.1.6 Chapter 6: Pharmacogenetic Variability of Glutathione-S-Transferases and Other ADME Enzymes in Patients Receiving Busulfan

The final experimental analysis on busulfan pharmacology examined genetic differences in the population. A linear regression analysis in 217 patient for a panel of 67 SNPs (on an exploratory panel of ADME enzymes) failed to identify an effect on busulfan clearance. A similar logistic regression analysis was also unsuccessful at determining an association between the tested SNPs and the incidence of SOS in the study population. An assessment on the various copy number variations in GSTM1, GSTT1 and GSTT2 enzymes also failed to identify any differences in busulfan clearance or SOS incidence in the study population.

However, a separate analysis on a subset of 193 patients identified a significantly higher clearance in patients with the GSTA1*A allele compared to GSTA1*A*B (0.22 L/h/kg vs 0.19 L/h/kg, $P = 0.004$) and GSTA1*B (0.22 L/h/kg vs 0.18 L/h/kg, $P = 0.0003$) alleles. No similar effect on SOS incidence was observed. The further division of GSTA1 polymorphisms was ineffective at better describing busulfan clearance or SOS incidence. This was the first study that characterised the six GSTA1 haplotyples into the activity groups to analyse busulfan clearance.

## 7.2 CLINICAL IMPACT AND TRANSLATION

The research described in this thesis aimed to address several issues pertinent to high dose busulfan therapy. Inter-occasion and inter-individual variability during transplantations is a lingering problem that has not been overcome by the intravenous formulation. While the pharmacokinetic variability is known to be wider in children than adults, there has been little progress in identifying a solution to overcome the variability in achieving targets. The results from this thesis demonstrate daily TDM in children is essential to tightly target busulfan cumulative AUCs.

However, this thesis also adds to literature where the relationship observed is of busulfan use and SOS, but not busulfan AUC or cAUC and SOS.[105] The relationship between high busulfan $C_{max}$ on day 1 and SOS is an intriguing outcome discussed in this thesis. The first dose of busulfan is calculated purely on body-size and pharmacokinetic monitoring performed on this dose forms the basis of subsequent dose adjustments. Therefore, any patient specific differences in the metabolism of busulfan may be masked when calculating the dose-adjusted cAUC. A high $C_{max}$ could be used to flag high-risk patients who could be offered additional prophylaxis or aggressive monitoring to diagnose SOS at the earliest. However, the timing of such a sample is crucial and a missed sampling time would not allow the use of this information.

Although we failed to develop a clear model for predicting SOS hazard in patients, the multivariate analysis did reveal a combination of likely factors that may play a role in the development of SOS. A clinical evaluation of risk factors as suggested in the EBMT guidelines may still be the most comprehensive method of predicting SOS risk.

Lastly, the genetic influence of glutathione S transferase polymorphisms (mainly in GSTA1) demonstrated a clear effect on pharmacokinetic variability where patients with the GSTA1*B/*B genotype had a lower clearance across all ages. The novel method of incorporating the several diplotypes created from multiple combinations of SNPs into activity groups proved ineffective in teasing out differences in clearance across the four groups. Moving forward, GSTA1 polymorphisms using the C-69-T single nucleotide polymorphism alone could be incorporated into practice to identify patients with a lower clearance.

### 7.3 LIMITATIONS

A large retrospective analysis of a heterogenous population of patients undergoing a complicated and challenging medical procedure is not without its limitations. Firstly, the very nature of the analysis was retrospective, meaning that there was little scope to action any recommendations that resulted from the study. Changes to practice, and to guidelines for treatment and management were observed and taken into account wherever possible, but there were still many inconsistencies that could not be evaluated. Some of these include the change in targets for cAUC in conditioning regimens over the years, or the reintroduction of Q6H dosing of busulfan for 16 doses in a small number of patients for a limited time. Changes to SOS diagnosis and severity guidelines also made analyzing SOS incidence data more challenging.

The second limitation of this analysis lies in the vast heterogeneity of the population. Patients were transplanted for over fifty different conditions using over thirty busulfan-based protocols. Generalisations and groupings were made for ease of analysis, but there may have been data loss as part of the process. All types of non-Hodgkin's Lymphomas

were grouped together for the analysis of patient populations and cases of SOS. There may be a possibility that one or more of the NHL subtypes had a higher incidence of SOS and went unnoticed in the analysis. Every patient is unique and carries innumerable sources of heterogeneity. The alternative to overlooking heterogeneity was dividing the patients into smaller sub-populations to remove some of the confounding factors.

The subdivision of patients brings to light the third limitation of this study, which is sample-size and study power. The retrospective nature of the study allowed for data collection from as many patients as possible to enhance study power. However, to appropriately calculate a statistically significant difference ($P < 0.05$), using small effect-sizes (10% or less) would require study populations in excess of 785 patients. This would have not been possible in the study times of all the three retrospective trials combined. The subdivision of patients therefore adds to the problem of sample size as the differences in effect would need to be far greater to bear significance or power. However, the small but significant differences that were shown in this analysis warrant further investigation to recruit homogenous groups of patients through large multi-center collaborative approaches to thoroughly analyse differences, resulting from factors such as concomitant medications or genotype.

Continuing with issues of sample-size, there were limitations in analyzing the genotype data. Having a small number of patients affected the ability to investigate genes with low minor allele frequencies, resulting in the exclusion of several SNPs from linear and logistic regression analysis. Similar issues of power and sample-size were encountered for the previous exploratory analysis by Ten Brink *et al*. To counteract this issue, the entire dataset was used without further division of training and validation cohorts. Even so, patient numbers needed to be significantly higher, possibly explaining the lack of a

conclusive result from the logistic regression and the unexpected findings of the linear regression analysis.

While this is not an exhaustive list of limitations of this analysis, it certainly brings to light several unavoidable issues encountered during this study. The results of from these retrospective analyses and the limitations offer a learning opportunity to better shape prospective studies and future retrospective studies, in terms of patient selection and clarity of endpoints.

## 7.4 FUTURE DIRECTIONS

The large dataset collected for this research provided the opportunity to explore several aspects of busulfan therapy, with more content than for one mere PhD student to handle. There were some smaller analyses that sparked interest but were not fit for incorporation into this thesis. This section describes some of the 'could have' and 'would have' elements for further analysis, which may serve as a point of continuation from this thesis:

### 7.4.1 The Impact of Dimethylacetamide SOS Incidence

Dimethylacetamide (DMA) is a solvent included in the formulation of intravenous busulfan. Busulfan is available as 60 mg/10 mL dissolved in 33% DMA (v/v). Therefore, cumulative doses of busulfan administered to patients can result in the administration of several mL of DMA into the patients. As a known hepatotoxin, the contribution of DMA to SOS incidence is of interest. There have been limited studies on DMA pharmacokinetics in busulfan formulations, but these have failed to demonstrate any effect of on SOS.[179] Plasma samples were stored at the Children's Hospital Westmead for all patients as part of the

retrospective analysis, which will provide an excellent opportunity for the analysis of DMA pharmacokinetics.

Two methods were also validated for detecting DMA in plasma, one using UPLC and the other, unpublished using LC-MS/MS analysis. Stability of DMA in plasma stored at -20 C was also demonstrated, giving confidence in the results of the analysis. However, given that previous studies appeared to demonstrate the safety of DMA safety,[179] [180] albeit with limited data, were considered sufficient to not continue with the analysis.

### 7.4.2 A Parametric Time to Event Analysis of SOS Incidence Post Busulfan Therapy

The inconclusive results from Chapter 5 were in part due to the proportional nature of the Cox regression analysis. Hazard of developing SOS is a complicated process which peaks post transplantation but decreases overtime. Furthermore, the relationship of covariates such as age or weight would not be completely proportional with the hazard of developing SOS and therefore alternative relationships would need to be tested for the analysis of SOS hazards.

Parametric time to event analysis offers an elegant solution to this problem by fitting survival to exponential distributions of survival, with the flexibility to test various time-varying hazards of developing SOS. There are three essential components of a time to event analysis:

The likelihood of not having SOS post-transplant (S(t)) at any given time (t) was described as an exponential function of the cumulative hazard (H(t)), integrated from the rate of instantaneous hazard (h(t)) between time 0 to time t.

*Equation 7-1*

$$S(t) = e^{-H(t)}$$

where:

*Equation 7-2*

$$H(t) = \int_0^t h(t)\, dt$$

The probability density (f(t)) of having SOS at any given time (t) was described as a function of survival and instantaneous rate of hazard:

*Equation 7-3*

$$f(t) = S(t) \times h(t)$$

The incidence of SOS post transplantation is a single event per individual, and therefore a parametric time to event analysis would be sufficient for investigation. Such an analysis is possible using non-linear mixed effects modeling using the program NONMEM as described in Chapter 3. The insights provided from a parametric time to event analysis may be able to better incorporate the covariates identified in the univariate Cox regression analysis and help construct a model for the prediction of SOS incidence.

### 7.4.3 Prospective Validation of the Findings from this Study

As reiterated in this chapter, several important findings in this research require prospective validation. The predictive capacity of $C_{max}$ for SOS development or accounting for GSTA1 polymorphism effect on clearance prior to busulfan dosing should be assessed prospectively to improve busulfan pharmacokinetic and pharmacodynamic outcomes.

### 7.4.4 The Association between HLA Variants and SOS

The Human Leukocyte Antigens (HLA) are part of the major histocompatibility complex (MHC) which are a group of glycoproteins found on chromosome 6. Donor HLA for groups A, B, C, DRB1 and DQB1 are matched to the patient HLA profile as a selection requirement for transplant. Mismatched- HLA between patient and donor have been reported as a risk factor for SOS.[181]

Variations in HLA have been previously been associated with adverse immune responses, such as Steven-Johnsons Syndrome in patients using the anti-epileptic drug, carbamazepine. Given the immune-response component in SOS, and an established increased risk of SOS in patients with HLA-mismatched transplants,[111] there may be merit in investigating the association between HLA-variants and SOS development in transplant patients. This interaction to the best of our knowledge has not been reported in literature.

## 7.5 CONCLUDING REMARKS

IN CONCLUSION, this research adds to the repository of information on the use of busulfan prior to haematopoietic stem cell and bone marrow transplants. Although substantial research has been conducted on busulfan since the first reported use in

transplants, there are still several questions left unanswered. An improved understanding of the pathophysiology of SOS and further research into curative treatment may help improve outcomes for patients. Until then, daily TDM in children offers a feasible solution to better control busulfan exposure. A high $C_{max}$ may be indicative of susceptibility to SOS and GSTA1 polymorphisms can be used to better guide dosing in patients based on differences in clearance; although the latter two findings warrant prospective validation.

# APPENDICES

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**Appendix 1. Code for NONMEM pop-PK analysis**

**;; 1. Based on: Run…**

**;; 2. Description: …**

**;; x1. Author: …**

**;; 3. Label:**

**;ID Patient ID**

**;HOSP Participating Institution**

**;CMT Compartment**

**;OCC Occasion of Dosing**

**;FLG Flag for DV 1 = DOSING, 2 = PK, 3 = SOS, 4 = LAST DATE OF FOLLOW UP, 5 = RELAPSE if observed**

**;EVID Event ID**

**;MDV Missing DV**

**;FLAG Flagged samples ignored from analysis (<LLOQ, Contamination etc)**

**;DUR Duration of Infusion**

**;RATE Rate of Infusion**

**;AMT Dose of Busulfan administered**

**;TAU Frequency of Dosing (h)**

**;TIME Time (clock time)**

**;ODV Untransformed (Original) DV (ug/mL)**

**;LNDV=DV Log-Tranformed DV**

**;CONUMOL Busulfan concentration uMol**

**;SEX = Sex (M/F) ;HGT = Height (cm)**

**;WGT = weight (kg) ;AIBW = Adjusted Ideal Bodyweight (kg) ;BUWGT = Weight of Busulfan dosing (kg)**

**;BSA = Body Surface Area (m2)**

**;AGE = Age at transplant (days)**

**;AGILENT = Instrument for Bu detection (Agilent / Other)**

**;TXTYPE = Transplant Type (Autologous/ Allogeneic)**

**;SERO ;FLU ;MEL ;CPHOS ;PMOL ;MDZOLE ;FLUZOLE ;ANTIVIR ;DEFIB =**

**;Concomitant Serotherapy, fludarabine, melphalan, cyclophosphamide, paracetamol, metronidazole, fluconazole, antivirals, defibrotide**

**;CONDT = COnditioning regimen**

**;CANCER = Diagnosis ( Cancer vs non cancer)**

**;ALB = Pre-transplant Albumin levels (g/L)**

**;SOS**

**$PROBLEM PK**

**$INPUT …**

**$DATA … IGNORE=@ IGNORE=(FLAG.EQ.1) IGNORE=(OCC.GT.5);**

**$SUBROUTINES ADVAN1 TRANS2**

**$PK**

**;:::::::::::::::: Inter-Occasion Variability on Clearance::::::::::::::::::**

**IF(OCC.EQ.1) IOVCL=ETA(3)**

**IF(OCC.EQ.2) IOVCL=ETA(4)**

**IF(OCC.EQ.3) IOVCL=ETA(5)**

**IF(OCC.EQ.4) IOVCL=ETA(6)**

**IF(OCC.EQ.5) IOVCL=ETA(7)**

**;:::::::::::::::: Inter-Occasion Variability on Volume::::::::::::::::::**

**IF(OCC.EQ.1) IOVV=ETA(8)**

**IF(OCC.EQ.2) IOVV=ETA(9)**

**IF(OCC.EQ.3) IOVV=ETA(10)**

**IF(OCC.EQ.4) IOVV=ETA(11)**

**IF(OCC.EQ.5) IOVV=ETA(12)**

**;:::::::::::::::::Inter-Individual Variability on Clearance and Volume::::::::::::::::::**

**PPVCL=IOVCL+ETA(1)**

**PPVV=IOVV+ETA(2)**

**;;;;;;;;;;;;;;;; Population values of PK Parameters ::::::::::::::::::**

**TVCL=THETA(1)*EXP(PPVCL)**

**TVV= THETA(2)*EXP(PPVV)**

**TVD1=DUR**

**;;;;;;;;;;;;;;;; Factor for BodySize::::::::::::::::::**

**FSIZE=(AIBW/70)**(3/4)**

**;;;;;;;;;;;;;;;; Factor for Maturity::::::::::::::::::**

**TM= THETA(3)**

**HILL=THETA(4)**

**FMAT= 1/(1+((AGE/(365*TM))**(-HILL)))**

**;;;;;;;;;;;;;;;; Individual PK Parameters ::::::::::::::::::**

**CL=TVCL*FMAT*FSIZE**

**V= TVV*(AIBW/70)**

**S1=V/1**

**D1=TVD1**

**;;;;;;;;;;;;;;;; Solver time to calculate Time After Dose ::::::::::::::::::**

**IF (AMT.GT.0)THEN**

**TDOS=TIME**

**TAD=0.0**

**ENDIF**

**IF(AMT.EQ.0) TAD=TIME-TDOS**

**K=CL/V ;Elimination Constant**

**AUC = DOSE/CL ; Area Under the Curve (mg.h/L)**

**CLNORM=CL/WGT ; Clearance normalised to bodyweight**

**;;;;;;;;;;;;;;;; CMAX at First and Subsequent Busulfan doses ::::::::::::::::::**

**IF(OCC.EQ.1) CMAX= (DOSE/(CL*DUR))*(1-EXP(-K*DUR))**

**IF(OCC.GT.1) CMAX= (DOSE/(CL*DUR))*((1-EXP(-K*DUR))/(1-EXP(-K*TAU)))**

**;;;;;;;;;;;;;;;; Age Categories ::::::::::::::::::**

**IF((AGE/365).LT.2) AGECAT=1 ; Infants and Toddlers**

**IF((AGE/365).GE.2.AND.(AGE/365).LT.10) AGECAT=2 ; Children**

**IF((AGE/365).GE.10.AND.(AGE/365).LT.25) AGECAT=3 ; Adolescents and Young Adults**

```
IF((AGE/365).GE.25) AGECAT=4 ;Adults


;;;;;;;;;;;;;;;; For TTE table sheet::::::::::::::::::

IF(FLG.EQ.3) DVID=1

IF(FLG.NE.3) DVID=0


;;;;;;;;;;;;;;;; Proportional Error model for Log-transformed Data ::::::::::::::::::

$ERROR (OBSERVATIONS ONLY)

IPRED = 0

IF(F.GT.0) IPRED = LOG(F)

Y = IPRED + ERR(1)


$THETA

(13.8) ;CL

(48.1) ; V

 (0.377) ; TM50

(0.634) ; HILL


$OMEGA BLOCK(2)

 0.049  ; CL
```

**0.0297 0.0232 ; V**

**$OMEGA BLOCK(1) 0.0126 ;ETA3 BOVCL**

**$OMEGA BLOCK(1) SAME ;ETA4 BOCL**

**$OMEGA BLOCK(1) SAME ;ETA5 BOVCL**

**$OMEGA BLOCK(1) SAME ;ETA6 BOVCL**

**$OMEGA BLOCK(1) SAME ;ETA7 BOVCL**

**$OMEGA BLOCK(1) 0.0081 ;ETA8 BOVV**

**$OMEGA BLOCK(1) SAME ;ETA9 BOVV**

**$OMEGA BLOCK(1) SAME ;ETA10 BOVV**

**$OMEGA BLOCK(1) SAME ;ETA11 BOVV**

**$OMEGA BLOCK(1) SAME ;ETA12 BOVV**

**$SIGMA**

**0.00725 ;ERR**

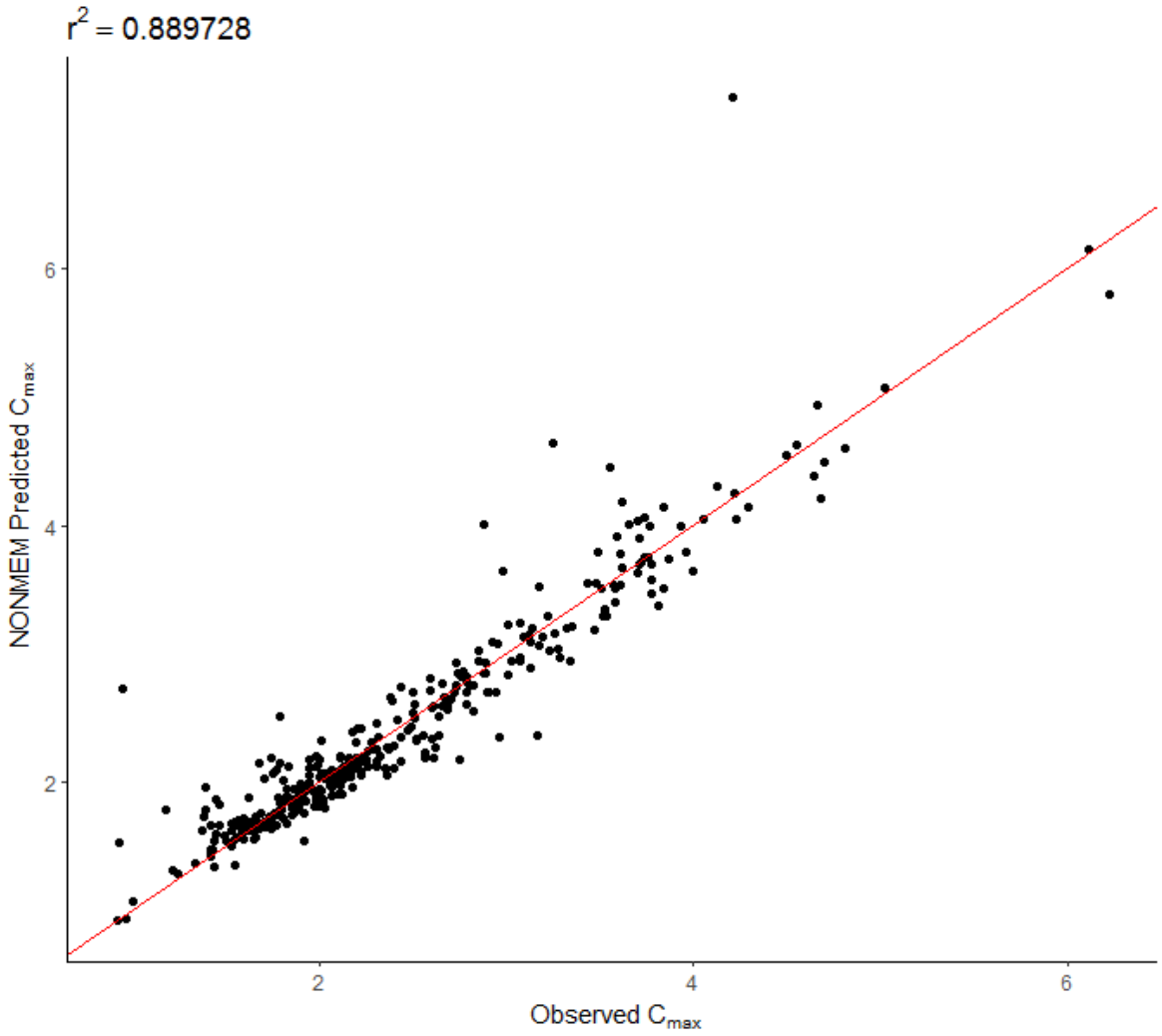
**$COV**

**$EST MAX=9990 SIG=3 PRINT=1 METHOD=COND INTER NOABORT**

**$TABLE…**

**Appendix 2. NONMEM vs. Observed $C_{max}$**

**Day 1 $C_{max}$ estimated from NONMEM compared to observed $C_{max}$ in patients.**




**<u>Appendix 2 shows good correlation between observed and predicted $C_{max}$ (r2 = 0.89), although some patients are overpredicted by the model compared to observed concentrations.</u>**

**Appendix 3.** Summary of SNP association with busulfan $CL_{NORM}$, where major allele (A1) was described for the number of patients included, with $\beta$ coefficients (slope), 5$^{th}$ percentile and 95$^{th}$ percentile confidence intervals, Z statistic and corresponding *P* value. Significant associations ($P < 0.01$) were demarked with an (*).

| Gene | SNP | Major SNP | Patients | $\beta$-Coefficient | 5 – 95 Percentile Confidence Interval | Wald *Z*-Statistic | *P* - Value |
|---|---|---|---|---|---|---|---|
| ABCB1 | rs1045642 | T | 217 | 0.00309 | -0.006 - 0.012 | 0.6681 | 0.505 |
| ABCB1 | rs1128503 | T | 217 | 0.004061 | -0.004 - 0.013 | 0.9434 | 0.347 |
| ABCB1 | rs3213619 | C | 215 | -0.00182 | -0.024 - 0.021 | -0.1596 | 0.873 |
| ABCC2 | rs2273697 | A | 217 | -0.00474 | -0.015 - 0.006 | -0.8731 | 0.384 |
| ABCC2 | rs3740066 | A | 216 | -0.00191 | -0.011 - 0.007 | -0.4002 | 0.689 |
| ABCC2 | rs717620 | A | 216 | -0.00125 | -0.013 - 0.011 | -0.2009 | 0.841 |
| ABCG2 | rs2231142 | A | 216 | 0.00716 | -0.007 - 0.021 | 1.022 | 0.308 |
| COMT | rs165599 | G | 217 | -0.00168 | -0.01 - 0.007 | -0.3784 | 0.706 |
| COMT | rs4680 | A | 217 | -0.00179 | -0.01 - 0.007 | -0.4235 | 0.672 |
| COMT | rs737865 | C | 217 | 0.004488 | -0.005 - 0.014 | 0.8922 | 0.373 |
| CYP1A1 | rs1048943 | G | 217 | -0.0044 | -0.019 - 0.01 | -0.6005 | 0.549 |
| CYP1A1 | rs1799814 | A | 217 | -0.02506 | -0.05 - -0.003 | -2.178 | 0.031 |
| CYP1A2 | rs762551 | C | 217 | -0.0028 | -0.012 - 0.006 | -0.6196 | 0.536 |
| CYP2A6 | rs1801272 | A | 216 | -0.02078 | -0.047 - 0.005 | -1.567 | 0.119 |
| CYP2A6 | rs28399433 | G | 217 | -0.00121 | -0.016 - 0.014 | -0.159 | 0.874 |
| CYP2B6 | rs3745274 | T | 217 | 0.004312 | -0.005 - 0.013 | 0.948 | 0.344 |
| CYP2B6 | rs8192709 | T | 217 | -0.01722 | -0.035 - 0.001 | -1.863 | 0.064 |
| CYP2C19 | rs3758581 | A | 217 | -0.01523 | -0.033 - 0.002 | -1.713 | 0.088 |
| CYP2C19 | rs4244285 | A | 217 | 0.0116 | 0.002 - 0.022 | 2.253 | 0.025 |
| CYP2C19 | rs4986893 | A | 217 | 0.001267 | -0.035 - 0.038 | 0.06775 | 0.946 |
| CYP2C8 | rs10509681 | C | 216 | -0.00162 | -0.016 - 0.012 | -0.2251 | 0.822 |
| CYP2C8 | rs1058930 | G | 217 | 0.003102 | -0.016 - 0.022 | 0.3142 | 0.754 |
| CYP2C8 | rs11572080 | A | 217 | -0.0016 | -0.016 - 0.012 | -0.2223 | 0.824 |
| CYP2C9 | rs1057910 | C | 217 | -0.01571 | -0.033 - 0.001 | -1.822 | 0.070 |
| CYP2C9 | rs1799853 | T | 217 | -0.00477 | -0.018 - 0.008 | -0.7085 | 0.479 |
| CYP2D6 | rs1080985 | G | 216 | -0.00788 | -0.018 - 0.002 | -1.597 | 0.112 |
| CYP2D6 | rs28371725 | A | 216 | -0.00273 | -0.018 - 0.013 | -0.3448 | 0.731 |

| Gene | SNP | Major SNP | Patients | β-Coefficient | 5 – 95 Percentile Confidence Interval | Wald *Z*-Statistic | *P* - Value |
|---|---|---|---|---|---|---|---|
| CYP2D6 | rs5030656 | D | 217 | 0.01013 | -0.016 - 0.036 | 0.7617 | 0.447 |
| CYP2E1 | rs2070673 | A | 210 | -0.01148 | -0.022- -0.001 | -2.191 | 0.030 |
| CYP3A4 | rs35599367 | T | 216 | 0.009994 | -0.013 - 0.033 | 0.8593 | 0.391 |
| CYP3A5 | rs776746 | A | 214 | -0.00098 | -0.014 - 0.012 | -0.1505 | 0.881 |
| DPYD | rs1801265 | C | 217 | 0.00125 | -0.01 - 0.012 | 0.224 | 0.823 |
| GSTP1 | rs1138272 | T | 217 | 0.002472 | -0.016 - 0.021 | 0.2682 | 0.789 |
| GSTP1 | rs1695 | G | 216 | -0.00607 | -0.015 - 0.003 | -1.353 | 0.178 |
| NAT1 | rs4986782 | A | 217 | -0.02975 | -0.063 - 0.004 | -1.74 | 0.083 |
| NAT2 | rs1041983 | T | 217 | -0.00501 | -0.014 - 0.004 | -1.094 | 0.275 |
| NAT2 | rs1208 | G | 216 | 0.001123 | -0.007 - 0.01 | 0.2568 | 0.798 |
| NAT2 | rs1799929 | T | 217 | 0.00131 | -0.007 - 0.01 | 0.2992 | 0.765 |
| NAT2 | rs1799930 | A | 217 | -0.00664 | -0.015 - 0.002 | -1.481 | 0.140 |
| NAT2 | rs1799931 | A | 216 | 0.007521 | -0.01 - 0.025 | 0.8367 | 0.404 |
| NAT2 | rs1801280 | C | 217 | 0.00196 | -0.006 - 0.01 | 0.4547 | 0.650 |
| SLC15A2 | rs1143671 | T | 217 | 0.000173 | -0.008 - 0.009 | 0.04029 | 0.968 |
| SLC15A2 | rs1143672 | A | 217 | 0.000173 | -0.008 - 0.009 | 0.04029 | 0.968 |
| SLC15A2 | rs2257212 | A | 216 | 0.000144 | -0.008 - 0.009 | 0.03342 | 0.973 |
| SLC15A2 | rs2293616 | T | 217 | 0.000173 | -0.008 - 0.009 | 0.04029 | 0.968 |
| SLC22A1 | rs12208357 | T | 217 | -0.00158 | -0.019 - 0.016 | -0.1787 | 0.858 |
| SLC22A1 | rs2282143 | T | 217 | -0.00122 | -0.026 - 0.024 | -0.0957 | 0.924 |
| SLC22A1 | rs34059508 | A | 217 | -0.00634 | -0.036 - 0.024 | -0.4162 | 0.678 |
| SLC22A1 | rs4646281A | A | 217 | 0.000739 | -0.007 - 0.009 | 0.1818 | 0.856 |
| SLC22A1 | rs628031 | A | 217 | 0.001028 | -0.007 - 0.009 | 0.2534 | 0.800 |
| SLC22A1 | rs72552763 | T | 217 | 0.004436 | -0.009 - 0.018 | 0.6497 | 0.517 |
| SLC22A2 | rs316019 | T | 217 | 0.000484 | -0.013 - 0.014 | 0.07111 | 0.943 |
| SLCO1B1 | rs2306283 | G | 216 | -0.00392 | -0.012 - 0.004 | -0.9445 | 0.346 |
| SLCO1B1 | rs4149056 | C | 217 | -0.00039 | -0.013 - 0.012 | -0.0603 | 0.952 |
| SLCO1B3 | rs4149117 | T | 216 | -0.0002 | -0.011 - 0.011 | -0.0368 | 0.971 |
| SLCO1B3 | rs7311358 | G | 208 | -0.00082 | -0.012 - 0.01 | -0.15 | 0.881 |
| TPMT | rs1142345 | G | 216 | -0.0277 | -0.05 - -0.006 | -2.476 | 0.014 |
| TPMT | rs1800460 | A | 217 | -0.0219 | -0.052 - 0.008 | -1.441 | 0.151 |

| Gene | SNP | Major SNP | Patients | *β*-Coefficient | 5 – 95 Percentile Confidence Interval | Wald *Z*-Statistic | *P* - Value |
|---|---|---|---|---|---|---|---|
| UGT1A1 | rs4124874 | C | 217 | -0.0047 | -0.013 - 0.004 | -1.075 | 0.284 |
| UGT1A1 | rs4148323 | A | 217 | -0.01451 | -0.041 - 0.012 | -1.059 | 0.291 |
| UGT2B15 | rs1902023 | T | 216 | 0.004538 | -0.004 - 0.013 | 1.086 | 0.279 |
| UGT2B7 | rs7662029 | A | 217 | -0.00181 | -0.01 - 0.007 | -0.4113 | 0.681 |
| UGT2B7 | rs7668258 | T | 217 | -0.00181 | -0.01 - 0.007 | -0.4113 | 0.681 |
| VKORC1 | rs17708472 | A | 209 | 0.004801 | -0.006 - 0.016 | 0.8404 | 0.402 |
| VKORC1 | rs7294 | A | 216 | -0.00543 | -0.014 - 0.003 | -1.199 | 0.232 |
| VKORC1 | rs9923231 | T | 217 | 0.001699 | -0.006 - 0.01 | 0.4116 | 0.681 |
| VKORC1 | rs9934438 | A | 215 | 0.001684 | -0.006 - 0.01 | 0.404 | 0.687 |

**Appendix 4.** Summary of SNP association with SOS incidence, where major allele (A1) was described for the number of patients included, with Odds ratio, 5 percentile and 95 percentile confidence intervals, Wald Z-statistic and corresponding *P* value. Significant associations (*P* < 0.01) were demarked with an (*).

| Gene | SNP | Major SNP | Patients | Odds Ratio | 5 – 95 Percentile Confidence Interval | Z Statistic | *P* - Value |
|---|---|---|---|---|---|---|---|
| ABCB1 | rs1128503 | T | 217 | 0.821 | 0.504 - 1.338 | -0.791 | 0.429 |
| ABCB1 | rs3213619 | C | 215 | 0.544 | 0.116 - 2.555 | -0.772 | 0.44 |
| ABCB1 | rs1045642 | T | 217 | 0.892 | 0.54 - 1.473 | -0.446 | 0.656 |
| ABCC2 | rs717620 | A | 216 | 0.549 | 0.261 - 1.153 | -1.585 | 0.113 |
| ABCC2 | rs2273697 | A | 217 | 1.153 | 0.634 - 2.099 | 0.467 | 0.641 |
| ABCC2 | rs3740066 | A | 216 | 0.92 | 0.546 - 1.552 | -0.312 | 0.755 |
| ABCG2 | rs2231142 | A | 216 | 1.556 | 0.745 - 3.251 | 1.177 | 0.239 |
| COMT | rs165599 | G | 217 | 1.486 | 0.914 - 2.417 | 1.598 | 0.11 |
| COMT | rs737865 | C | 217 | 1.108 | 0.641 - 1.914 | 0.367 | 0.713 |
| COMT | rs4680 | A | 217 | 0.967 | 0.608 - 1.538 | -0.142 | 0.887 |
| CYP1A1 | rs1799814 | A | 217 | 0.481 | 0.101 - 2.285 | -0.92 | 0.357 |
| CYP1A1 | rs1048943 | G | 217 | 0.668 | 0.256 - 1.739 | -0.827 | 0.408 |
| CYP1A2 | rs762551 | C | 217 | 1.002 | 0.609 - 1.649 | 0.007 | 0.994 |
| CYP2A6 | rs28399433 | G | 217 | 1.346 | 0.605 - 2.991 | 0.729 | 0.466 |
| CYP2A6 | rs1801272 | A | 216 | 0.639 | 0.13 - 3.148 | -0.55 | 0.582 |
| CYP2B6 | rs8192709 | T | 217 | 0.506 | 0.114 - 2.243 | -0.897 | 0.37 |
| CYP2B6 | rs3745274 | T | 217 | 1.233 | 0.746 - 2.04 | 0.817 | 0.414 |
| CYP2C19 | rs4244285 | A | 217 | 0.735 | 0.395 - 1.369 | -0.971 | 0.332 |
| CYP2C19 | rs3758581 | A | 217 | 1.422 | 0.566 - 3.573 | 0.749 | 0.454 |
| CYP2C19 | rs4986893 | A | 217 | 0.453 | 0.05 - 4.129 | -0.703 | 0.482 |
| CYP2C8 | rs11572080 | A | 217 | 1.813 | 0.841 - 3.907 | 1.519 | 0.129 |
| CYP2C8 | rs10509681 | C | 216 | 1.796 | 0.833 - 3.872 | 1.494 | 0.135 |
| CYP2C8 | rs1058930 | G | 217 | 0.505 | 0.138 - 1.845 | -1.033 | 0.302 |
| CYP2C9 | rs1799853 | T | 217 | 1.593 | 0.766 - 3.313 | 1.245 | 0.213 |
| CYP2C9 | rs1057910 | C | 217 | 1.661 | 0.682 - 4.047 | 1.117 | 0.264 |
| CYP2D6 | rs5030656 | D | 217 | 2.797 | 0.782 - 10.01 | 1.581 | 0.114 |
| CYP2D6 | rs1080985 | G | 216 | 1.256 | 0.737 - 2.14 | 0.838 | 0.402 |

| Gene | SNP | Major SNP | Patients | Odds Ratio | 5 – 95 Percentile Confidence Interval | Z Statistic | *P* - Value |
|---|---|---|---|---|---|---|---|
| CYP2D6 | rs28371725 | A | 216 | 1.012 | 0.441 - 2.322 | 0.029 | 0.977 |
| CYP2E1 | rs2070673 | A | 210 | 1.245 | 0.681 - 2.277 | 0.712 | 0.477 |
| CYP3A4 | rs35599367 | T | 216 | 1.153 | 0.337 - 3.946 | 0.227 | 0.82 |
| CYP3A5 | rs776746 | A | 214 | 0.933 | 0.469 - 1.854 | -0.198 | 0.843 |
| DPYD | rs1801265 | C | 217 | 1.389 | 0.76 - 2.538 | 1.068 | 0.286 |
| GSTP1 | rs1138272 | T | 217 | 2.381 | 0.936 - 6.057 | 1.821 | 0.069 |
| GSTP1 | rs1695 | G | 216 | 0.959 | 0.581 - 1.583 | -0.165 | 0.869 |
| NAT1 | rs4986782 | A | 217 | 1.415 | 0.249 - 8.025 | 0.392 | 0.695 |
| NAT2 | rs1799931 | A | 216 | 0.299 | 0.067 - 1.326 | -1.589 | 0.112 |
| NAT2 | rs1799930 | A | 217 | 1.32 | 0.807 - 2.161 | 1.105 | 0.269 |
| NAT2 | rs1799929 | T | 217 | 0.822 | 0.496 - 1.362 | -0.762 | 0.446 |
| NAT2 | rs1041983 | T | 217 | 1.069 | 0.642 - 1.78 | 0.256 | 0.798 |
| NAT2 | rs1208 | G | 216 | 1.017 | 0.625 - 1.655 | 0.067 | 0.946 |
| NAT2 | rs1801280 | C | 217 | 0.996 | 0.612 - 1.621 | -0.016 | 0.987 |
| SLC15A2 | rs2257212 | A | 216 | 1.325 | 0.826 - 2.128 | 1.166 | 0.244 |
| SLC15A2 | rs1143671 | T | 217 | 1.325 | 0.825 - 2.13 | 1.165 | 0.244 |
| SLC15A2 | rs1143672 | A | 217 | 1.325 | 0.825 - 2.13 | 1.165 | 0.244 |
| SLC15A2 | rs2293616 | T | 217 | 1.325 | 0.825 - 2.13 | 1.165 | 0.244 |
| SLC22A1 | rs34059508 | A | 217 | 3.501 | 0.839 - 14.61 | 1.719 | 0.086 |
| SLC22A1 | rs4646281A | A | 217 | 0.756 | 0.476 - 1.201 | -1.185 | 0.236 |
| SLC22A1 | rs628031 | A | 217 | 0.762 | 0.481 - 1.209 | -1.152 | 0.249 |
| SLC22A1 | rs2282143 | T | 217 | 1.558 | 0.424 - 5.733 | 0.668 | 0.504 |
| SLC22A1 | rs72552763 | T | 217 | 1.273 | 0.6 - 2.7 | 0.629 | 0.529 |
| SLC22A1 | rs12208357 | T | 217 | 0.998 | 0.384 - 2.591 | -0.004 | 0.997 |
| SLC22A2 | rs316019 | T | 217 | 1.965 | 0.997 - 3.873 | 1.95 | 0.051 |
| SLCO1B1 | rs4149056 | C | 217 | 2.291 | 1.136 - 4.622 | 2.316 | 0.021 |
| SLCO1B1 | rs2306283 | G | 216 | 1.123 | 0.71 - 1.778 | 0.497 | 0.619 |
| SLCO1B3 | rs7311358 | G | 208 | 0.818 | 0.42 - 1.592 | -0.591 | 0.554 |
| SLCO1B3 | rs4149117 | T | 216 | 0.864 | 0.459 - 1.626 | -0.453 | 0.65 |
| TPMT | rs1142345 | G | 216 | 1.808 | 0.57 - 5.737 | 1.005 | 0.315 |
| TPMT | rs1800460 | A | 217 | 1.701 | 0.381 - 7.586 | 0.696 | 0.486 |

| Gene | SNP | Major SNP | Patients | Odds Ratio | 5 – 95 Percentile Confidence Interval | Z Statistic | *P* - Value |
|---|---|---|---|---|---|---|---|
| UGT1A1 | rs4148323 | A | 217 | 0.536 | 0.081 - 3.529 | -0.649 | 0.517 |
| UGT1A1 | rs4124874 | C | 217 | 1.034 | 0.628 - 1.703 | 0.133 | 0.894 |
| UGT2B15 | rs1902023 | T | 216 | 0.653 | 0.404 - 1.056 | -1.737 | 0.082 |
| UGT2B7 | rs7662029 | A | 217 | 0.762 | 0.466 - 1.247 | -1.082 | 0.279 |
| UGT2B7 | rs7668258 | T | 217 | 0.762 | 0.466 - 1.247 | -1.082 | 0.279 |
| VKORC1 | rs7294 | A | 216 | 1.419 | 0.853 - 2.359 | 1.348 | 0.178 |
| VKORC1 | rs9934438 | A | 215 | 0.805 | 0.504 - 1.287 | -0.906 | 0.365 |
| VKORC1 | rs9923231 | T | 217 | 0.824 | 0.517 - 1.313 | -0.816 | 0.415 |
| VKORC1 | rs17708472 | A | 209 | 0.811 | 0.421 - 1.559 | -0.629 | 0.529 |

**Appendix 5. List of Excluded SNPs from linear and Logistic regression**

ABCB1(rs1045642)

ABCB12(rs1128503)

ABCB13(rs2032582)

ABCB14(rs3213619)

ABCC2(rs2273697)

ABCC25(rs3740066)

ABCC26(rs56199535)

ABCC27(rs56220353)

ABCC28(rs56296335)

ABCC29(rs717620)

ABCG2(rs2231142)

ABCG210(rs72552713)

COMT(rs165599)

COMT11(rs4680)

COMT12(rs737865)

CYP1A1(rs1048943)

CYP1A113(rs1799814)

CYP1A114(rs1800031)

CYP1A115(rs41279188)

CYP1A116(rs56313657)

CYP1A117(rs72547509)

CYP1A118(rs72547510)

CYP1A2(rs12720461)

CYP1A219(rs2069514)

CYP1A220(rs56107638)

CYP1A221(rs762551)

CYP2A6(CYP2A6_A7conversion)

CYP2A622(CYP2A6E1)

CYP2A623(CYP2A6E2)

CYP2A624(rs1801272)

CYP2A625(rs28399433)

CYP2A626(rs28399444)

CYP2A627(rs28399447)

CYP2A628(rs28399454)

CYP2A629(rs28399468)

CYP2A630(rs4986891)

CYP2A631(rs5031016)

CYP2A632(rs5031017)

CYP2B6(rs12721655)

CYP2B636(rs28399499)

CYP2B637(rs34097093)

CYP2B638(rs3745274)

CYP2B639(rs8192709)

CYP2C19(rs12248560)

CYP2C1943(rs28399504)

CYP2C1944(rs3758581)

CYP2C1945(rs41291556)

CYP2C1946(rs4244285)

CYP2C1947(rs4986893)

CYP2C1948(rs55640102)

CYP2C1949(rs56337013)

CYP2C1950(rs72552267)

CYP2C1951(rs72558186)

CYP2C8(rs10509681)

CYP2C852(rs1058930)

CYP2C853(rs11572080)

CYP2C854(rs11572103)

CYP2C855(rs72558195)

CYP2C856(rs72558196)

CYP2C9(rs1057910)

CYP2C957(rs1799853)

CYP2C958(rs2256871)

CYP2C959(rs28371685)

CYP2C960(rs28371686)

CYP2C961(rs56165452)

CYP2C962(rs72558187)

CYP2C963(rs72558188)

CYP2C964(rs72558190)

CYP2C965(rs7900194)

CYP2C966(rs9332130)

CYP2C967(rs9332131)

CYP2C968(rs9332239)

CYP2D6(CYP2D6intr1E3)

CYP2D669(dup4125_4133)

CYP2D670(rs1065852)

CYP2D671(rs1080985)

CYP2D672(rs28371706)

CYP2D673(rs28371725)

CYP2D674(rs35742686)

CYP2D675(rs3892097)

CYP2D676(rs5030655)

CYP2D677(rs5030656)

CYP2D678(rs5030862)

CYP2D679(rs5030863)

CYP2D680(rs5030865)

CYP2D681(rs5030867)

CYP2D682(rs72549346)

CYP2D683(rs72549347)

CYP2D684(rs72549349)

CYP2D685(rs72549352)

CYP2D686(rs72549353)

CYP2D687(rs72549354)

CYP2D688(rs72549356)

CYP2D689(rs72549357A)

CYP2D690(rs72549357B)

CYP2E1(rs2070673)

CYP2E194(rs72559710)

CYP3A4(rs35599367)

CYP3A495(rs4646438)

CYP3A496(rs55785340)

CYP3A497(rs67666821)

CYP3A5(rs10264272)

CYP3A598(rs41279854)

CYP3A599(rs41303343)

CYP3A5100(rs55965422)

CYP3A5101(rs776746)

DPYD(rs1801265)

DPYD102(rs1801266)

DPYD103(rs1801268)

DPYD104(rs3918290)

DPYD105(rs72549309)

GSTT2(GSTT2bE1)

NAT1(rs4986782)

NAT1118(rs4986989)

NAT1119(rs5030839)

NAT1120(rs55793712)

NAT1121(rs56172717)

NAT1122(rs56318881)

NAT1123(rs56379106)

NAT2(rs1041983)

NAT2124(rs1208)

NAT2125(rs1799929)

NAT2126(rs1799930)

NAT2127(rs1799931)

NAT2128(rs1801279)

NAT2129(rs1801280)

NAT2130(rs1805158)

SLC15A2(rs1143671)

SLC15A2131(rs1143672)

SLC15A2132(rs2257212)

SLC15A2133(rs2293616)

SLC22A1(rs12208357)

SLC22A1134(rs2282143)

SLC22A1135(rs34059508)

SLC22A1136(rs34130495)

SLC22A1137(rs34305973)

SLC22A1138(rs35167514)

SLC22A1139(rs36103319)

SLC22A1140(rs4646277)

SLC22A1141(rs4646278)

SLC22A1142(rs4646281A)

SLC22A1143(rs4646281B)

SLC22A1144(rs55918055)

SLC22A1145(rs628031)

SLC22A1146(rs72552763)

SLC22A2(rs316019)

SLC22A2147(rs8177504)

SLC22A2148(rs8177508)

SLC22A2149(rs8177516)

SLC22A2150(rs8177517)

SLC22A6(rs11568626)

SLCO1B1(rs2306283)

SLCO1B1151(rs4149056)

SLCO1B1152(rs55737008)

SLCO1B1153(rs56061388)

SLCO1B1154(rs56101265)

SLCO1B1155(rs56199088)

SLCO1B1156(rs59502379)

SLCO1B1157(rs72559745)

SLCO1B3(rs4149117)

SLCO1B3158(rs7311358)

SLCO2B1(rs2306168)

SULT1A1(rs1801030)

SULT1A1159(rs72547527)

SULT1A1160(rs9282861)

TPMT(rs1142345)

TPMT164(rs1800460)

TPMT165(rs1800462)

TPMT166(rs1800584)

TPMT167(rs56161402)

UGT1A1(rs34993780)

UGT1A1168(rs35350960)

UGT1A1169(rs4124874)

UGT1A1170(rs4148323)

UGT1A1171(rs55750087)

UGT2B15(rs1902023)

UGT2B7(rs7662029)

UGT2B7175(rs7668258)

VKORC1(rs17708472)

VKORC1176(rs7294),

VKORC1177(rs9923231)

VKORC1178(rs9934438

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