PERSONALIZING THE CONDITIONING REGIMEN IN HAEMATOPOIETIC STEM CELL TRANSPLANTATION

ASWIN ANAND PAI

PhD THESIS
2023

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCE AND TECHNOLOGY, THIRUVANANTHAPURAM

An Institution of National Importance established by an Act of the Indian Parliament (Act No.52 of 1980)

Dept. of Science and Technology, Govt. of India
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DECLARATION BY THE STUDENT

I, **Aswin Anand Pai,** hereby certify that I had personally carried out the work depicted in the thesis entitled **"Personalizing the Conditioning Regimen in Haematopoietic Stem Cell Transplantation"**, except where due acknowledgement has been made in the text.

No part of the thesis has been submitted for the award of any degree or diploma prior to this date.

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*Clearance was obtained from the Institutional Ethics Committee for carrying out the study.

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APPROVAL OF THESIS

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PERSONALIZING THE CONDITIONING REGIMEN IN HAEMATOPOIETIC STEM CELL TRANSPLANTATION

Submitted by

Aswin Anand Pai

for the degree of

Doctor of Philosophy

of

Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum

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(Name & Signature of the Guide)	external examiner

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- Ratan Tata.

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TABLE OF CONTENTS

Page No

Declaration by student……...…..i

Certificate by guide……...…..ii

Approval of thesis……...……iii

Acknowledgements……...…..iv

List of figures……...…… viii

List of tables…….....xi

Abbreviations…….....……...…....xii

Synopsis……..……...….xv

1. Introduction…….....1

1.1. Rationale and Hypothesis……4

1.2. Objectives……6

1.3. Brief overview of thesis chapters……6

1.3.1. Literature review…….....….6

1.3.2. Materials and Methods……...…..…6

1.3.3. Results……...…....7

1.3.4. Discussion……...…..7

1.3.5. Summary, Conclusion, and Bibliography…….....7

2. Review of Literature……...8

2.1. Thalassemias…….....9

2.2. Beta Thalassemia……..9

2.2.1. Epidemiology of β-thalassemia…….....9

2.2.2. Prevalence of β-thalassemia……...…....10

2.2.3. Populations at Risk for β-thalassemia……....10
2.2.4. β-thalassemia in India…….……....11
2.3. Pathophysiology of β-thalassemia…..……..11
2.4. Clinical Phenotypes in β-thalassemia…….……....14
2.5. Screening and Diagnosis…….....……..15
2.6. Clinical Implications…….....……17
2.7. Management of β-thalassemia…….....20
2.7.1. Transfusion Therapy…….....20
2.7.2. Iron Chelation…….....…20
2.7.3. Gene Therapy…….....22
2.7.4. Luspatercept Therapy…….....23
2.7.5. Induction of fetal hemoglobin production.....25
2.7.6. HCT for β-thalassemia.....26
2.7.6.1. History of HCT in β-TM.....27
2.7.6.2. Pre-HCT Stratification in β-TM.....27
2.7.6.3. Conditioning in HCT for β-TM.....29
2.7.6.3.1. Historical Conditioning for HCT β-TM.....29
2.7.6.3.2. Evolution of conditioning regimen for HCT in β-TM.....31
2.7.6.3.3. Advent of Thiotepa/Treosulfan/Fludarabine regimen (TFT) 33
2.7.6.3.4. Introduction to the Use of the TFT Regimen in β-TM HCT.....34
2.7.6.3.5. Clinical studies in β-TM HCT setting with TFT regimen.....34

2.8. Stem Cell Sources.....37
2.8.1. HCT from HLA-Matched Sibling Donor.....37
2.8.2. Alternate Donor Transplantation for HCT in β-TM.....39
2.9. Post-transplant Care and Management of Iron Overload…40
2.10. Life after HCT in β-TM.....42
2.10.1. Mixed Chimerism.....42
2.10.2. Gonadal Function, puberty, and Infertility.....43
2.10.3. Long-term Complications and Quality of Life Post HCT in β-TM.....44
2.11. Pharmacology of TFT Conditioning.....45
2.11.1. Components of TFT Regimen.....45
2.11.1.1. Thiotepa.....45
2.11.1.1.1. Structure and Mechanism of Action.....45
2 11.1.1.2. ThioT Pharmacokinetics.....47
2.11.1.2. Fludarabine.....45
2.11.1.2.1. Structure and Mechanism of Action.....49
2.11.1.2.2. Flu Pharmacokinetics.....50
2.11.1.2.3. Flu Pharmacogenetics.....50
2.11.1.2.4. Dose-exposure-response Studies on Flu.....51
2.11.1.3. Treosulfan.....53
2.11.1.3.1. Structure and Mechanism of Action.....53
2.11.1.3.2. Treo Pharmacokinetics.....54
2.11.1.3.3. Treo Dose Exposure Response Relationship Studies.....57

2.12. Endothelial Dysfunction and Role of EASIX in HCT…..59
2.13. Metabolomics in HCT. ……..…....61

3. Patients and Methods……....63
3.1. Patients……....63
3.2. Blood sample collection and Processing……64
3.2.1. Blood Sampling for Pharmacokinetic Analysis…..……64
3.2.2. Blood Sampling for Pharmacogenomic Analysis……...64
3.2.3. Blood Sampling for Metabolomic Studies…….……65
3.3. Pharmacogenomic Analysis…….……....65
3.3.1. SNP Profiling using DMET Array …….……...65
3.3.2. Discovery cohort…….……...66
3.3.3. Validation Studies ….…….…….…..67
3.3.4. Screening of Selected SNPs in prospective samples.…68
3.3.5. Hardy-Weinberg Equilibrium……68
3.4. Assay of Flu & Treo-S, S-EBDM levels using LC-MS/MS……69
3.4.1. Treo & S, S-EBDM level measurement……70
3.4.1.1. Reagents and Chemicals……70
3.4.1.2. Sample Processing for Treo-S, S-EBDM Assay..71
3.4.1.3. Quantification of Treo and S, S-EBDM using LC-MS/MS……..……71
3.4.2. Validation of Treo and S, S-EBDM Assay……...72
3.4.2.1. Accuracy and Intra-day Precision……...73
3.4.2.2. Linearity……..73
3.4.2.3. Recovery……...……..74

3.4.2.5. Limit of Detection and Lower Limit of Quantification ……..……..75
3.4.3. Flu level measurement by LC-MS/MS…….……75
3.5. Population Pharmacokinetics (PopPK)……...77
3.5.1. Flu Pharmacokinetics…….……...77
3.5.2. Treo and S, S-EBDM PK…….….78
3.6. Pre-HCT EASIX Scoring……78
3.7. Clinical Definitions and Study Endpoints…….……..79
3.7.1. Whole blood Chimerism Analysis…….……80
3.7.1.1. Procedure…….……81
3.8. Pharmacodynamic Modelling for Treo & S, S-EBDM…...82
3.9. Global Plasma Metabolomic Profiling…….……...83
3.9.1. Plasma Metabolite Extraction…….……..83
3.9.2. LC-MS/MS-based Global Metabolomics……..83
3.9.3. MS Data Acquisition…….84
3.9.4. Processing of untargeted metabolomics data……84
3.9.5. Database searches for Metabolite assignment……..,85
3.9.6. Data Analysis…….85
3.9.7. Statistical Analysis for global metabolomic profiling...85
3.10. Statistical analyses…….……86

4. Results……87
4.1. Evaluation of dose-exposure response relationship to Treo and S, S-EBDM in patients with β-TM undergoing HCT.87

4.1.1. Patient demographics…….87
4.1.2. Treo & S, S-EBDM levels…….….88
4.1.3. Assessment of systemic Treo & S, S-EBDM exposure using PopPK model……88
4.1.4. Role of Genetic Polymorphisms in explaining variability in Treo and S, S-EBDM Exposure…….…….91
4.1.5. HCT outcomes……...……93
4.1.6. Impact of Treo & S, S-EBDM exposure on HCT outcomes……..…….94
4.1.7. Influence of Genetic Polymorphisms on HCT Outcomes……..…….95
4.1.8. Pharmacodynamic Modeling and Optimal Treo exposure Prediction…….……96
4.2. Impact of Fludarabine exposure on HCT outcomes……...99
4.2.1. Patient demographics……99
4.2.2. Flu levels……100
4.2.3. Assessment of Flu systemic exposure using PopPK Model……...……..101
4.2.4. HCT outcomes……103
4.2.5. Role of Flu Exposure and genetic variants on HCT outcomes……105
4.3. Endothelial Activation and Stress IndeX (EASIX) – measured pre-HCT predict transplantation-related mortality in patients with β-TM……..……106
4.3.1. Patient demographics……..106
4.3.2. EASIX-PreHCT Scoring…….107

4.3.3. HCT outcomes……107
4.3.4. ROC Analysis Using EASIX- PreHCT for Predicting TRM+100……108
4.3.5. Analysis of Predictors of TRM+100 in Class III β-TM cohort……..……109
4.4. A pilot study to identify biomarkers of sinusoidal obstruction syndrome using global plasma metabolomic profiling……115
4.4.1. Patient Demographics…….……115
4.4.2. Treo-based regimen alters plasma metabolome Postconditioning……..…116
4.4.3. Pre-conditioning plasma metabolomic profile predicts SOS……117
4.4.4. Post-conditioning plasma metabolomic profile predicts SOS……120

5. Discussion……122
5.1. Evaluation of dose-exposure response relationship to Treo & S, S-EBDM in patients with β-TM undergoing HCT....122
5.2. Impact of Fludarabine exposure on HCT outcomes……..130
5.3. Endothelial Activation and Stress IndeX (EASIX) – measured pre-HCT predict transplantation-related mortality in patients with β-TM……...132
5.4. A pilot study to identify biomarkers of sinusoidal obstruction syndrome using global plasma metabolomic profiling……134

5.5. Study Limitations and Future Directions……137

6. Summary and Conclusion…….138

7. Bibliography……...…141

8. List of publications……164

9. Appendices……..167

LIST OF FIGURES

Figure No	Figure Caption	Page No
Fig 2.1	Pathophysiology of β-Thalassemia	13
Fig 2.2	Classification of β-Thalassemia	16
Fig 2.3	Pathophysiological and Clinical Manifestations of β-Thalassemia.	18
Fig 2.4	Current and future therapeutic modalities for β-thalassemia	25
Fig 2.5	Proposed strategy to guide decisions on HCT for β-TM patients with consistent access to adequate supportive care	30
Fig 2.6	Analysis of day 28 chimerism and survival in different subsets of β-TM	35
Fig 2.7	Five-year OS comparing TFT with Bu/Cy regimens in older children with β-TM (>12 years)	36
Fig 2.8	Mechanism of iron-related organ/tissue damage in Thalassemia patients	41
Fig 2.9	Evolution of chimerism after HCT	43
Fig 2.10	Structure of Thiotepa and its active Metabolite Tepa	45
Fig 2.11	Possible mechanisms of interaction of Thiotepa with DNA	46
Fig 2.12	Metabolic biotransformation of Thiotepa and Tepa	48
Fig 2.13	Metabolism of Fludarabine to its active metabolite Fludarabine triphosphate	49
Fig 2.14	Structural Analogy between Treosulfan and Busulfan	53
Fig 2.15	Metabolism and mechanism of action of Treosulfan and its active metabolites S, S-EBDM and S, S-DEB	54
Fig 2.16	Endothelial challenges during alloHCT	59
Fig 3.1	Workflow employed in Fludarabine/Treosulfan Pharmacogenetic studies	67
Fig 3.2	Representative Chromatogram showing Treosulfan, metabolite S, S-EBDM, and 4′-Aminoacetophenone	72
Fig 3.3	Linearity and range of Treosulfan and S, S-EBDM Assay	74
Fig 3.4	Representative Chromatogram showing Flu and 5-Fluorocytidine	76

Figure No	Figure Caption	Page No
Fig 3.5	Study design of Fludarabine & Treosulfan-S, S-EBDM PK-PD studies	76
Fig 3.6	Whole blood chimerism analysis examples for Complete chimerism, Mixed chimerism, and Rejection	81
Fig 4.1.1	Plots showing Treosulfan and S, S-EBDM concentrations vs. time points in the study cohort	89
Fig 4.1.2	Basic goodness-of-fit plots of the final Treosulfan and ‘S, S-EBDM PopPK model	90
Fig 4.1.3	Correlation between Treosulfan with S, S-EBDM exposure, and Treosulfan Clearance with age	91
Fig 4.1.4	*NQO1/ GSTA1*B* genotypes influence Treosulfan and ‘S, S-EBDM Exposure	93
Fig 4.1.5	Association between Treosulfan exposure in patients with and without graft rejection	94
Fig 4.1.6	Association between Treosulfan/S, S-EBDM exposure ratio in patients with and without hepatic SOS	95
Fig 4.1.7	Impact of *NQO1* polymorphism on 1-year OS and Thalassemia free survival (TFS)	96
Fig 4.1.8	Impact of *GSTA1*B* polymorphism on 1-year OS and TFS	96
Fig 4.1.9	Treosulfan AUC and the probability of 1-year TFS and OS	98
Fig 4.2.1	Interindividual variation in Fludarabine Cmax.	100
Fig 4.2.2	Basic goodness-of-fit plots of the final Fludarabine PopPK model	102
Fig 4.2.3	Associations between Fludarabine Clearance with Age and *NT5E* Polymorphism	103
Fig 4.2.4	No significant association between Fludarabine exposure and clinical outcomes.	105
Fig 4.3.1	ROC curve for EASIX Pre-Tx as a predictor of TRM at day 100	109
Fig 4.3.2	TRM+100 curve for class III patients TM using the proposed EASIX-PreTx cut-off of 0.85	111
Fig 4.3.3	Kaplan-Meier survival curves for OS based on the proposed EASIX Pre-Tx cutoff (0.85)	114
Fig 4.4.1	Partial least squares discriminant analysis (PLS-DA) indicating effective group discrimination at the end of conditioning.	116
Fig 4.4.2	Volcano plot of differential metabolites between pre and end of conditioning groups	117
Fig 4.4.3	PLS-DA model comparing metabolomic profiles among patient samples without SOS (Pre-conditioning)	118

Figure No	Figure Caption	Page No
Fig 4.4.4	Volcano plot of differential metabolites in patients with SOS (Pre-conditioning)	118
Fig 4.4.5	Metabolite pathway enrichment analysis to identify pathways enriched in the patient group with SOS compared to no SOS.	119
Fig 4.4.6	PLS-DA model comparing metabolomic profiles among patient samples without SOS (End of conditioning)	120
Fig 4.4.7	Volcano plot of differential metabolites in patients with SOS (End of conditioning).	121
Fig 5.1.1	Pharmacodynamic model fit of the quadratic expression describing the change in probability of success with increasing Treosulfan Exposure	127

LIST OF TABLES

Table No	Table Caption	Page No
Table 3.1	Primer Sequences and Conditions for screening selected SNPs in prospective samples	68
Table 3.2	Mass transitions and optimized MS/MS parameters for quantification of Treo, S, S-EBDM, and 4′-Aminoacetophenone	71
Table 3.3	Representative table showing MS validation parameters – Inter-day Accuracy and Precision for Treosulfan and S, S-EBDM assay	73
Table 3.4	MS Parameters for Metabolite peak identification, alignment, and Annotation	85
Table 4.1.1	Patient Demographics for Treo-S, S-EBDM PK studies	87
Table 4.1.2	Frequency of genetic variants with potential association with Treo and metabolite S, S-EBDM exposure	92
Table 4.1.3	Cox proportional hazards model for 1-year Mortality and Graft Rejection	97
Table 4.1.4	Univariate and Multivariate Cox regression analysis for Predictors of 1-year TFS	98
Table 4.2.1	Patient Demographics for Flu PK Studies	99
Table 4.2.2	PopPK of Flu in Patients with β-TM Undergoing HCT	101
Table 4.2.3	HCT outcomes	104
Table 4.3.1	Table 4.3.1 Patient Characteristics of the entire cohort for the EASIX Study	106
Table 4.3.2	HCT outcomes of the entire cohort for the EASIX study	107
Table 4.3.3	Patient characteristics for all class III patients with β-TM undergoing HCT where EASIX-PreTx was available	109
Table 4.3.4	HCT outcomes for all class III patients with β-TM undergoing HCT where EASIX-PreTx was available.	111
Table 4.3.5	Comparison of patients who had TRM at day 100 versus those who did not among the class III β-TM where EASIX was available	112
Table 4.3.6	Univariate and multivariate analysis for predictors of TRM+100 amongst class III patients	113
Table 4.4.1	Patient Demographics for Global metabolomic profiling	115
Table 5.1.1	Comparison of Treo PK with previous reports	125

LIST OF ABBREVIATIONS

S.No	ABBREVIATION	FULL FORM
1	OD	Optical Density
2	5-FC	5-flurocytidine
3	AAP	4′-Aminoacetophenone
4	aGVHD	Acute Graft Versus Host Disease
5	AKT	Protein kinase B
6	alloHCT	Allogeneic Hematopoietic Stem Cell Transplantation
7	ALT/AST	Alanine transaminase/Aspartate transaminase
8	AML	Acute myeloid leukemia
9	AUC	Area under the curve; exposure
10	BCL11A	B-cell leukemia/lymphoma Transcription Factor A)
11	BM	Bone marrow
12	BSA	Body surface area
13	Bu	Busulfan
14	CC	Complete chimerism
15	CES2	Carboxylesterase 2
16	cGVHD	Chronic Graft Versus Host Disease
17	CL	Clearance
18	Cmax	Maximum/peak) plasma concentration
19	CV	Coefficient of variation
20	CWRES	conditional weighted residuals
21	Cy	Cyclophosphamide
22	DCK	Deoxycytidine Kinase
23	DMET	Drug-Metabolizing Enzymes and Transporters
24	DNA	Deoxyribonucleic acid
25	EASIX	Endothelial activation and stress index
26	EDTA	Ethylenediaminetetraacetic acid
27	EMR	Electronic Medical Record
28	EOI	End of Infusion
29	Flu	Fludarabine
30	GDF	Growth differentiation factor
31	GOF	Goodness-of-fit

32	GSTs	Glutathione S-transferases
33	GVHD	Graft Versus Host Disease
34	HbA	Haemoglobin A
35	HbE	Haemoglobin E
36	HCT	Haematopoietic Stem Cell Transplantation
37	HLA	Human Leukocyte Antigen
38	HPLC	High Performance Liquid Chromatography
39	HSCs	Haematopoietic Stem Cells
40	HWE	Hardy-Weinberg Equilibrium
41	IFC	Integrated Fluidic Circuit
42	IRB	Institute Review Board
43	IS	Internal standard
44	JAK	Janus kinase
45	KLF1	Krueppel-like factor 1
46	LC-MS/MS	Liquid chromatography- Tandem mass spectrometry
47	LDH	Lactate Dehydrogenase
48	LLOQ	Lower Limit of Quantification
49	LOD	Limit of Detection
50	LSM	Limited Sampling Model
51	MAF	Minor allele frequency
52	MC	Mixed chimerism
53	MCH	Mean corpuscular hemoglobin
54	MCV	Mean corpuscular volume
55	MDS	Myelodysplastic syndromes
56	Mel	Melphalan
57	MIP	Molecular Inversion Probe
58	MRM	Multiple reaction monitoring
59	MSD	Matched sibling donor
60	mTOR	mammalian target of rapamycin
61	MUD	Matched unrelated donor
62	NQO1	NAD(P)H Quinone Dehydrogenase 1
63	NRM	Non relapse mortality
64	NT5E	5'-Nucleotidase Ecto

65	OFV	Objective function value
66	OS	Overall survival
67	PBSC	Peripheral blood stem cells
68	PCR	Polymerase chain reaction
69	PD	Pharmacodynamics
70	PG	Pharmacogenetics
71	PK	Pharmacokinetics
72	PLS-DA	Partial least squares-discriminant analysis
73	PopPK	Population Pharmacokinetic model
74	Q1/3	Quadrupole mass filter
75	QC	Quality Control
76	RFLP	Restriction fragment length polymorphism
77	ROC	receiver operating characteristic curve
78	RRTs	Regimen related toxicities
79	S, S-DEB	Diepoxide {(2S,3S)-1,2:3,4-diepoxybutane
80	S, S-EBDM	Monoepoxide {(2S,3S)-1,2-epoxy-3,4-butanediol 4-methanesulfonate
81	SAEM	Stochastic Approximation Expectation Maximisation
82	SNP	Single Nucleotide Polymorphism
83	SOS	Sinusoidal obstruction syndrome
84	STR	Short tandem repeats
85	TDM	Therapeutic drug monitoring
86	TFS	Thalassemia free survival
87	TFT	Thiotepa/Fludarabine/Treosulfan
88	ThioT	Thiotepa
89	Treo	Treosulfan
90	TRM	Transplant-related mortality
91	UPN ID	Unique Patient Number ID
92	V	Volume
93	VNTR	Variable number of tandem repeats
94	VWF	von Willebrand factor
95	WBC	White Blood Cell
96	α-Hb	Alpha-globin
97	β-TM	Beta-Thalassemia Major

PERSONALIZING THE CONDITIONING REGIMEN IN HAEMATOPOIETIC STEM CELL TRANSPLANTATION

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SYNOPSIS

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Background

Allogeneic hematopoietic stem-cell transplantation (HCT) remains the only curative modality for patients with β-thalassemia major (TM), despite significant progress in gene therapy. At our center, the introduction of a toxicity-reduced conditioning TFT regimen containing Treosulfan (Treo), Fludarabine (Flu), and Thiotepa (ThioT) and a peripheral blood stem cell graft in patients with class III TM has significantly improved transplant outcomes, resulting in a notable reduction in early Transplant Related Mortality (TRM) from 46% (with the historical Busulfan (Bu)/Cyclophosphamide (Cy) regimen) to 13%. However, challenges related to mixed chimerism, graft rejection, and regimen-related toxicities (RRTs) continue to be significant concerns, which limit the success of HCT in these patients. Therefore, an unmet need exists to optimize the TFT regimen for HCT in TM.

One way to personalize the conditioning regimen is to study Pharmacokinetics (PK) and identify the dose-exposure response relationship for all the drugs used. This information will aid in establishing the optimal dosage range for each drug, allowing the doses to be adjusted accordingly to achieve the desired therapeutic range associated with improved outcomes and minimal adverse effects. Regrettably, unlike Bu/Cy, there is a scarcity of studies that have proposed such a therapeutic range for Flu and Treo in patients with TM undergoing HCT. Previous dose-exposure-response studies for Flu/Treo were primarily conducted in cohorts of non-uniform underlying diagnoses, inconsistent TFT dosing, and varying GVHD prophylaxes. Recent efforts by many groups suggest the feasibility of individualizing Flu/Treo dosing. However, no such attempt has been made in a uniform cohort of patients such as those with TM.

Genetic variants in genes encoding for drug-metabolizing enzymes and transporters might explain the variability in PK, thereby affecting treatment responses, including drug efficacy and safety. Further, if such a genotype-phenotype association is established, it could aid a priori predicting patients' response to drugs and the personalized dosing to prevent drug-related adverse effects from the first dose. Pharmacogenetics has been extensively investigated in alkylating drugs such as Bu and Cy, and limited studies for Flu. However, to our knowledge, no pharmacogenomic studies related to Treo in patients undergoing HCT. Identifying genetic markers to predict the risk of developing HCT complications could help to tailor conditioning regimens and improve HCT outcomes.

We hypothesize that the PK of Flu /Treo and their metabolites influence HCT outcomes in patients with TM. Genetic variants in Drug Metabolising enzymes and transporters genes can explain the variability in Flu/Treo PK and HCT outcomes. Personalizing the TFT regimen in HCT for patients with TM could be feasible.

Objectives of the Study

1. Establishment of a robust analytical methodology to evaluate Fludarabine and Treosulfan Pharmacokinetics in β-thalassemia patients undergoing HCT.

2. To analyze the Pharmacokinetics (PK) of Flu/Treo and their metabolites in patients with β-thalassemia undergoing HCT and to evaluate the dose-exposure-response relationship.

3. Exploratory analysis of genetic variants in the genes coding for drug-metabolizing enzymes/ transporters to explain the variability in Flu/Treo PK.

Patients and Methods

Patients

All consecutive patients with TM receiving ThioT/Flu/Treo (TFT) regimen before undergoing HCT between 2011 and 2021 were included in the study after obtaining written informed consent or assent from the patients/parents, respectively. The Institutional review board approved this study (IRB No: 9411, dated 29-04-2015).

Sample Collection for Flu/Treo PK

Heparinized peripheral blood samples were collected at predetermined time points just before (0hr, Pre), at the end of infusion (EOI) & 2, 4, and 24hrs after Flu/Treo infusion based on the Limited sampling model (LSM) reported previously by us. For Treo PK samples, Blood samples were immediately adjusted to a pH of 5.5 by adding 50µL of 1M citric acid/mL of blood to avoid Treo's artificial ex vivo conversion to S, S-EBDM. PK samples for measuring Flu levels do not require any stabilization steps. The samples were centrifuged at 13,000 rpm for 5 mins to obtain plasma and stored at –80^{o}C until further analysis.

LC-MS/MS assays for quantification of Flu/Treo levels

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) was employed to quantify both Flu, Treo, and S, S-EBDM levels in plasma samples. LC-MS/MS assay was standardized for measuring Treo and S, S-EBDM in plasma samples, and the method was validated for their specificity, linearity, precision, accuracy, and recovery before their application in clinical samples. For the measurement of Flu, an already validated LC-MS/MS assay in our laboratory was used.

Screening for polymorphisms

Peripheral blood was collected in EDTA tubes before the start of conditioning from all patients, and DNA was extracted using the Qiagen Gentra kit. Genotyping for 1936 polymorphisms in the DMET (Drug metabolizing enzymes and transporter) genes was performed by the DMET array (Affymetrix UK Ltd, High Wycombe, UK). Genotypes were called with DMET console version 1.1 using the Dynamic Genotype Boundaries algorithm (Affymetrix UK Ltd). For the Validation cohort, SNPs were determined by FLUIDIGM technology (integrated fluidic circuits (IFCs) (Standard BioTools Inc. California, USA).

Population Pharmacokinetic Modeling (PopPK)

PopPK modeling for Flu was done using non-linear mixed effects analysis performed via Monolix (version 5.1.0) using the Stochastic Approximation Expectation-Maximization (SAEM) method. For Treo and S, S-EBDM PopPK was estimated using non-linear mixed effects modeling via nlmixr2 in R(4.3.0) using the SAEM method.

Clinical Endpoints and Definitions

HCT outcomes such as RRTs, engraftment, Rejection, GVHD, donor-recipient chimerism status, and survival status were documented longitudinally in medical charts and electronically. The data were collected through a retrospective chart review at the end of the follow-up for the analysis. The study's primary objective was to evaluate the dose-exposure-response relationship of Flu, Treo, and its epoxy metabolite- S, S-EBDM in attempting to derive a therapeutic range in this uniform cohort of patients with high-risk TM. The primary endpoints included 1-year Thalassemia-free Survival (TFS) and Overall Survival (OS). Secondary

endpoints included Neutrophil engraftment, Chimerism status, RRTs, Graft rejection, GVHD, Early Transplant related mortality (TRM D+30), and late TRM (TRM+100).

Pharmacodynamic (PD) Modeling

PD modeling was performed to identify the cut-off and therapeutic window for the drugs. All PD analyses were done using R (version 4.3.0, R Foundation for Statistical Computing, Vienna, Austria).

Statistical Analysis

All statistical analyses were performed by R Statistical software (version 4.3.0; R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism software (version 8.4.3; GraphPad Software Inc, San Diego, CA, USA), and IBM SPSS statistics 21.0 (IBM Corp. Armonk, NY, USA).

Significant Findings of the Study

- Our study is one of the most extensive single-center studies to evaluate the Dose-exposure response relationship to Flu, Treo, and S, S-EBDM in a uniform cohort of TM patients undergoing HCT. Previous reports on dose-exposure-response studies on Treo-S, S-EBDM have been evaluated in non-uniform cohorts with mixed underlying diagnoses and inconsistent Flu/Treo dosing with or without ThioT. Studies on Flu PK so far have not addressed Flu PK in the TFT regimen. The uniformity in diagnosis, TFT dosing, and GVHD prophylaxis contribute to unbiasedness in the present study's findings, which is also the major highlight.
- We developed a simple, rapid, cost-effective MS-based assay to quantify Treo and S, S-EBDM in patient plasma samples. The assay also fulfilled the validation requirements (Linearity, Accuracy, Precision, Selectivity, Matrix effect, and Carry-over) for quantitative analysis of Treo and S, S-EBDM in biosamples. The validated assay was sensitive, could detect very low drug concentrations, and required a minimal amount of plasma sample for routine analysis.
- Our study reveals that Flu PK does not predict HCT outcomes in patients with high-risk TM. Although we observed a wide inter-individual variability (IIV) in Flu

exposure, we did not find any impact of Flu exposure on both early and late HCT outcomes. We speculate that the dose-exposure relationship to Flu may be better captured if the pharmacodynamic endpoints, such as lymphosuppression or immune recovery, were compared in patients with TM receiving a TFT regimen.

- We evaluated the role of Treo and S, S-EBDM in a large uniform cohort of patients with TM. We observed that patients with higher Treo exposure were associated with a lower incidence of graft rejection and better survival (1-year OS & TFS), while higher Treo exposure was not associated with RRTs, especially Sinusoidal Obstruction Syndrome (SOS) and Mucositis or any other TRM.
- We assessed the impact of the metabolite-S, S-EBDM on HCT outcomes which has not been explored so far. We did not observe the effect of S, S-EBDM exposure on early and late HCT outcomes, including survival.
- We also explored the role of prodrug-Treo to its metabolite-S, S-EBDM exposure ratio on HCT outcomes. Patients who developed SOS had significantly higher Treo to S, S-EBDM exposure ratio compared to those who did not develop SOS. The patient group with the highest Treo to S, S-EBDM exposure ratio had an increased incidence of SOS on quartile analysis.
- PD modeling to identify the optimal cut-off for better OS and TFS showed Treo exposure ≥1660 mg*hr/L to be significantly associated with better 1-year TFS and a trend to better 1-year OS. It is also worth noting that the patient group with the highest Treo exposure (> 2400 mg*hr/L) did not experience any adverse HCT outcomes, including RRTs, Rejection, or other TRM.
- To identify pharmacogenetic variants that could explain variability in Flu/Treo PK, we performed an exploratory analysis using a commercially available DMET microarray in a retrospective patient cohort. While no genetic variants were associated with Flu PK, four genetic polymorphisms showed significant association with Treo PK (3'UTR variants in *GSTA4*, *NQO1*, a missense variant in *GSTZ1,* and an intronic variant in *CES2* genes). We validated these four polymorphisms in addition to *GSTA1*B* promoter polymorphism in the present study cohort.
- Our validation studies identified only *GSTA1*B* and *NQO1* polymorphisms to retain the association with Treo and S, S-EBDM PK. Other genetic variants did not show any association with Treo or S, S-EBDM PK.
- We tested the influence of all five genetic polymorphisms on HCT outcomes. We identified both *GSTA1*B* and *NQO1* polymorphisms to impact the survival post-HCT.

Patients with NQO1 and GSTA1*B variant genotypes had significantly inferior 1-year OS and TFS. Other genetic polymorphisms did not influence HCT outcomes, including OS and TFS.

- We further evaluated the impact of *GSTA1*B* and *NQO1* polymorphisms in a large retrospective cohort of patients with TM, and only the *GSTA1*B* variant genotype significantly impacted poor survival post-HCT.

Implications of the Study Findings

- The validated Treo and S, S-EBDM assay in our laboratory could be helpful for routine Therapeutic Drug Monitoring (TDM). The advantages of the assay, such as short run-time and the need for minimal plasma samples, make it an ideal methodology for TDM.
- The present proof of concept study established the feasibility of optimal Treo dosing to achieve better HCT outcomes, especially in preventing graft rejection and early TRM. The study findings demonstrate the feasibility of Treo TDM.
- Our study findings revealed that high Treo levels are beneficial as there is no evidence of toxicities associated with higher Treo exposure (as observed in the present study and consistent with previous studies). Therefore, the proposed TDM strategy could help personalize the TFT regimen for optimal HCT outcomes, especially for high-risk patients with TM.
- Pharmacogenetic markers- *NQO1* and *GSTA1*B* could aid in establishing pre-emptive Treo dosing. However, further studies evaluating genotype-based dosing are warranted.
- *GSTA1*B* could be a prognostic biomarker for HCT with a TFT regimen.

1. INTRODUCTION

Over the past five decades, the life expectancy of patients with β-Thalassemia Major (β-TM) has significantly increased through optimal supportive care, regular blood transfusions, and adequate iron chelation therapy. Nevertheless, the lifelong nature of treatment poses a considerable challenge to the quality of life for patients with β-TM. Virtually all patients with β-TM must carefully plan their lives around regular transfusions, and even those receiving optimal care face a non-negligible risk of complications from either the transfusion therapy itself or the resulting iron overload. However, achieving transfusion independence is only possible for a small fraction of patients. It is important to note that most patients with β-TM reside in developing countries like ours, where access to safe and adequate transfusion therapy and regular chelation is often limited to a minority of individuals (Kattamis et al., 2022; Mathews et al., 2017; Taher et al., 2021). Allogeneic hematopoietic stem cell transplantation (alloHCT) remains the most established and potentially curative treatment for patients with β-TM (Algeri et al., 2023; Mathews et al., 2017).

The long-term goal of alloHCT in β-TM is to replace the patient's bone marrow with healthy and fully functional hematopoietic stem cells (HSCs). These transplanted HSCs should have the ability to support efficient erythropoiesis, thereby preventing the onset of significant organ dysfunction and complications. Therefore, performing HCT early during childhood is advisable to mitigate comorbidities arising from chronic transfusions. Significant improvements in overall HCT outcomes in β-TM have been achieved through advancements in high-resolution donor Human leukocyte antigens (HLA)-typing, the selection of conditioning regimens, graft-versus-host

disease (GVHD) prophylaxis, and enhanced supportive care measures (Algeri et al., 2023; Oikonomopoulou and Goussetis, 2021).

The conditioning regimen in β-TM should possess the ability to eliminate an expanded bone marrow (BM) and offer adequate immunosuppression to achieve stable donor graft engraftment, all while minimizing excessive toxicity on tissues/organs that have been chronically damaged by iron overload (Algeri et al., 2023; Mathews and Savani, 2014; Mulas et al., 2022). Introducing a toxicity-reduced conditioning regimen containing TFT- Treosulfan (Treo), Fludarabine (Flu), and Thiotepa (ThioT) for alloHCT in our center has significantly improved HCT outcomes in patients with class III β-TM. The outcomes of the transplant have notably improved, leading to a remarkable decrease in early Transplant Related Mortality (TRM) from 46% with the historical Busulfan (Bu)/Cyclophosphamide (Cy) regimen to 13% with TFT (Mathews et al., 2013). However, challenges such as mixed chimerism, graft rejection, and regimen-related toxicities (RRTs) continue to be significant concerns that hamper the success of HCT in these patients (George et al., 2015). Therefore, optimizing the TFT regimen for HCT in β-TM is critical to address these challenges effectively.

The conditioning regimen can be personalized by investigating the Pharmacokinetics (PK) of the drugs used and establishing the dose-exposure response relationship to help determine the optimal dosage range for each drug, enabling adjustments to achieve the desired therapeutic range. By doing so, improved outcomes can be achieved while minimizing adverse effects. Unfortunately, unlike the well-studied Bu/Cy regimen (Gaziev et al., 2010), there is a lack of studies proposing a therapeutic range for Flu and Treo, specifically in patients with β-TM undergoing HCT. Previous dose-exposure-response studies (Chiesa et al., 2020; J. B. Langenhorst et al., 2019;

Stoep et al., 2022; Takahashi et al., 2021) for Flu/Treo were predominantly conducted in cohorts with diverse underlying diagnoses, inconsistent TFT dosing, and varied GVHD prophylaxis. Recent research efforts by multiple research groups (Chiesa et al., 2020; J. B. Langenhorst et al., 2019) have indicated the feasibility of individually tailoring Flu/Treo dosing. However, no such attempt has been made for patients with β-TM.

Genetic polymorphisms in drug-metabolizing enzymes and transporters may contribute to the variability in PK, consequently impacting treatment responses, including both drug efficacy and safety. Establishing a genotype-phenotype association in this context could offer the potential to predict, in advance, patients' response to drugs and enable personalized dosing strategies to prevent drug-related adverse effects from the very first dose. Pharmacogenetics has been extensively studied for alkylating drugs like Bu and Cy (Balasubramanian et al., 2009a, 2009b; Goekkurt et al., 2007; Hassan and Andersson, 2013; Muñiz et al., 2022; Pinto et al., 2009; B. Poonkuzhali et al., 2001; Poonkuzhali et al., 2004; Srivastava et al., 2004; Takahashi et al., 2022), with limited research conducted for Flu (Mohanan et al., 2017; Nguyen et al., 2021; Sanghavi et al., 2016). However, to our knowledge, no pharmacogenomic studies have explored the relationship between genetic polymorphisms and the response to Flu/Treo in patients with β-TM undergoing alloHCT. Identifying genetic variants that predict the risk of developing HCT complications would be valuable for tailoring conditioning regimens and ultimately improving HCT outcomes.

Soluble biomarkers have been shown to aid in the early detection of complications associated with HCT and can potentially guide treatment decisions

(Balakrishnan et al., 2023). Presently, plasma and serum are the most commonly utilized biomarkers, effectively offering insights into systemic disorders that frequently impact transplant recipients, such as GVHD (Balakrishnan et al., 2020; Bidgoli et al., 2022). Growing evidence suggests that endothelial dysfunction plays a significant role in numerous life-threatening complications associated with HCT, including GVHD and RRTs (Luft et al., 2021). Recent studies demonstrated that Endothelial Activation and Stress Index (EASIX) could be a biomarker to predict GVHD, RRTs, and survival post-HCT (Jiang et al., 2021; Luft et al., 2017, 2020). The role of EASIX as a biomarker in Treo based regimen has not been addressed so far. Recent advances in metabolomics have revolutionized our understanding of complex human diseases and aid in identifying biomarkers corresponding to specific biochemical changes, especially during a complex therapy such as HCT (Reikvam et al., 2015, 2016, 2023). To our knowledge, there are no reports on the plasma metabolomics changes post-conditioning in patients with β-TM undergoing HCT receiving TFT conditioning.

1.1 Rationale and Hypothesis

At our center, TFT based conditioning regimen has been used to reduce TRM and graft rejection for high-risk β-TM since 2009. We have previously demonstrated that using a TFT regimen in patients with high-risk TM led to improved HCT outcomes. However, caveats such as mixed chimerism, rejection, and toxicities hinder the success of HCT in this regimen. Personalizing the conditioning regimen can improve HCT outcomes. There is very sparse data on dose-exposure-response to TFT conditioning

in high-risk β-TM, and the impact of Flu/Treo exposure on long-term HCT outcomes in β- TM has not been assessed before.

We hypothesized that:

1 The interpatient difference in the exposure of Flu/Treo and their metabolites explain the difference in HCT outcomes. Evaluating dose-exposure-response to Flu/Treo in β-TM-HCT could demonstrate the feasibility of optimal Flu/Treo dosing.

2 Genetic variants in Drug Metabolising enzymes & transporters (DMET) genes can explain variability in Flu/Treo PK and outcomes.

3 Identifying predictive biomarkers of HCT outcomes in the context of TFT conditioning could help personalize therapy.

4 Plasma metabolomics could help identify biomarkers for predicting HCT outcomes in patients with β-TM.

Our laboratory focuses on studying the role of the drugs used in conditioning regimens in HCT and how to personalize these regimens. We have previously studied Bu (Balasubramanian et al., 2009, 2019; Chandy et al., 2005; B. Poonkuzhali et al., 2001), Cy (Balasubramanian et al., 2009, 2012; Poonkuzhali et al., 2004), Mel (Pai et al., 2020), Flu (Mohanan et al., 2017), and Treo (Mohanan et al., 2018). Therefore, we could use the laboratory expertise in the PK and PG of the conditioning regimen.

1.2. Objectives of the Study

1 *Establishment of analytical methodology to evaluate Flu and Treo levels in plasma.*

2 *Evaluating the PK of Flu/Treo conditioning regimen in β-TM patients undergoing HCT and assessing their dose-exposure-response relationship, if any.*

3 *Analysis of genetic variants in the genes encoding drug metabolizing enzymes and transporters using an exploratory approach to explain variability in Flu/Treo PK.*

1.3 Brief overview of the thesis chapters

1.3.1. Literature review

Following the introduction, Chapter 2 is the literature review, which elaborates on the disease (β-TM), its pathology and molecular mechanisms, and the treatment options. The primary focus is on alloHCT as a curative modality for β-TM. Different conditioning regimens used in β-TM- alloHCT are addressed, and the role of the TFT regimen in alloHCT for β-TM is elaborated. PK, as well as dose-exposure-response studies for Flu as well as Treo, are described. A brief review of the role of metabolomics and biomarkers in alloHCT is detailed towards the end.

1.3.2. Materials and Methods

Analytical methodologies for measuring Flu, Treo, and S, S-EBDM are described in detail in the methodology section. The study design, the techniques and methods used for PG experiments, and data analysis are outlined. PopPK and PD modeling are also briefed. The pilot Global metabolomics study methodology is described in a detailed manner. An outline of statistical methods used in the study is also detailed.

1.3.3. Results

The results section is divided into four parts based on each objective, as listed above. The first part addresses the dose-exposure-response relationship to Treo and its metabolite S, S-EBDM. This part also highlights PG biomarkers relevant to Treo PK that could help further optimize the regimen. The second part emphasizes the role of Flu exposure on alloHCT outcomes. The third part highlights the role of EASIX, a biomarker that could predict alloHCT outcomes. The last part describes a pilot global metabolomic study to predict biomarkers for SOS.

1.3.4. Discussion

The significance of the major results obtained in this study is described in detail in this section. The results obtained in this study are compared with the existing scientific data, along with potential implications for clinical translation.

1.3.5. Summary, Conclusion, and Bibliography

The key observations from this doctoral work are summarized in this chapter, and the ongoing work and future directions are listed. The references cited in the text are listed in Bibliography.

2. REVIEW OF LITERATURE

2.1. Thalassemias

Thalassemias are a heterogeneous group of recessively inherited disorders characterized by an irregular, decreased production of normal α or β globin subunits of hemoglobin A (HbA). The condition arises from an imbalanced production of globin chains, resulting in ineffective erythropoiesis, increased hemolysis, and abnormal iron homeostasis (Kattamis et al., 2022; Taher et al., 2021). The genes responsible for producing β globin are located on chromosome 11, whereas the α globin genes can be found on chromosome 16 (Ali et al., 2021; Bajwa and Basit, 2023; Kattamis et al., 2022; Taher et al., 2021).

Thalassemia is classified into two main types, α, and β-thalassemia, based on the specific genetic mutation and the globin-chain subunits affected within the hemoglobin tetramer. Patients with thalassemia exhibit a wide range of clinical phenotypes, from nearly normal without complications to severe forms that require lifelong transfusion support. Allogeneic hematopoietic stem cell transplantation (alloHSCT) is still considered the only curative modality for this condition (Algeri et al., 2023; Ali et al., 2021; Oikonomopoulou and Goussetis, 2021).

New agents have shown promise in improving anemia and reducing transfusion dependence, and initial results from gene therapy approaches are encouraging. However, access to health resources remains unequal worldwide, a significant concern as most patients reside in underserved areas.

2.2 Beta Thalassemia

β-thalassemia is characterized by the absence or reduced production of the β-globin chain (Ali et al., 2021; Galanello and Origa, 2010). It was initially defined by Cooley and Lee in 1925 (COOLEY et al., 1927). β-thalassemia occurs due to genetic mutations that result in the replacement of a single nucleotide, small deletions or insertions within the β-globin gene or its neighboring flanking sequence, or in rare cases, substantial deletions. These mutations ultimately cause a reduction in the production of β-globin chains and HbA. β-thalassemia is further classified based on decreased (β^+) or absent (β^0) production of globin chains, which can lead to microcytic and hypochromic anemia, as well as a range of syndromic forms (Lei et al., 2019). Over 350 documented mutations have been associated with β-thalassemia, often classified based on severity. The term β^+ indicates milder mutations that result in a partial reduction in the synthesis of β-globin chains. In contrast, β^0 describes more severe mutations that can completely halt the production of β-globin chains (Taher et al., 2021).

2.2.1 Epidemiology of β-thalassemia

The prevalence of hemoglobinopathies worldwide is significant, with an estimated 1.5% of the population carrying a β-thalassemia allele and 5% carrying an α-thalassemia allele, making it the most common monogenic disorder (Kattamis et al., 2020; Modell and Darlison, 2008). Over 90% of patients reside in tropical regions spanning sub-Saharan Africa, the Mediterranean, the Middle East, the Indian subcontinent, and Southeast Asia (Modell and Darlison, 2008; Weatherall, 2012). Coinheritance of hemoglobin E with β-thalassemia is mainly found in eastern regions of India, Bangladesh, Myanmar, and Southeast Asia. Developed countries' registries

have accurately captured the disease burden and its changes, reflecting the impact of prevention programs and global population movements that have spread thalassemia to non-endemic regions, including northern Europe and North America. Although data collection is slower in low- and middle-income countries, it is imperative to do so as over 80% of β-thalassemia patients reside in these regions (Kattamis et al., 2020; Weatherall, 2018).

2.2.2 Prevalence of β-thalassemia

β-thalassemia is prevalent among individuals of Mediterranean, African, and South Asian ancestry. The following are the prevalence rates of β-thalassemia gene mutations among various population groups across different regions of the world:

Americas: The gene mutation affects 0-3% of the population.

Eastern Mediterranean: The gene mutation affects 2-18% of the population.

Europe: The gene mutation affects 0-19% of the population.

Southeast Asia: The gene mutation affects 0-11% of the population.

Sub-Saharan Africa: The gene mutation affects 0-12% of the population.

Western Pacific: The gene mutation affects 0-13% of the population.

2.2.3 Populations at Risk for β-thalassemia

β-thalassemia is more common in tropical and subtropical regions worldwide, especially in areas where malaria is or has been prevalent. The reason for this correlation is not fully understood, but it is believed that individuals carrying the genetic mutation may have increased protection against malaria (Vlok et al., 2021).

The southern regions of Italy and Greece are the most affected areas in Europe. In Asia, the Maldives has an exceptionally high prevalence of β-thalassemia, with around 16% of the population reported to have the condition. Other countries in tropical regions with higher rates of β-thalassemia include India and Thailand (Kattamis et al., 2020).

2.2.4 β-thalassemia in India

Around 50 million Indians are carriers of thalassemia; each year, approximately 12,000 children are born with β-thalassemia (Choudhry, 2017). However, several ethnic groups have a much higher prevalence (4–17 %) (Colah et al., 2017).

2.3 Pathophysiology of β-thalassemia

The pathophysiology of β-thalassemia is central to the disruption of the balance between α-like and non-α-like (β- and γ-) globin chain production, resulting in hemolysis and ineffective erythropoiesis. In β-thalassemia, the excess α-globin chains lead to unstable α-tetramers, which induce significant oxidative stress, causing damage to the cytoskeleton and impaired cellular function (Kattamis et al., 2022). The extent of the imbalance between α-chains and non-α chains primarily depends on the severity of the molecular defects in both the α-globin and β-globin genes. Molecular changes in genes involved in hemoglobin switching, such as KLF1 or BCL11A, can influence the clinical phenotype (Prakobkaew et al., 2014). Ineffective erythropoiesis is characterized by increased apoptosis of early red blood cell precursors, disrupted erythroid differentiation, and enhanced proliferation. Studies on ineffective erythropoiesis have revealed altered signaling pathways, including upregulation of

JAK/AKT/mTOR and SMAD2/3, as well as dysregulated concentrations of various ligands involved in erythropoiesis regulation (such as bone morphogenetic proteins and growth differentiation factors of the TGF-β superfamily).

Additionally, molecular chaperones like HSP70 and α-Hb stabilizing protein play a protective role (Oikonomidou and Rivella, 2018). Chronic anemia leads to increased erythropoietin production, further promoting ineffective erythropoiesis, bone marrow expansion, and extramedullary hematopoiesis, particularly in the spleen. The enlarged spleen traps red blood cells, exacerbating anemia (Kattamis et al., 2022).

Disrupted iron homeostasis is a central characteristic of thalassemia pathophysiology. Ineffective erythropoiesis significantly influences hepcidin regulation more than iron concentration alone. Despite elevated iron levels, hepatic hepcidin production is suppressed by ineffective erythropoiesis, mediated by erythroid regulators like GDF-15 and erythroferrone. Low hepcidin levels enhance iron availability, leading to increased dietary absorption and release from reticuloendothelial cells, eventually resulting in iron overload and iron-induced damage to cells and organs (Kattamis et al., 2006; Rivella, 2019). The overview of the pathogenesis of β-thalassemia is illustrated in Fig 2.1

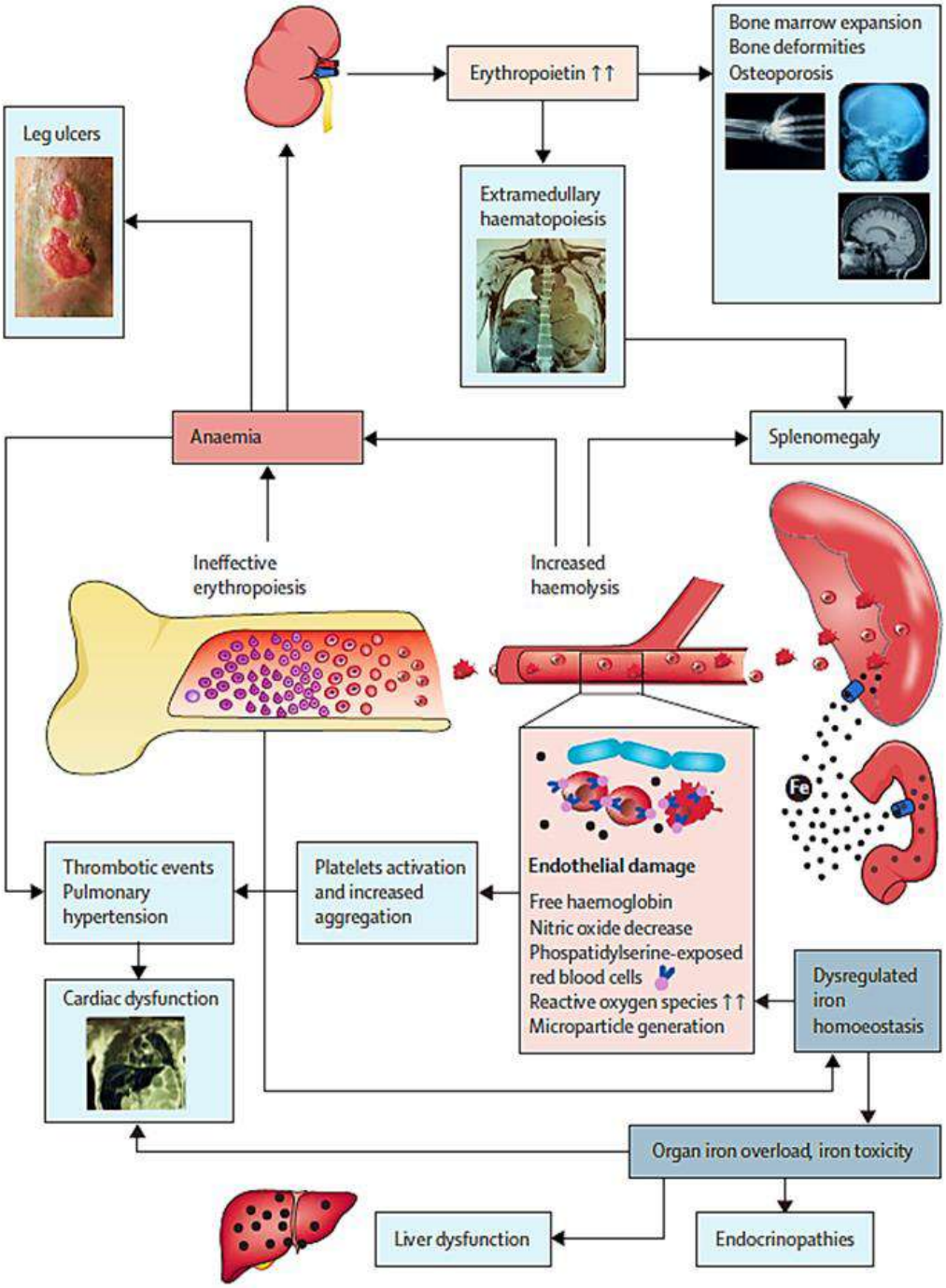


Fig 2.1 Pathophysiology of β-TM
Globin chain imbalance leads to free-chain precipitation, cytoskeleton alterations, intramedullary apoptosis, and intravascular and extravascular hemolysis. Suppressed hepcidin production leads to iron overload. Endothelial damage and a permissive microenvironment lead to platelet aggregation. (Kattamis et al., 2022)

2.4 Clinical Phenotypes in β-thalassemia

From a clinical perspective, β-thalassemia can be classified based on the severity of anemia and the need for regular red blood cell transfusions (Musallam et al., 2021). β-thalassemia minor, resulting from the inheritance of one mutation in a heterozygous state, is typically asymptomatic, with minimal, microcytic, and hypochromic anemia. β-thalassemia major (β-TM) patients require lifelong regular red blood cell transfusions, typically beginning in early childhood. β-thalassemia intermedia presents with less severe anemia, and patients may not require transfusions or only need sporadic transfusions. However, some individuals with β-thalassemia intermedia may eventually benefit from initiating regular transfusions, while patients with β-TM may discontinue their transfusion regimen, often after undergoing splenectomy. The decision to start chronic transfusions is not always straightforward. Various factors, including the personal perspectives of healthcare providers and the availability of local healthcare resources, can influence it.

An alternative categorization approach can be employed to address these complexities. Instead of classifying patients solely as β-TM or intermedia based on their long-term medical history, patients can be grouped as either transfusion-dependent thalassemia or non-transfusion-dependent β-thalassemia depending on whether they currently require regular red blood cell transfusions or not and are anticipated to continue to need them. This characterization provides a more detailed understanding of the patient's situation. Still, it is not absolute due to the potential for patients to shift categories, and the introduction of new therapies that reduce the need for transfusions may require the development of new terminology (Musallam et al., 2021; Weatherall,

2012). The current classification based on transfusion dependency is illustrated in Fig 2.2.

Hemoglobin E (HbE) is an abnormal hemoglobin resulting from a single point mutation in the β-globin gene and behaves similarly to a β+ mutation. When HbE mutation is co-inherited with a β-thalassemia mutation, it is classified as HbE– β-thalassemia. The severity of HbE–β-thalassemia can vary from mild to moderate (similar to β-thalassemia intermedia) to severe (similar to β-TM), depending on genetic modifiers mentioned earlier (Fucharoen and Weatherall, 2012; Musallam et al., 2013). The remarkable adaptability of children with HbE–β-thalassemia to low hemoglobin levels, attributed to a better erythropoietin response in early life, may delay or reduce the need for transfusion (O'Donnell et al., 2007).

2.5 Screening and Diagnosis

Conventionally, β-thalassemia screening is done by carefully examining the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) from the complete blood count using an autoanalyzer. This approach offers rapid and cost-effective results for identifying cases that require further investigation. Individuals carrying β-thalassemia and α^0-TM typically exhibit MCV levels below 79 fl and MCH levels below 27 pg. The diagnosis of β-thalassemia and detection of pathological variants generally are performed using high-performance liquid chromatography (HPLC) or capillary electrophoresis. Molecular methods have become the gold standard for confirming the diagnosis, identifying variant hemoglobins, elucidating complex cases, and enabling prenatal diagnosis (Lee et al., 2019; Traeger-Synodinos et al., 2015).

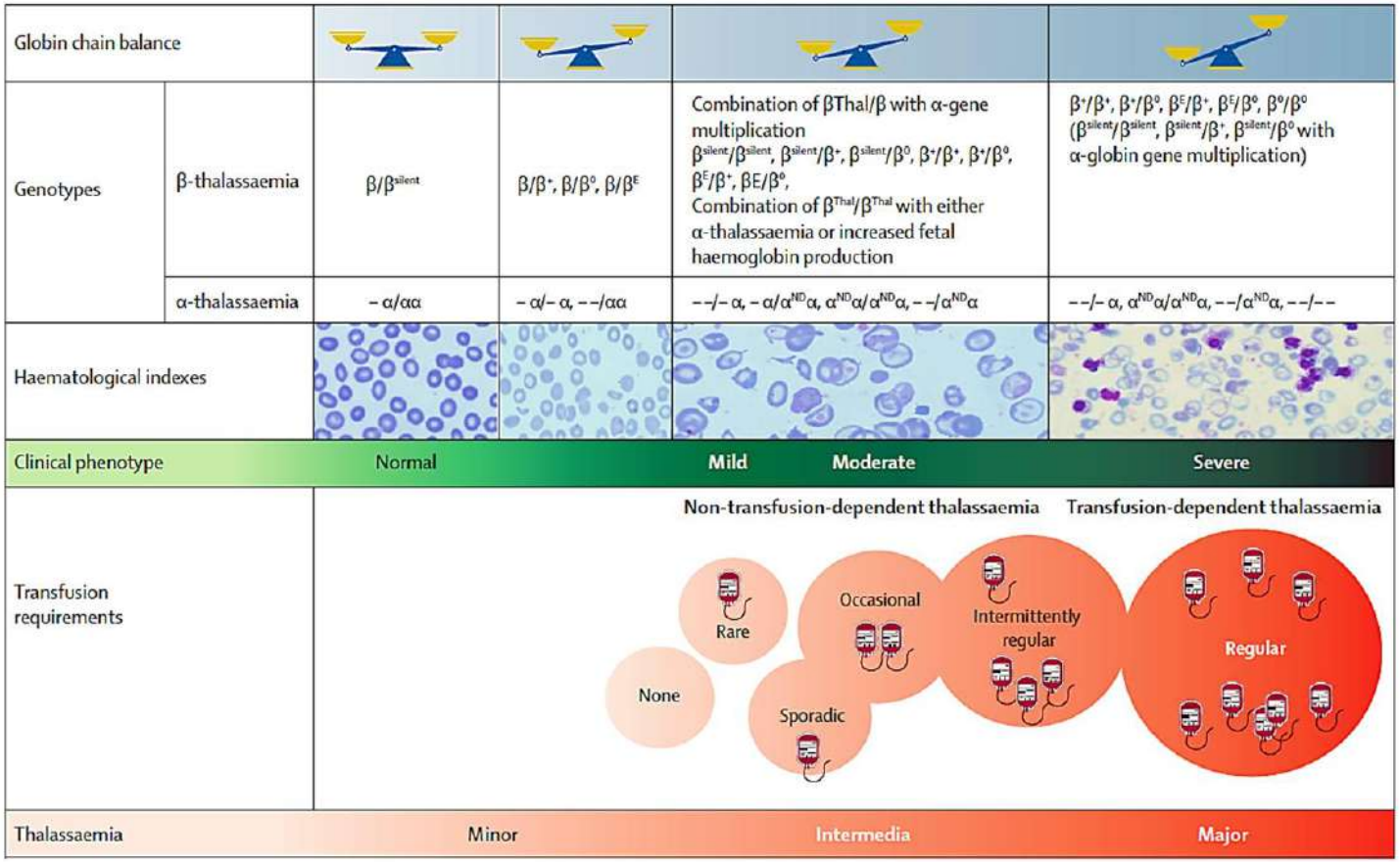


Fig 2.2 Classification of thalassemia
Hematological and clinical phenotypes correlate with genotypes and severity of globin chain imbalance, ND=non-deletional.
(Kattamis et al., 2022)

2.6 Clinical Implications

Chronic anemia resulting from ineffective erythropoiesis and peripheral hemolysis in β-thalassemia can give rise to various complications, including growth and developmental delay, fatigue, leg ulcers, and the potential for organ failure in adolescents and young adults (Rund and Rachmilewitz, 2005; Taher et al., 2018). Anemia has also been found to impact psychological well-being independently (Mihailescu et al., 2020).

Ineffective erythropoiesis leads to expansion of the bone marrow, leading to bone changes, pain, and deformities, which are responsible for characteristic craniofacial protrusions observed in β-thalassemia patients. Additionally, compensatory extramedullary foci, including the spleen and liver (hepatosplenomegaly), become activated and may undergo hematopoiesis. In some cases, these foci can grow into extramedullary hematopoietic pseudotumors. If these pseudotumors develop in critical areas like the paraspinal canal or chest, they can cause severe compression and may require emergency management (Rund and Rachmilewitz, 2005; Taher et al., 2018).

Peripheral hemolysis in β-thalassemia leads to the expression of prothrombotic markers on the surface of red blood cells, contributing to a hypercoagulable state (Eldor and Rachmilewitz, 2002). Platelet activation, microparticles, and other coagulation abnormalities further promote this state. Clinically, these disorders can manifest as venous and arterial thrombosis, pulmonary hypertension, and cerebrovascular events, including silent infarcts, which become more prevalent with aging (Manfrè et al., 1999; Musallam et al., 2012; Taher et al., 2010).

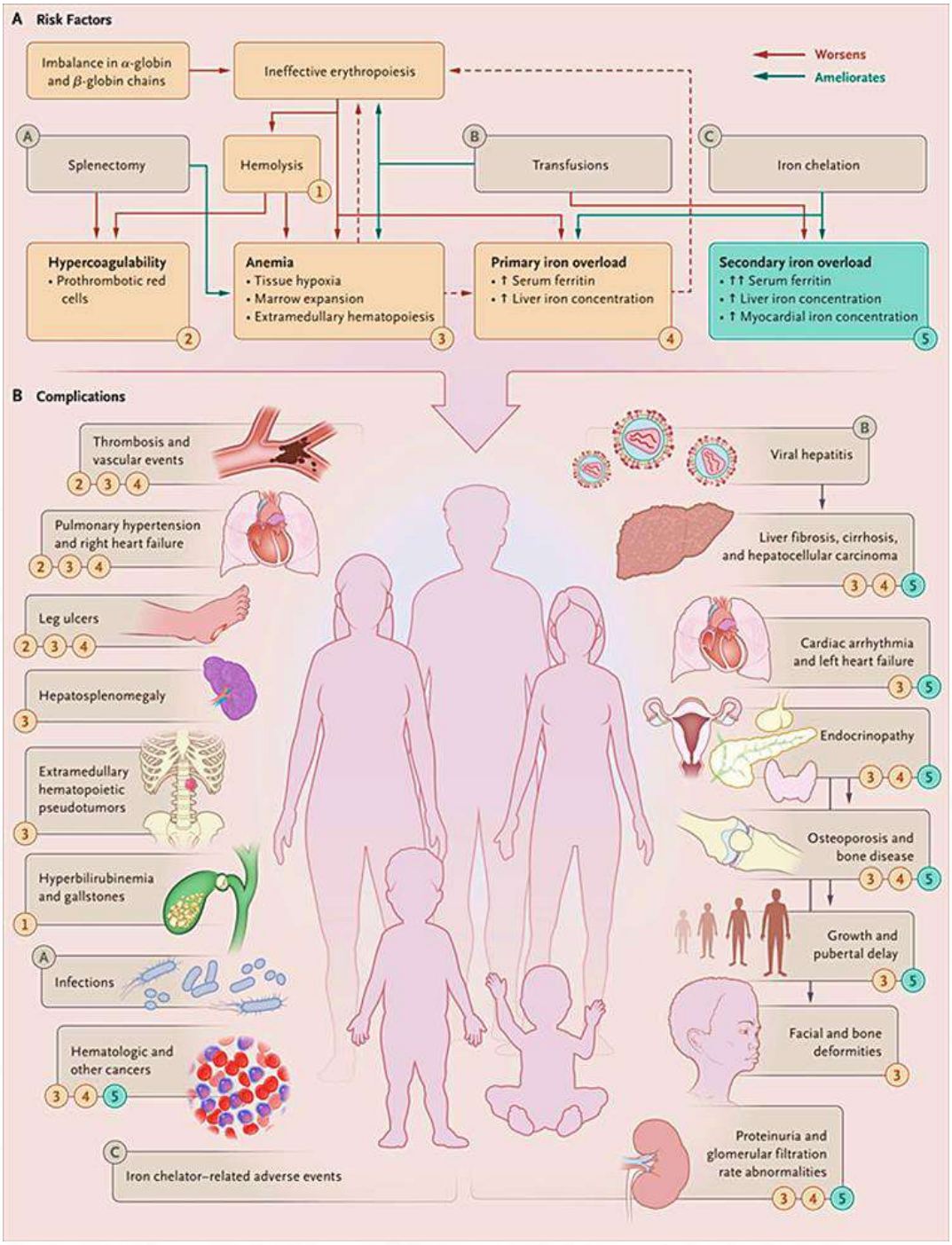


Fig 2.3 Pathophysiological and Clinical Manifestations of β-Thalassemia. Circled letters and numbers indicate complications with causal risk factors. (Kattamis et al., 2022)

Patients with more severe manifestations of β-thalassemia major or intermediate tend to accumulate iron within the reticuloendothelial system due to blood exchange. Excessive iron deposition occurs in multiple endocrine tissues, including the hepatic parenchyma, and gradually affects the heart tissues. Research indicates that maintaining consistent and long-term control of plasma labile iron levels and total iron overload, starting from initiating transfusion therapy, may be necessary to prevent complications (Aydinok et al., 2015). Despite advancements in iron chelation therapy, many patients still experience substantial iron accumulation in their myocardium and liver. As a result, these complications lead to the development of comorbidities that affect multiple organs, with the heart, liver, and endocrine glands being particularly vulnerable (Betts et al., 2020; Bonifazi et al., 2017; Chuncharunee et al., 2019). The pathophysiological and clinical manifestations of β-Thalassemia are shown in Fig 2.3. Advancing age remains a significant risk factor for complications in β-thalassemia (Pinto et al., 2019; Taher and Cappellini, 2018). Many clinical complications, such as vascular and hepatic diseases, develop gradually over several years. In addition to hepatic cancer, hematologic and solid cancers have been reported in older patients with β-thalassemia, which can be attributed to chronic stress in the bone marrow and iron overload (Taher and Cappellini, 2018). With improved survival among these patients, other complications associated with advancing age, such as heart disease, diabetes, renal disease, and cancers linked to non-thalassemia population risk factors, can also arise. Advancing age can also bring about social and psychological challenges related to marriage, work, and social integration (Taher and Cappellini, 2018). Additionally, the burden of the disease, long-term treatment requirements, and psychiatric disorders

can substantially impair the quality of life in individuals with β-thalassemia, leading to increased healthcare needs (Patel et al., 2019).

2.7 Management of β-thalassemia

2.7.1 Transfusion Therapy

The primary treatment approach for β-TM involves lifelong red blood cell transfusions to improve anemia and suppress ineffective erythropoiesis. Individuals with β-TM typically do not survive beyond 10 to 15 years without transfusions. The timing of transfusion initiation depends on the severity of the disease, and for β-TM, it typically begins within the first two years of life. In patients who require regular transfusions, the goal is to maintain pretransfusion hemoglobin levels within the target range of 9 to 10.5 g per deciliter (11 to 12 g per deciliter in patients with heart disease) (Taher et al., 2021). While there may be regional differences in transfusion protocols (Lal et al., 2018), significant progress in donor blood screening and preparation has reduced rates of alloimmunization and blood-borne infections in most countries (Cappellini et al., 2014).

2.7.2 Iron Chelation

Transfusion-dependent β-thalassemia patients require regular monitoring for iron overload, which can be achieved through serum ferritin measurements and hepatic and myocardial MRI scans. Iron chelation therapy should be initiated promptly after the transfusion of 10 packed red-cell units or when the serum ferritin level exceeds 1000 ng per milliliter (Taher et al., 2021). Three iron chelators are available for managing iron overload: subcutaneous deferoxamine and the oral agents, deferiprone and

deferasirox (available as dispersible and film-coated tablets). Deferoxamine and deferasirox are approved for patients older than two years (Cappellini et al., 2014), while deferiprone is approved as a second-line therapy for patients older than six years. However, recent randomized trials have shown the efficacy and safety of deferiprone in younger patients as well (Maggio et al., 2020). Extensive data support the ability of all three chelators, whether used alone or in combination (such as deferoxamine and deferiprone), to reduce systemic, hepatic, and myocardial iron overload. However, there are limited direct comparisons between oral chelators (Cappellini et al., 2011; Maggio et al., 2002; Pennell et al., 2006, 2012, 2014; Tanner et al., 2007). The magnitude of reduction in iron overload varies depending on the specific organ and chelation agent, and higher doses may be required to reverse cardiac siderosis. Oral chelators have advantages over deferoxamine in terms of treatment adherence, and the new film-coated deferasirox tablet has shown improved patient-reported outcomes compared to the dispersible form (Cappellini et al., 2014; Taher et al., 2017). Iron chelators should be chosen based on local guidelines, clinical judgment, and the patient's iron overload profile. Successful treatment involves adjusting the dose according to ongoing iron intake, regular monitoring, addressing adherence issues, and managing adverse events. Continuous parenteral deferoxamine remains the preferred option for patients with existing cardiac dysfunction, and there is available data on the potential benefits of combining deferoxamine with deferiprone (Cappellini et al., 2014).

Patients with non-transfusion-dependent β-thalassemia and a hemoglobin level below 10 g per deciliter are at an increased risk of complications (Taher et al., 2015). Due to the potential risk of secondary iron overload, it is not recommended to prescribe

lifelong regular transfusion therapy for these patients. There is a lack of data on new therapies explicitly targeting anemia in this patient population. Therefore, further studies are needed to evaluate the effectiveness of such treatments. It is essential to monitor all patients with non-transfusion-dependent β-thalassemia for iron overload using serum ferritin or liver iron measurements, starting from age 10, when iron-related complications may arise. Iron chelation therapy is recommended for patients with serum ferritin levels of 800 ng per milliliter or higher or a liver iron concentration of 5 mg per gram or higher. The target concentrations of ferritin and liver iron are 300 ng per milliliter or lower and 3 mg per gram or lower, respectively (Taher et al., 2013). While there are available data on deferoxamine and deferiprone (Calvaruso et al., 2015), it is worth noting that deferasirox is the only iron chelator currently approved specifically for this patient population based on data from a randomized phase 2 trial, which demonstrated significant reductions in serum ferritin and liver iron concentrations over a two-year treatment period (AT Taher et al., 2013).

2.7.3 Gene Therapy

Gene therapy involves rectifying the faulty production of β-globin chains by extracting hematopoietic stem cells from an individual with β-thalassemia. These cells are genetically modified using viruses to introduce external β-like-globin transgenes that facilitate proper gene expression. The initial gene therapy treatment, betibeglogene autotemcel (LentiGlobin BB305), has obtained conditional marketing authorization in Europe for patients aged 12 or older who have transfusion-dependent β-thalassemia, a non-β^0/β^0 genotype and are eligible for a transplant but lack a matched sibling donor. The authorization was granted based on findings from two phase 1–2 studies involving

22 patients. These patients were infused with hematopoietic stem cells modified ex vivo using the LentiGlobin BB305 vector. This vector encodes a hemoglobin variant, HbA, with a T87Q amino acid substitution that helps prevent sickling (Thompson et al., 2018). Among the 22 patients, 15 ceased to require transfusions following the gene therapy (12 out of 13 patients with a non-β^0/β^0 genotype and 3 out of 9 patients with a β^0/β^0 genotype). The remaining patients exhibited a reduced annualized red-cell volume and a decreased number of transfusions compared to before the gene therapy. Adverse events associated with the treatment were consistent with those observed in autologous HCT.

Results from two ongoing phase 3 trials involving children and adults with β^0/β^0 or non-β^0/β^0 transfusion-dependent β-thalassemia are still pending (ClinicalTrials.gov numbers, NCT02906202 and NCT03207009). Several other vectors and gene therapy approaches have also undergone evaluation. In phase 1–2 trial with patients having transfusion-dependent β-thalassemia and β^0 or severe β^+ mutations, intrabone administration of hematopoietic stem cells modified with the β-globin-expressing (GLOBE) lentiviral vector led to reduced transfusion requirements in three adults and complete independence from transfusions in three out of four assessed children (Marktel et al., 2019).

2.7.4 Luspatercept Therapy

Luspatercept (ACE-536) is the most recently authorized medication in the United States and Europe for treating adults with β-thalassemia who require transfusions. It is a genetically engineered fusion protein that combines a modified section of the human activin receptor type IIB with the Fc domain of human IgG1. These components work

together to bind to specific ligands from the transforming growth factor β superfamily, inhibit SMAD2/3 signaling, and enhance the maturation of red blood cells (Guerra et al., 2019; Suragani, Cadena, et al., 2014; Suragani, Cawley, et al., 2014).

Based on promising results from a phase 2 study, a recent phase 3 clinical trial called BELIEVE was conducted (Piga et al., 2019). This double-blind trial involved adults with transfusion-dependent β-thalassemia randomly assigned to receive subcutaneous Luspatercept (1.00 to 1.25 mg per kilogram of body weight) every three weeks (224 patients) or a placebo (112 patients). The trial demonstrated that Luspatercept, over a fixed 12-week period, reduced the need for transfusions by at least 33% in 21.4% of the Luspatercept group, compared to 4.5% in the placebo group (Cappellini et al., 2020). Moreover, Luspatercept also led to parallel decreases in serum ferritin levels without causing clinically significant changes in liver or myocardial iron concentrations. However, Luspatercept was associated with some adverse events, including transient bone pain, arthralgia, dizziness, hypertension, and hyperuricemia, which occurred more frequently than with the placebo. The Luspatercept-treated patients also experienced higher rates of thrombosis, although these events were primarily observed in individuals with known risk factors. It is recommended to monitor patients for signs and symptoms of thrombotic events. Luspatercept is gradually being incorporated into local management protocols for transfusion-dependent β-thalassemia while awaiting data on its long-term use and use in children. However, the use of Luspatercept in India is limited by its poor availability and high cost.

The current treatment modalities are represented in Fig 2.4.

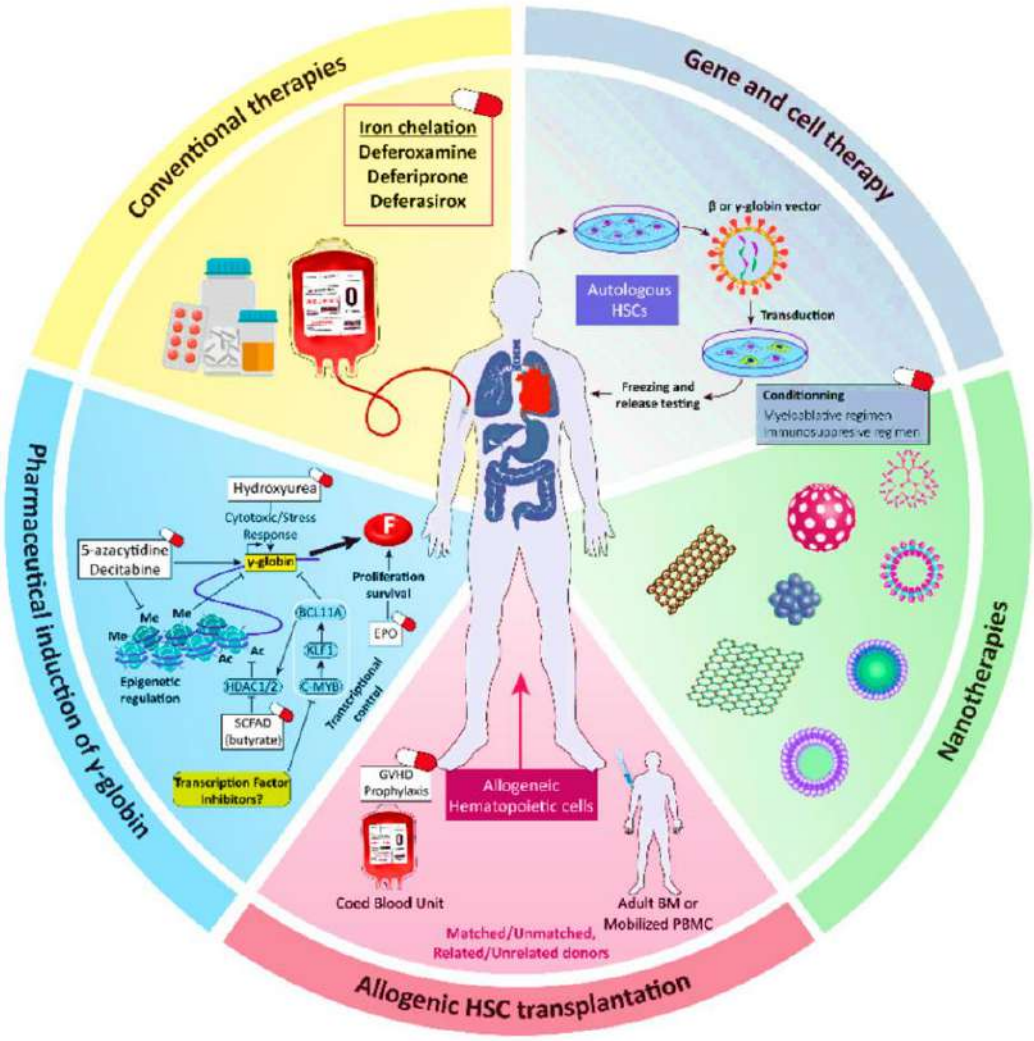


Fig 2.4 Current and future therapeutic modalities for β-thalassemia
(Rahimmanesh et al., 2022)**.**

2.7.5 Induction of fetal hemoglobin production

The induction of fetal hemoglobin production has been explored as a therapeutic approach in individuals with long-term manifestations of β-thalassemia to improve the lifespan of red blood cells. One of the drugs used to stimulate fetal hemoglobin production is hydroxyurea. Hydroxyurea has been used for the treatment of both sickle cell disease and β-thalassemia. It promotes the production of γ-globin and improves

hematological parameters and measurable signs in patients with β-thalassemia intermedia (Tari et al., 2018; Wilber et al., 2011).

Hydroxyurea acts as a cytotoxic compound during the synthesis phase of the cell cycle and inhibits ribonucleotide reductase (Finotti et al., 2015). It regulates and enhances the expression of the fetal hemoglobin gene GATA-2, associated with apoptosis and the cell cycle while suppressing the expression of the GATA-1 gene. Additionally, hydroxyurea can induce progenitor cell proliferation and increase erythropoietin levels (Pace et al., 2015).

2.7.6 HCT for β-thalassemia

AlloHCT, which involves using stem cells from HLA-matched related donors, is an established and effective cure for children suffering from transfusion-dependent β-thalassemia. Analysis from Europe between 2000 and 2010 reveals a 91% OS rate and an 83% EFS rate for children who received transplants from MSD donors (Baronciani et al., 2016). A study involving 50 transplant centers in China, India, and the USA demonstrates that HCT outcomes were most favorable for children under 6, with an EFS of 86%. Children aged 7 to 15 had an acceptable EFS of 80%, while patients aged 16 to 25 had a lower EFS of 63% (Li et al., 2019). The OS and EFS rates did not significantly differ between transplants from HLA-matched related and unrelated donors, suggesting that matched unrelated donor transplants are a viable option for young children.

While novel gene-addition therapy approaches have yielded promising outcomes, their broad implementation is constrained by affordability hurdles from high costs, resulting in the recent suspension of European commercialization. Concurrently, the progress in

high-resolution donor HLA-typing, selection of conditioning regimens, GVHD prophylaxis, and supportive care measures have consistently enhanced the overall outcome of HCT in transfusion-dependent alloHCT, which involves using stem cells from HLA-matched related donor, is an established and effective cure for children suffering from transfusion-dependent β-thalassemia, especially β-TM.

2.7.6.1 History of HCT in β-TM

The first HCT for β-TM was performed in Seattle by Thomas *et al.* in 1981 (Donnall Thomas et al., 1982). During the early 1980s, the transplantation procedure was only available at a handful of centers globally. However, from December 17, 1981, to January 31, 2003, more than 1000 consecutive patients between the ages of 1 and 35 underwent transplantation in Pesaro(G. Lucarelli et al., 1985; G Lucarelli et al., 1985; Lucarelli et al., 1984, 1987, 1990, 1992, 1992, 1993, 1996, 1999). Following the groundbreaking efforts of the Seattle and Pesaro groups, this therapeutic approach has become widely implemented worldwide.

2.7.6.2 Pre-HCT Stratification in β-TM

The objective of HCT in β-TM is to replace the patient's marrow with healthy functional hematopoietic stem cells (HSCs) capable of supporting effective erythropoiesis before significant organ dysfunction and complications occur (Algeri et al., 2023). With this goal in mind, HCT is ideally performed early, preferably during early childhood, to avoid the burden of comorbidities associated with chronic transfusions. Numerous studies have demonstrated that patients who undergo transplantation at a young age have better OS and EFS than late adolescents and adults.

An extensive analysis conducted by the European Group for Blood and Marrow Transplant (EBMT) suggests that 14 years serves as a cutoff for achieving optimal outcomes in HCT for β-TM (Baronciani et al., 2016; Li et al., 2019).

To assess the risk of transplant-related complications and the probability of successful HCT outcomes in β-TM, the Pesaro group developed a well-established scoring system in the late 1980s. This scoring system considers three variables: (1) quality of chelation therapy (regular versus non-regular), (2) presence and severity of hepatomegaly (determined by a palpable liver edge more than 2 cm below the costal margin), and (3) the extent of liver fibrosis observed in a pretransplant liver biopsy examination. Patients are classified into risk classes based on the presence or absence of these risk factors. Patients without risk factors are classified as belonging to risk class 1, while patients with all three risk factors are classified as belonging to risk class 3 (Lucarelli et al., 1990).

The Pesaro classification system has certain limitations. Firstly, it applies explicitly to the pediatric population and has not been validated for adult use (Lucarelli et al., 1992). Additionally, two of the three variables used in the scoring system, chelation quality and hepatomegaly, can be subject to variability in interpretation both within and between observers. Efforts to develop a scoring system based on precise and quantitative measures of iron overload, which would provide greater accuracy and applicability, have not been successful thus far (Angelucci et al., 2017). Another limitation of the Pesaro classification is that it does not account for a subgroup of patients at very high risk, which is more prevalent in developing countries. As HCT becomes increasingly available in these regions, it becomes crucial to identify and appropriately modify HCT protocols for this high-risk group. It has been suggested

that patients above seven years of age with liver sizes of 5 cm represent a very high-risk subgroup within the conventional Class III group, referred to as Class III Vellore high-risk or Class III VHR (Mathews et al., 2007). Despite these limitations, the Pesaro classification holds undeniable merit as it highlights the crucial role of patients' quality of medical care before transplantation in determining the outcome after AlloHCT. It is a powerful demonstration of the impact of pre-HCT medical care on post-HCT results. The proposed algorithm for HCT in β-TM is illustrated in Fig 2.5.

2.7.6.3 Conditioning in HCT for β-TM

2.7.6.3.1 Historical Conditioning for HCT in HCT for β-TM

The first alloHCT in β-TM was performed by Thomas *et al.* in 1982 (Donnall Thomas et al., 1982). They used a conditioning regimen comprising a single dose of Bu (5mg/kg intravenously) and Cy (50mg/kg intravenously) on four successive days, followed by alloHCT. Eventually, a myeloablative conditioning regimen of Bu/Cy with total body or lymphoid irradiation (TBI/TLI-200cGy, Bu-4mg/kg, and Cy-50mg/kg for four successive days) or without (Bu-3.5 mg/kg and Cy-50 mg/kg for four consecutive days) was routinely However, the latter regimen (Bu/Cy) became the standard conditioning as the former regimen was not recommended for patients with β-TM undergoing HCT because of the high rate of TRM.

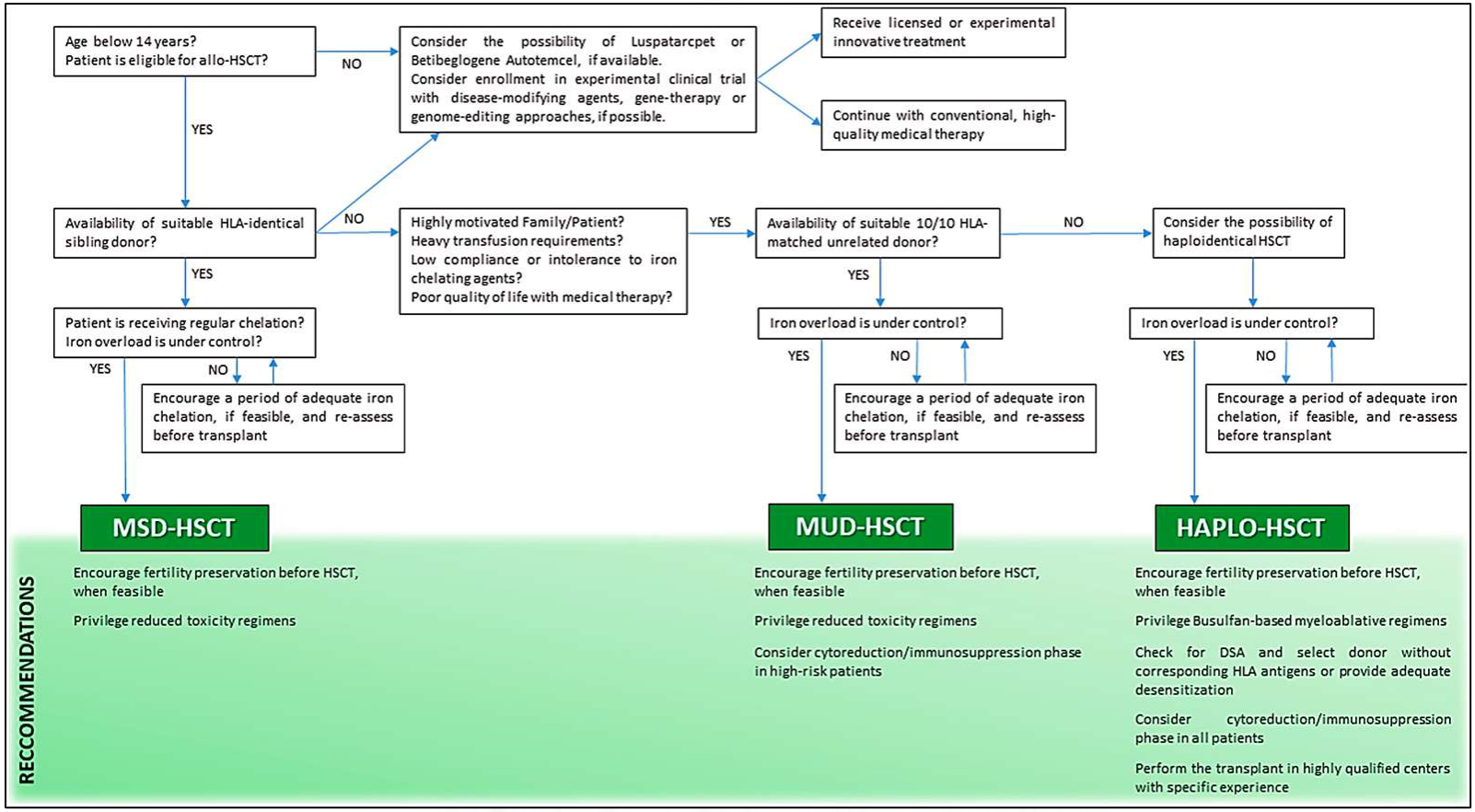


Fig 2.5 Proposed strategy to guide decisions on HCT for β-TM patients with consistent access to adequate supportive care. (Algeri et al., 2023)

The earliest large study on Bu/Cy regimen in β-TM-HCT reported OS and Thalassemia Free Survival (TFS) rates of 82% and 75%, respectively (Lucarelli et al., 1990). However, HCT outcomes, including TFS and OS, were inferior in class III patients compared to Class I/II patients with β-TM undergoing HCT, probably because Class III patients with β-TM have a high risk of graft rejection and regimen-related toxicity (RRTs), especially sinusoidal obstruction syndrome (SOS), leading to multiple systems organ failure and death. These complications are due to the pre-existing high degree of alloimmunization and iron overload-related organ damage in this high-risk cohort. Therefore, this led to optimizing the conditioning regimen for class III patients with β-TM undergoing HCT (Lucarelli et al., 1990).

2.7.6.3.2 Evolution of conditioning regimen for HCT in β-TM

Inferior Outcomes in Class III patients with β-TM led to the evaluation of novel conditioning regimens that could improve HCT outcomes. The initial strategy developed by Lucarelli et al. to reduce TRM in Class III patients entailed reducing the cumulative dose of CY from 200 mg/kg to 160 mg/kg. While this approach successfully lowered mortality associated with TRM, it also increased graft rejection from 13% to 35% (Lucarelli et al., 1996). In another study by Hussein et al., HCT outcomes were improved with a RIC regimen comprising 4 mg/kg/day of Bu, 35 mg/m^2/day of Flu, and one 500 cGy TLI resulting in good OS and TRM rates of 100% and 0%, respectively. The acute and chronic GVHD rates were acceptable at 11% and 8%, respectively. However, there was a relatively high incidence of 20.6% graft failure (Hussein et al., 2013). Subsequent attempts with the RIC regimen augmented graft rejection risk (Horan et al., 2005; Iannone et al., 2003).

At our center, In our center, we evaluated two different busulfan regimens to investigate their impact on graft rejection (Chandy et al., 2005b). We also incorporated anti-lymphocyte globulin (ALG) in one of the regimens to observe its influence on graft rejection and GVHD. While we successfully established a correlation between graft rejection and busulfan pharmacokinetics, we did not observe any effect of ALG on graft rejection or GVHD in this study. Furthermore, we could not address the cohort's relatively high incidence of RRTs. Gaziev et al. conducted a study that demonstrated the potential of intravenous Bu with therapeutic dose monitoring (TDM) and adjustments as a promising strategy to decrease RRTs and the risk of graft rejection (Gaziev et al., 2010). However, in another study by Chiesa *et al.*, the TDM approach reduced RRTs. Still, it did not manage to decrease the risk of graft rejection in children with advanced disease (Chiesa et al., 2010).

Sodani et al. reported the first successful attempt to improve clinical outcomes in Class III patients by developing a modified conditioning regimen by reducing Cy dosage to 160 mg/kg (Sodani et al., 2004). However, based on their initial unfavorable experience, they augmented Pre-transplant immunosuppression (PTI) by introducing Flu and AZA to the conditioning regimen. Starting from day 45, they used therapeutic interventions such as intensive chelation, hyper-transfusion therapy, hydroxyurea, and growth factors. In a relatively small series of 33 consecutive Class III patients, they achieved a graft rejection rate of less than 10% and an event-free survival exceeding 85%. In another study conducted by Anurathapan et al., a novel approach involving administering one or two courses of PTI with a combination of Flu and dexamethasone (Dexa) one to two months before RIC consisting of Flu, Bu, and ATG (Anurathapan et al., 2013). This study demonstrated similar GVHD and TRM rates but no graft

failure incidence. Also, in a German survey of children and adolescents undergoing MSD-HSCT for β-TM, promising outcomes were observed when Cy was replaced entirely with Flu. During a follow-up period of 25 months, no GVHD or mortality was reported, and all patients remained in good health (Sauer et al., 2007).

A recent study in India reported excellent outcomes with 100% OS and TFS with MUD-PBSC protocol involving PTI with HU, Flu, TTP, and Cy. Only one patient experienced GVHD, albeit with a relatively short follow-up period (Kharya et al., 2021). Recent investigations in the Haplo-HCT β-TM setting demonstrated improvements in OS and TFS with MAC or RIC regimen with T-cell depletion and PTI, noting that the incidence of graft failure and GVHD remained significant and could not be overlooked (Anurathapan et al., 2016; Pession et al., 2011).

2.7.6.3.3 Advent of Thiotepa/Treosulfan/Fludarabine regimen (TFT)

While not improving TFS, Bu-based regimens have been associated with RRTs (SOS, obliterans bronchiolitis, and thrombotic thrombocytopenic purpura), particularly in high-risk class III β-TM patients. In recent years, Treosulfan (dihydroxy busulfan, Treo) has garnered significant interest as a potential substitute for Bu due to its favorable toxicity profile, particularly in the context of HCT for high-risk β-TM. Treo holds appeal due to its reported low hepatic toxicity and consistent pharmacokinetic profile. These characteristics address significant concerns associated with conventional Bu usage in this patient population. Bernado *et al.* published the initial report on using Treo in a conditioning regimen for β-TM in 2008. It was proven safe and effective in patients with β-TM (Bernardo et al., 2008). Treo is used increasingly

in class III high-risk patients with β-TM because of the substantial risk of hepatotoxicities such as SOS in iron-overloaded patients.

2.7.6.3.4 Introduction to the Use of the TFT Regimen in β-TM HCT

Bernado *et al.* initially published a report on using Treo in the conditioning regimen for β-TM in a small cohort of 20 patients (Bernardo et al., 2008). Among these patients, 45% were classified as Class III, and there were 18 matched unrelated stem cell transplants. Only two patients experienced a temporary increase in liver enzymes. The conditioning comprising Treo, Flu, and ThioT (TFT) was highly well-tolerated, resulting in complete chimerism in 17 cases.

2.7.6.3.5 Clinical studies in β-TM HCT setting with TFT regimen

Progressively, the same group presented their expanded findings involving 60 cases of β-TM, with a median age of 7 years. Among the 48 children, only 7% were classified as Class III, while the remaining 12 cases were adults. Among the patients, 40 (67%) received a transplant from an unrelated donor, and in 47 cases (79%), the source of stem cells was bone marrow. Consistent with the earlier report, the regimen exhibited excellent tolerance, with a low incidence of graft failure (<10%) and TFS of 84% (Bernardo et al., 2012). In contrast to the positive findings, a small series from India reported similar outcomes between a Treo-based regimen (n = 28) and a historical Bu-based regimen (n = 12). However, there were some notable differences between the two groups. The median age in the Bu group was 7 years, while in the Treo group, it was 9.6 years. Also, the Treo group had a higher proportion of Class III patients, with 75% falling into this category. Among them, 52.4% were classified as Class III HR.

In the Bu arm, 58% of patients were classified as Class III, but the specific number meeting the criteria for Class III HR is not provided. Unfortunately, information regarding the age and risk group of patients in the treosulfan arm who died due to RRTs (n = 4) and those with graft rejection (n = 2) is unavailable. It is important to note that the outcomes of Class III HR patients can significantly differ from those of the entire population, reported previously (Choudhary et al., 2013).

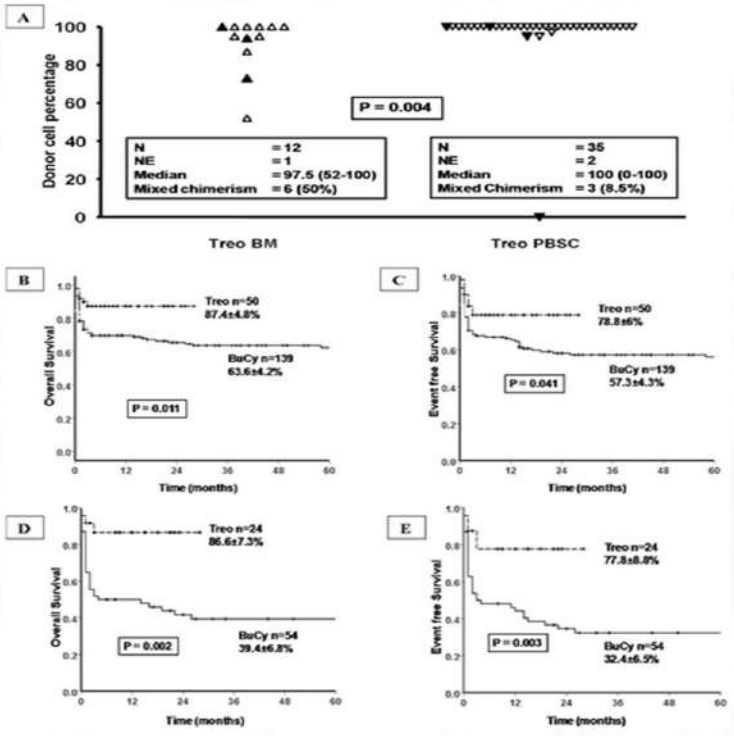


Fig.2.6. Analysis of day 28 chimerism and survival in different subsets. (A) Day 28 chimerism comparing patients conditioned with a TFT regimen and receiving either a BM or a PBSC graft; NE = not evaluated; indicates cases that had died before day 28. Filled triangles indicate cases with an event defined as either graft rejection or death. Empty triangles indicate cases that did not have an event defined as either graft rejection or death (B) OS of all Class III patients conditioned with either a TFT or Bu/Cy regimen (C) EFS of all Class III patients conditioned with either a TFT or Bu/Cy regimen (D) OS of all Class IIIHR patients conditioned with either a TFT or Bu/Cy regimen (E) EFS of all Class IIIHR patients conditioned with either a TFT or Bu/Cy regimen. (Mathews et al., 2013)

At our center, we demonstrated that using the TFT regimen improved outcomes in this Class III as a whole and the subset of Class III HR, reducing early TRM from 46% (with Bu/Cy) to 13%. We also observed a significant decrease in non-relapse mortality (NRM) and RRTs, especially SOS, in the class III HR compared with a historical control arm (Bu/Cy) with TFT conditioning. However, in the Class III HR group, there was a notably higher likelihood of developing mixed chimerism. However, this challenge could be overcome by employing a PBSC graft. TFT conditioning, and a PBSC graft, resulted in significantly improved OS and EFS, specifically for patients in the Class III HR group, without a notable increase in the risk of GVHD (Mathews et al., 2013), Fig 2.6.

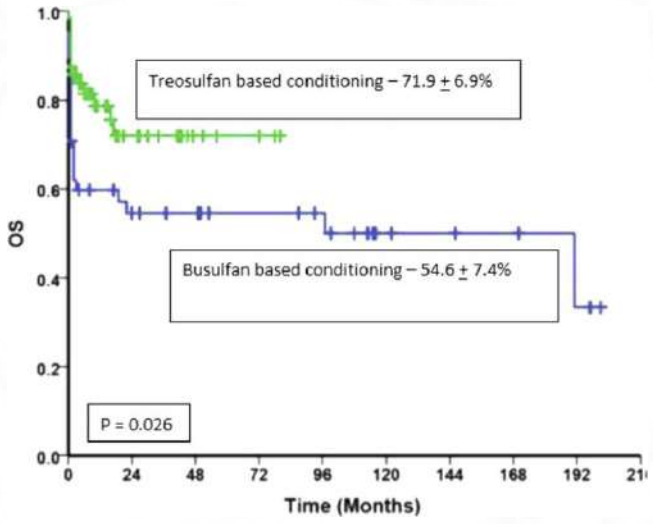


Fig.2.7 Five-year OS comparing TFT with Bu/Cy regimens in older children with β-TM (>12 years). (George et al., 2019)

In a subset analysis, we also observed that TFT conditioning is associated with a better OS following MSD-HCT in older children (>12 years) with β-TM compared to the historical cohort of patients who received Bu/Cy conditioning, Fig 2.7 (George et al.,

2019a). However, we also noticed that mixed chimerism was common with TFT conditioning (George et al., 2015).

A recent analysis demonstrated no differences in OS and the incidence of aGVHD and cGVHD between patients who underwent HCT after either a Flu/Bu or Treo-based conditioning regimen. However, the Treo cohort had a higher incidence of second HCT (HR 2.24 [95%CI: 1.21 to 4.13%]; P=0.01), and the most common reason for the second transplant was likely primary or secondary graft failure or graft loss (Lüftinger et al., 2022). Another recent retrospective study from India reported that Flu/Bu/Cy/ATG-based conditioning was effective and well-tolerated in high-risk β-TM HCT settings (Mehta et al., 2022).

2.8 Stem Cell Sources

2.8.1 HCT from HLA-Matched Sibling Donor

Over the past three decades, more than 2,000 patients with β-TM have undergone HCT from HLA-matched related donors. Early reports from the 2000s consistently showed OS rates above 90% and TFS rates above 80% in class I patients across multiple research groups (Isgrò et al., 2010; Lucarelli and Gaziev, 2008). These findings were further supported by a retrospective survey conducted by the EBMT, which included 1,061 patients treated with matched sibling donor (MSD) transplantation and reported OS and TFS rates of 91% and 83%, respectively. With additional refinements in patient preparation and the introduction of RIC regimens, the TFS rates have improved and now consistently approach or even exceed 90% in low-risk subjects transplanted in more recent years (Li et al., 2019; Locatelli et al., 2013)

As a result, alloHCT using an available MSD is now considered a standard-care procedure for subjects with thalassemia major and is increasingly offered at a younger age to prevent the development of organ damage. Although the survival rates have also increased above 90% in high-risk patients with the adoption of RIC regimens, the TFS rates remain suboptimal, ranging from 66% to 81% (Li et al., 2019).

While using BM-derived HSCs is the standard practice in MSD transplantation, alternative stem cell sources have been explored. The feasibility of using cord blood (CB) from HLA-identical siblings for HCT in patients with β-TM has been demonstrated since 2003. This approach has been associated with a reduced risk of acute and chronic graft-versus-host disease (aGVHD and cGVHD) as well as transplant-related mortality (TRM), provided that the CB unit has a sufficient number of nucleated cells (more than 3.5 × 10^7/kg) (Li et al., 2019). In an extensive retrospective analysis published in 2013, OS, TFS, aGVHD, and cGvHD rates were reported as 95%, 88%, 20%, and 12% for BM recipients (n=389) and 96%, 81%, 10%, and 5% for CB recipients (n=70), respectively (Li et al., 2019).

Promising results have also been observed with the co-transplantation of CB and BM-derived HSCs harvested from the same HLA-matched family donor. This combined infusion has improved hematopoietic recovery while preserving the protective effect of CB against GvHD (Tucunduva et al., 2015). Several research groups have evaluated using PBSCs from MSD to address the risk of graft failure in β-TM (Ghavamzadeh et al., 2008; Iravani et al., 2010; Mathews et al., 2013). PBSC grafts have been associated with faster engraftment and a low incidence of graft rejection (Ghavamzadeh et al., 2008; Mathews et al., 2013). However, this advantage is accompanied by an increased

risk of cGvHD, which can be particularly problematic in nonmalignant diseases (Iravani et al., 2010; Irfan et al., 2008).

In a large retrospective analysis conducted by the EBMT, the use of PBSCs was associated with significantly lower OS and EFS than BM and CB grafts (Baronciani et al., 2011). However, in updated results from 193 MSD-HCTs performed after "NF-08-TM" conditioning, impressive OS and TFS rates of 97.4% were reported, despite using PBSC grafts. Additionally, the incidence of grade II-IV aGVHD was remarkably low at 5.4%, and moderate/severe cGVHD occurred in only 3.9% of cases (He et al., 2020). It is important to consider ethical concerns related to the mobilization of minor donors when analyzing the results obtained using PBSCs in β-TM patients (He et al., 2020).

2.8.2 Alternate Donor Transplantation for HCT in β-TM

Early reports of unrelated donor HCT for β-TM demonstrated high rates of graft rejection and TRM, partially attributed to factors such as older recipient age, advanced disease, and less refined donor selection methods (Baronciani et al., 2016). T cell-depleted haploidentical transplants for thalassemia showed a disease-free survival (DFS) rate of 70% (Sodani et al., 2011). Registry results for unrelated umbilical cord blood transplantation in β-TM were poor, with DFS rates of 21% in β-TM recipients (Ruggeri et al., 2011).

However, current donor selection and transplant conditioning advancements have improved TFS rates in the early follow-up stages. Strategies such as the addition of hydroxyurea/azathioprine before conditioning in mismatched and matched related HCT (94% and 82% DFS, respectively), dexamethasone/fludarabine before

conditioning in haploidentical donor HCT (94% DFS), fludarabine, thiotepa, and treosulfan in unrelated donor HCT (82% DFS), and a reduced-intensity conditioning (RIC) combination of hydroxyurea, alemtuzumab, fludarabine, melphalan, and thiotepa (79% and 80% DFS for unrelated donor cord and bone marrow, respectively) have contributed to improved outcomes (Anurathapan et al., 2016; Bernardo et al., 2012; Gaziev et al., 2013; Shenoy et al., 2013; Shenoy and Thompson, 2016). Long-term follow-up is essential to assess the merits of alternate donor HCT approaches and evaluate their efficacy across different modalities.

2.9 Post-transplant Care and Management of Iron Overload

The management of iron overload remains an essential consideration after transplantation. Following a successful transplant and once the patient is stable with a hemoglobin (Hb) level above 100 g/L, the preferred method for iron removal is phlebotomy. This procedure can be repeated every 14 to 28 days, and a volume of 6 to 8 ml/kg can be removed in each session (Angelucci et al., 1997).

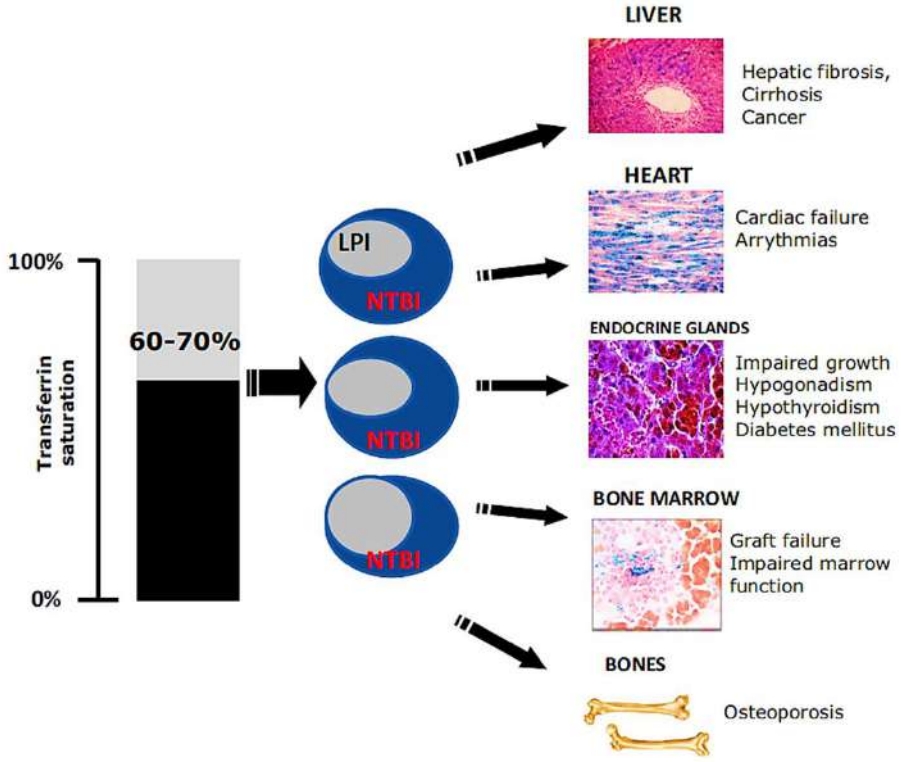


Fig 2.8 Mechanism of iron-related organ/tissue damage in thalassemia patients: When transferrin saturation levels exceed 60% to 70%, there is an elevation in the levels of non-transferrin-bound serum iron (NTBI) and labile plasma iron (LPI). This increase leads to iron-mediated harm to various organs such as the liver, heart, bone marrow, and bones in cases of long-term iron overload resulting from chronic transfusion therapy for thalassemia (Shenoy et al., 2018)**.**

However, there are specific scenarios where phlebotomy may not be feasible. For example, chelation therapy should be initiated in younger children with difficult venous access, inadequate hemoglobin levels, or when phlebotomy is not possible for older children. Chelation therapy may need to be continued for an extended period, possibly years until the ferritin level drops below 100 ng/ml. The choice of optimal pharmacological agents and chelation regimen following transplantation is yet to be defined. In addition to iron chelation, patients require careful attention to immunization, as well as monitoring for endocrine and organ dysfunction resulting from iron overload, Fig 2.8. A multidisciplinary team should thoroughly evaluate and

manage these aspects of alloHCT. Performing HCT at a younger age, ideally between 2 and 5 years, before any significant end-organ damage occurs, prevents long-term complications. This approach allows children to grow up and lead normal lives without the sequelae of the disease after a successful allo-SCT.

2.10 Life after HCT in β-TM

2.10.1 Mixed Chimerism

Mixed chimerism (MC) is a condition observed in a significant proportion (35-45%) of patients who undergo HCT in β-TM where both donor and host cells coexist in the post-transplant period (Andreani and Gregori, 2018; Fouzia et al., 2018). The evolution of MC in β-TM can vary over time. Some patients progress towards complete donor chimerism, while others develop a stable status known as persistent MC, where donor and host cells coexist for an extended period after HCT.

Interestingly, even patients with persistent low-level MC can exhibit normal hemoglobin levels without requiring transfusion support, and they do not experience iron accumulation or clinically significant erythroid hyperplasia (Andreani et al., 1996, 2000). It has been suggested that as little as 10% donor chimerism may be sufficient for a long-term cure (Alfred and Vora, 2011; Andreani et al., 2011). However, MC is a known risk factor for graft rejection, particularly if it occurs early after transplantation. Several studies have explored the trajectory of MC over time and its relationship with graft rejection, Fig 2.9.

The presence of specific biomarkers, such as T regulatory type 1 (Tr1) cells characterized by the co-expression of CD49b and LAG-3 and the ability to secrete IL-

10, has been investigated to predict the evolution of MC in β-TM patients after HCT (Andreani et al., 2014).

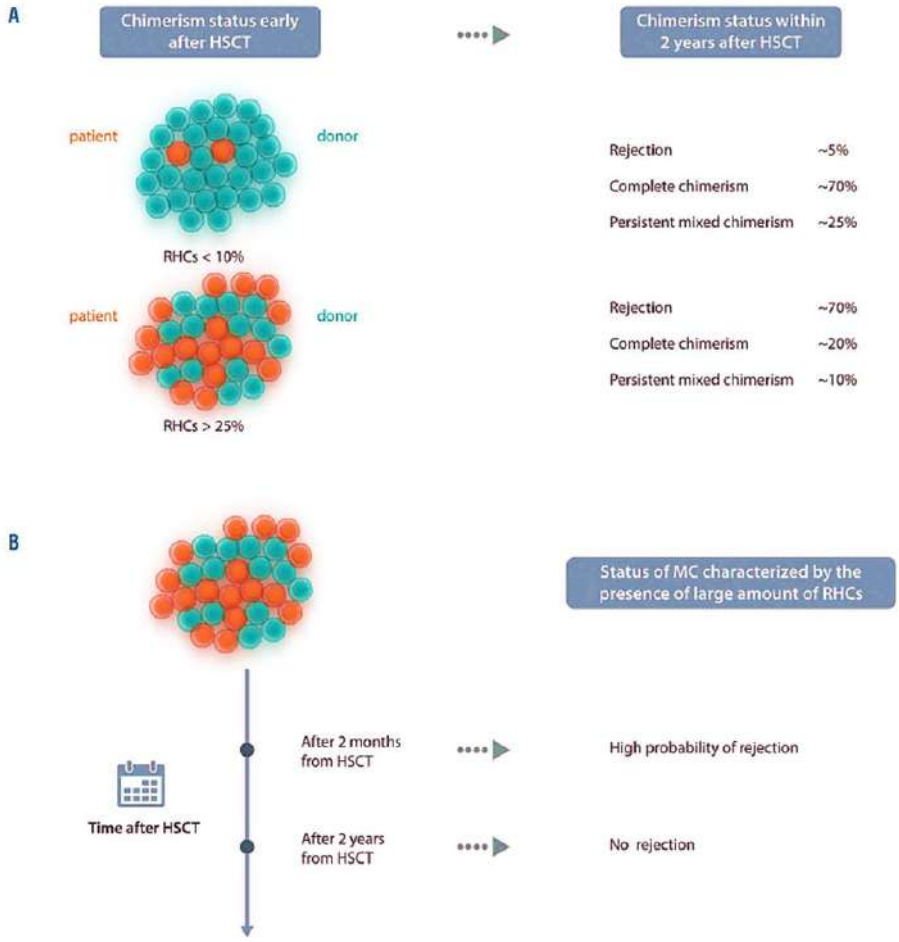


Fig 2.9 Evolution of chimerism after HCT. Early MC is associated with a higher risk of graft rejection, while late chimerism persists with a stable graft. RHCs: residual host cells. MC: mixed chimerism (Srivastava and Shaji, 2017)***.***

2.10.2 Gonadal Function, puberty, and Infertility

β-TM patients undergoing HCT experience dual toxicity on gonadal function, resulting from iron overload and myeloablative chemotherapy. In particular, the risk of ovarian failure is considerably high in girls of pubertal age, with rates ranging from 80% to 100% (E. Vlachopapadopoulou et al., 2005; Li et al., 2004; Sanctis et al., 1991).

However, if HCT is performed before puberty, the incidence of ovarian toxicity is lower. Males generally exhibit higher tolerance, and those who are pubertal during HCT typically maintain normal testosterone levels. Nonetheless, HCT during the prepubertal period can still affect puberty and testosterone levels in approximately 30% to 40% of recipients (E. Vlachopapadopoulou et al., 2005). Overall, the gonadal function is significantly impacted in thalassemia patients undergoing HCT, with distinct implications for females and males depending on pubertal status at the time of transplantation.

2.10.3 Long-term Complications and Quality of Life Post HCT in β-TM

Successful alloHCT allows for transfusion independence and resolution of ineffective erythropoiesis and results in long-term quality of life (QOL) comparable to that of the general population (Caocci et al., 2017; La Nasa et al., 2013). However, considering AlloHCT carries some morbidity and mortality risk, and β-TM can now be managed with conventional medical therapy for prolonged periods, it is crucial to have data on long-term real-life complications to assess the risk-benefit ratio of transplantation (Shenoy et al., 2018).

It is important to note that studies on transplant-related late effects in β-TM patients mainly involve those transplanted with Bu-based conditioning regimens, reflecting the results of transplants performed more than 20 years ago. Today, less toxic preparative regimens have been developed, but longer follow-up is required to evaluate their impact on the occurrence of late complications and fertility.

2.11 Pharmacology of TFT Conditioning

2.11.1 Components of TFT Regimen

The toxicity-reduced conditioning regimen comprises alkylating agents (ThioT and Treo) with a nucleotide analog (Flu). At our center, Flu is administered as 40 mg/m^2/day × 4 days over a 1-h infusion from day −5 to day −2 and Treo 14 g/m^2/day × 3 days at the rate of 5 g/h from day −5 to day −3. ThioT is given at a dose of 8 mg/kg/day and is administered on day −6 before HCT.

2.11.1.1 Thiotepa

2.11.1.1.1 Structure and Mechanism of Action

ThioT (N, N′, N″-triethylenethiophosphoramide) has been employed as an alkylating agent for treating solid tumors and hematological diseases for over 70 years (Shay and Sun, 1955; Zarafonetis et al., 1955). It is an alkylating agent frequently included in myeloablative regimens alongside Treo and Flu or Bu and Flu. It is administered at 8-10 mg/kg, typically given once at 8 mg/kg or for two consecutive days at 5 mg/kg. Due to its high lipophilicity, ThioT can effectively penetrate the blood-brain barrier, making it advantageous for its myeloablative properties and treating diseases involving the central nervous system like Lymphoma (Heideman et al., 1989).

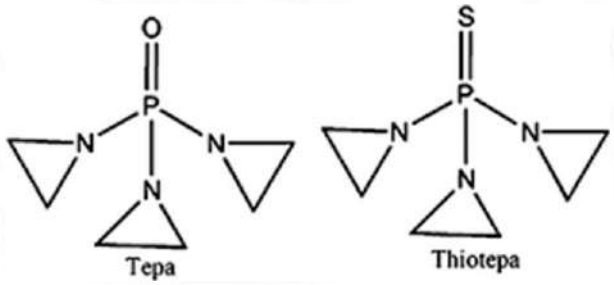


Fig 2.10 Structure of Thiotepa and its active metabolite Tepa. (Torabifard and Fattahi, 2013)

ThioT is rapidly metabolized to its active metabolite, triethylene phosphoramide (Tepa), which exhibits similar alkylating activity to ThioT, Fig 2.10. Two different pathways were observed in both in vivo and in vitro studies, demonstrating the alkylation of Guanine by ThioT and Tepa (Torabifard and Fattahi, 2013). However, the exact mechanism of action remains uncertain. ThioT, a polyfunctional alkylating agent, can create crosslinks with DNA molecules, as depicted in Figure 2.11.

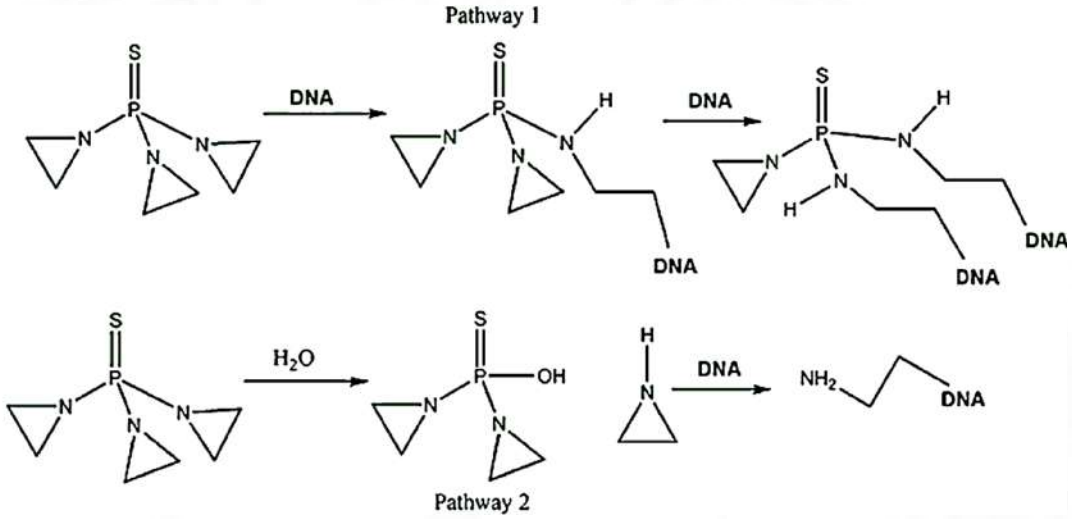


Fig 2.11 Possible mechanisms of interaction of Thiotepa with DNA. (Torabifard and Fattahi, 2013)

Pathway 1 exhibits two distinct reaction mechanisms. In the first mechanism, one of the aziridinyl groups acquires a proton, followed by a ring-opening reaction through nucleophilic attack by the N7 Guanine of DNA. The second mechanism involves a direct nucleophilic ring-opening of the aziridinyl group. In pathway 2, ThioT and Tepa release aziridine through hydrolysis, and the released aziridine subsequently interacts with the N7 Guanine of DNA. This reaction serves as a model for chemical modifications of DNA induced by alkylating agents. By cross-linking of DNA strands,

these compounds inhibit DNA, RNA, and protein synthesis (Hemminki, 1984; Musser et al., 1992).

2.11.1.1.2 ThioT Pharmacokinetics

Absorption

ThioT achieves its highest concentrations near the end of an intravenous infusion (EMA, 2018).

Distribution

Approximately 10% to 20% of ThioT binds to plasma proteins. In the pediatric population, the mean volume of distribution of ThioT was 30 L/m2 (44%) or 1.2 L/kg (47%) after a single intravenous infusion of ThioT at a dose of 5 mg/kg over 3 hours. In adults receiving intravenous ThioT as a bolus or infusion (20 mg to 250 mg/m2) for up to 4 hours, the mean volume of distribution ranged from 1.0 L/kg (30%) to 1.9 L/kg (17%). The estimated mean clearance of ThioT was 0.58 L/hr/kg (60%) or 13.8 L/hr/m2 (52%). The mean terminal elimination half-life was 1.7 hours (64%) for ThioT and 4 hours (29%) for its major active metabolite, N, N', N''-triethylene phosphoramide (Tepa), in the pediatric population. In adults receiving intravenous ThioT as a bolus or infusion (20 mg to 250 mg/m^2) for up to 4 hours, the mean ThioT clearance ranged from 14.6 L/hr/m^2 (23%) to 27.9 L/hr/m^2 (69%). In the adult population, the mean terminal elimination half-life ranged from 1.4 hours (7%) to 3.7 hours (14%) for ThioT and from 4.9 hours to 17.6 hours (20%) for Tepa (EMA, 2018).

Metabolism

ThioT is metabolized in the liver. The hepatic transformation of ThioT involves the enzymes CYP3A4 and CYP2B6, while the conjugation process is facilitated by

glutathione S-transferase (GSTs). Coadministration with inhibitors of CYP3A4 or CYP2B6 enzymes can potentially elevate plasma concentrations of ThioT while possibly decreasing the levels of its active metabolite, Tepa. Conversely, concurrent use of ThioT with inducers of CYP3A4 or CYP2B6 enzymes may lower plasma concentrations of ThioT and elevate the concentrations of Tepa, Fig 2.12 (Ekhart et al., 2009).

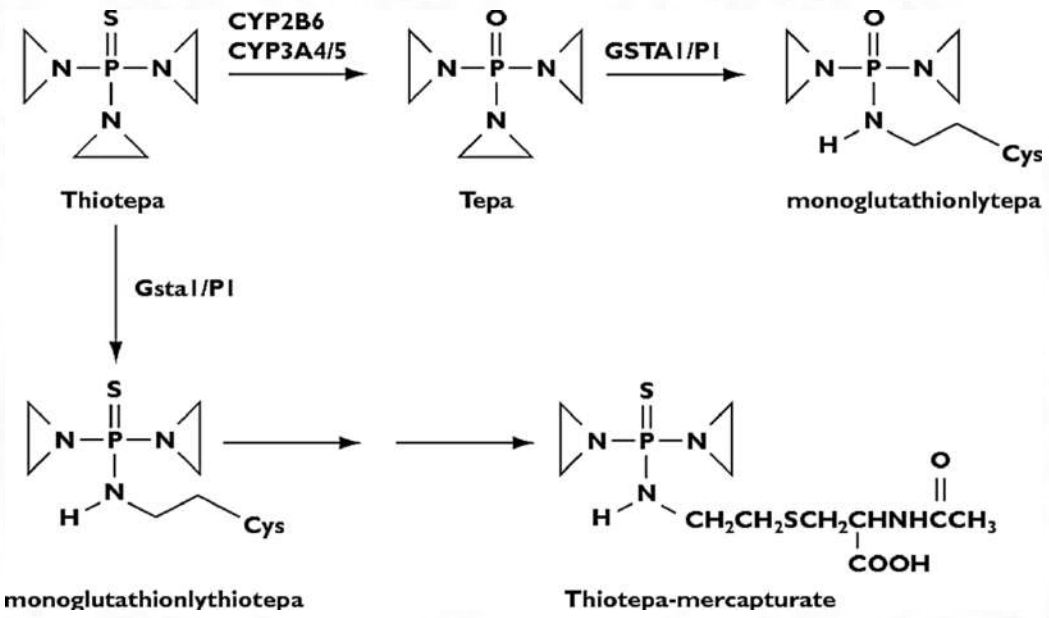


Fig 2.12 Metabolic biotransformation of thiotepa and Tepa (Ekhart et al., 2009)

<u>Excretion</u>

In adult and pediatric patients, less than 2% of the ThioT dose is excreted in the urine, while Tepa accounts for 11% or less of the dose (EMA, 2018).

It is to be noted that although the ThioT PK has been investigated in adults and children, there is a lack of studies explicitly examining its PK and dose-exposure-response in the context of AlloHCT.

2.11.1.2 Fludarabine

2.11.1.2.1 Structure and Mechanism of Action

Flu (9-β-D-arabinofuranosyl-2-fluoroadenine monophosphate), a purine analog, is a monophosphate derivative of adenosine arabinoside, used as an antineoplastic agent. Flu is generally intravenously administered as a prodrug, Flu monophosphate (F-ara-AMP), which is rapidly dephosphorylated to Flu by the action of 5'ectonucleotidase (NT5E/CD73), taken up by cells (Human equilibrate nucleoside transporter, hENT1,2 and Human Concentrative Nucleoside Transporter, hCNT3) and phosphorylated into active metabolite fludarabine triphosphate (F-ara-ATP) by several kinases including

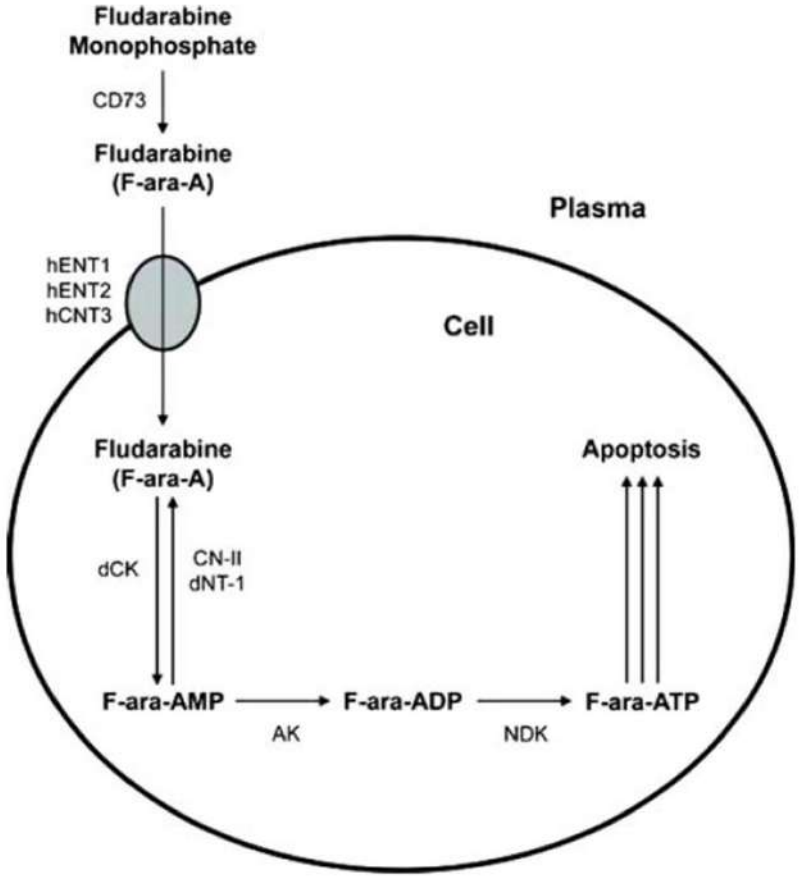


Fig 2.13 Metabolism of Fludarabine to its active metabolite Fludarabine triphosphate. (Woodahl et al., 2008)

deoxycytidine kinase (dCK), adenylate kinase (AK), and nucleoside diphosphate kinase (NDK). F-ara-ATP mediates cytotoxicity primarily by inhibiting DNA/RNA synthesis (Gandhi and Plunkett, 2002; Woodahl et al., 2008). The mechanism of action of Flu is represented in Fig 2.13.

2.11.1.2.2 Flu Pharmacokinetics

Shortly after intravenous infusion, the Flu undergoes rapid conversion to its active metabolite, 2-fluoro-ara-A (F-araA). After administering five daily doses of 25 mg 2-fluoro-ara-AMP/m^2 to patients via a 30-minute infusion, there is a moderate buildup of F-araA concentrations. Throughout a 5-day treatment regimen, plasma trough levels of F-araA increase approximately two-fold. The estimated terminal half-life of F-araA is about 20 hours. In-vitro experiments indicate Flu has a plasma protein binding range of 19% to 29%.

2.11.1.2.3 Flu Pharmacogenetics

All the genes encoding enzymes and transporters involved in Flu metabolism are highly polymorphic. Limited studies attempted to identify genetic variants that could explain variability in Flu PK. One study by Sanghavi et al. attempted to identify genes potentially involved in the bioactivation and transport of Flu but failed to identify any associations with the genetic variants screened (Sanghavi et al., 2016). Previously, we identified a 5′-UTR promoter polymorphism (rs2295890) in the *NT5E* gene encoding ectonucleotidase enzyme affecting Flu PK. We observed that the patients with variant genotypes exhibited significantly lower clearance of Flu than their counterparts with wild-type genotypes (Mohanan et al., 2017). A recent study by Nguyen et al. identified

SNPs rs3925058 in *CMPK1* and rs11853372 in the uptake transporter *SLC28A1* and a 3'-UTR SNP rs2037067 in *TENM3/DCTD* affecting Flu PK (Nguyen et al., 2021). All the above studies employed a candidate gene approach to identify genetic markers for flu metabolism.

2.11.1.2.4 Dose-exposure-response Studies on Flu

Long-Boyle and colleagues first conducted a Flu dose-exposure-response study on a group of 87 patients with various hematological disorders such as AML, NHL, or MDS, demonstrated a correlation between increased exposure to Flu and higher risks of TRM and OS (Long-Boyle et al., 2011). McCune et al. also observed an association between elevated plasma Flu exposure and NRM in 16 patients with malignant diseases (McCune et al., 2012). However, another study conducted by the same research group did not observe any association between Flu exposure and NRM or GVHD (McCune et al., 2015). We previously evaluated Flu PK in 53 patients diagnosed with aplastic anemia (75%) and Fanconi anemia (25%). Most patients received a Flu/Cy regimen (55%), while others were treated with Flu/Cy along with TBI (38%) or ATG (7%). All patients received a 30 mg/m2 Flu dose daily for six days. We did not observe any association between the Flu PK and engraftment, mixed chimerism, rejection, OS, or TRM. However, multivariate analysis showed that a cumulative AUC (cAUC)greater than 29.4 µM*h was associated with a higher risk of aGVHD (Mohanan et al., 2017).

Ivaturi et al. conducted a prospective PK study involving 133 pediatric patients who underwent transplantation for malignant (44%) and non-malignant (56%) conditions. They received Flu in various conditioning regimens and dosages. The study did not

find any association between Flu exposure and TRM. Although patients with a cAUC between 15 and 19 mg*h/L had the highest 1-year OS rate, this was not statistically significant. However, in the malignant subgroup, patients with a cAUC between 15 and 19 mg*h/L had a higher 1-year DFS than those with a cAUC <15 mg*h/L (82.6% vs. 52.8%). The authors propose a minimum exposure threshold of 15 mg*h/L for better HCT outcomes (Ivaturi et al., 2017).

Chung et al. performed pharmacokinetics of Flu in a group of 43 Korean pediatric patients, with a median age of 11.8 years (range 1.3-18.5). Most patients underwent transplantation for a malignant condition (72.1%). They received Flu combination with different agents, the most common regimen being Flu/Bu and etoposide, given at a daily dose of 40 mg/m^2 for six days. The authors performed exploratory analyses to investigate the relationship between Flu cAUC and various outcomes, including toxicities, GVHD, relapse, EFS, and survival. However, they did not observe any significant association between Flu cAUC and HCT outcomes (Chung et al., 2019).

Langenhorst et al. conducted a retrospective cohort analysis of 192 patients, including 119 adults and 73 children, with a median age of 36.2 years (range 0.23-74). All patients received a Flu/Bu conditioning regimen, primarily for malignant diseases (65%). The study revealed a correlation between higher Flu cAUC and increased incidence of NRM and between lower Flu cAUC and more graft failures. No impact on relapse was observed. Based on the findings, the authors determined that a target window of 15-25 mg*h/L for Flu cAUC was optimal to reduce the probability of an event. Patients in the above-optimal group (>25 mg*h/L) had a higher incidence of NRM, while patients in the under-optimal group (<15 mg*h/L) had an increased risk of graft failure and NRM (J. B. Langenhorst et al., 2019). A recent study also revealed

that Flu exposure alone was not strongly associated with NRM or OS. Still, increased exposure to both Flu and Cy was associated with a >16-fold higher NRM (Takahashi et al., 2021).

All the above-discussed studies present varying results. Additionally, including different conditioning regimens and Flu dosage schemes across studies makes the comparison of results challenging. A randomized phase II study is underway to explore the effects of individualized fludarabine conditioning on transplant-related outcomes in adult patients with hematological malignancies (van der Stoep et al., 2022).

These findings suggest no clear-cut evidence of a dose-exposure-response relationship for Flu in HCT, and the role of TDM in Flu is still unclear in a non-malignant setting. Although evidence for TDM for Flu is growing, more studies are needed to identify a single optimal target, especially in pediatric populations with non-malignancy such as β-TM.

2.11.1.3 Treosulfan

2.11.1.3.1 Structure and Mechanism of Action

Treo, a derivative of Bu that is soluble in water and contains two hydroxy groups (Fig 2.14), is an alkylating agent. It is increasingly used as part of the conditioning regimen owing to its impressive myeloablative and immunosuppressive properties.

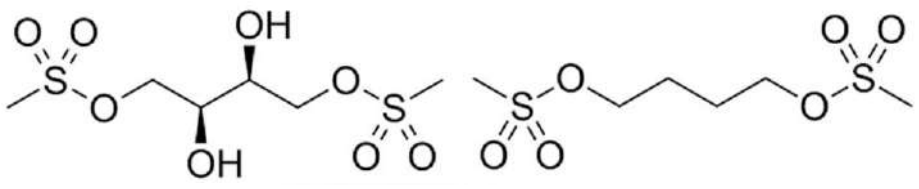


Fig 2.14 Structural Analogy between Treosulfan (left) and Busulfan (right)

Treo, administered as a prodrug, is converted non-enzymatically and at physiological temperature and pH 7.42 to its active metabolite monoepoxide (2S, 3S)-1,2-epoxy butane-3,4-diol-4-methanesulfonate (S, S-EBDM) and (2S,3S)- 1,2:3,4-diepoxybutane (S, S-DEB) that causes cytotoxicity (Feit et al., 1970). To date, there have been attempts to study the pharmacogenetics of Treo.

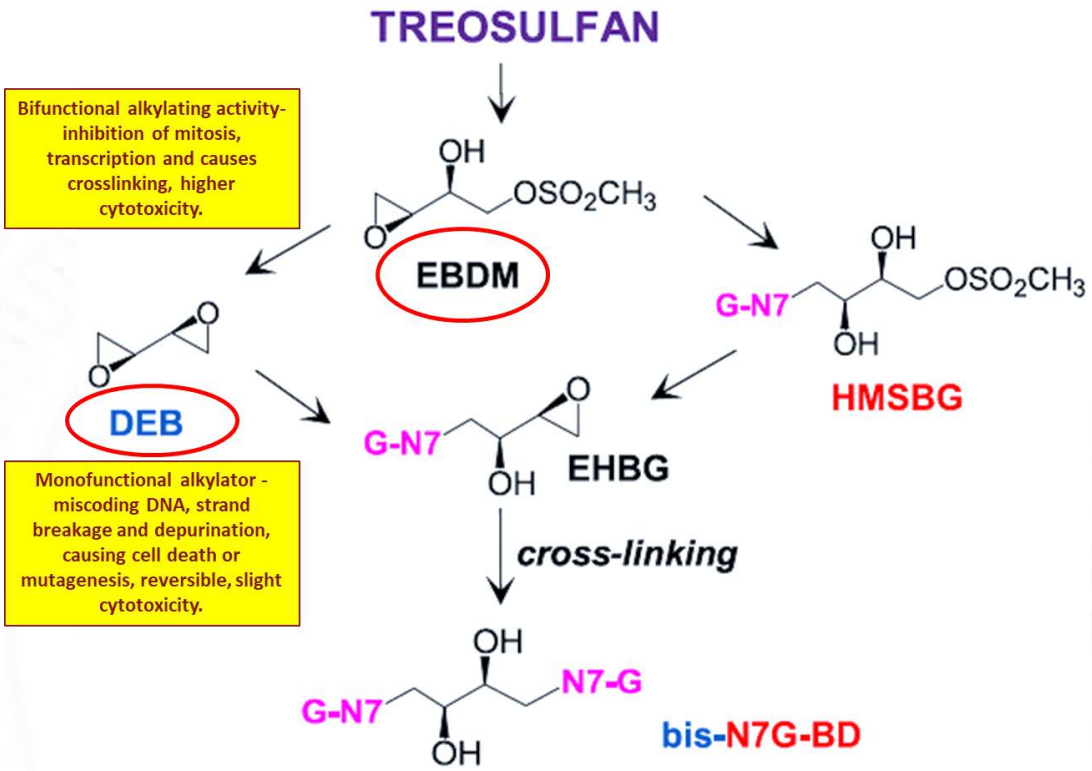


Fig 2.15 Metabolism and mechanism of action of Treosulfan and its active metabolites S, S-EBDM and S, S-DEB. Treosulfan converts nonenzymatically to the monoepoxide intermediate (EBDM), and then to (2S,3S)-1,2:3,4- diepoxybutane (DEB). The latter alkylates DNA forming mainly (2'S,3'S)-N-7-(2',3',4'-trihydroxybut-1'-yl)guanine (THBG) and (2S,3S)-1,4-bis-(guan-7'-yl)-2,3-butane-2,3-diol cross-link (bis-N7G-BD) via the intermediate epoxide adduct (EHBG).(Romański et al., 2019)

2.11.1.3.2 Treo Pharmacokinetics

Peak plasma Treo levels are reached at the end of intravenous administration. Treo is rapidly distributed in the body; however, its capacity to penetrate through the blood-brain barrier is limited. The volume of distribution in adult patients is about 20–47

liters. Treo non-enzymatically metabolizes to S, S-EBDM, and S, S-DEB. The terminal half-life (T1/2) is approximately 2 hours. T1/2 of S, S-EBDM, and S, S-DEB did not differ statistically from its prodrug Treo. About 14–40% of the Treo dose is excreted unchanged with the urine within 24 hours.

In 1998, Hilger RA et al. published a study on Treo PK in 18 adults with advanced or resistant ovarian or small-cell lung cancer. They employed reverse-phase high-performance liquid chromatography (HPLC) with refractometric detection to separate and detect Treo in plasma and urine. The study found that the terminal half-life of treosulfan ranged from 1.8 hours, and the area under the curve (AUC) and maximum plasma concentration (Cmax) values were significantly higher in recipients who received $10g/m^2$ compared to those who received $8g/m^2$. The urinary excretion of the drug's parent compound accounted for nearly 30% of the total dose administered over 48 hours, with approximately 25% excreted within the first 6 hours after administration (Hilger et al., 1998).

Scheulen et al. conducted further research and demonstrated that the AUC increased linearly to $56g/m^2$ in adult patients. Interestingly, the half-life, volume of distribution, and renal elimination were found to be independent of the Treo dose (Scheulen et al., 2000). Similarly, Beelen et al. in 2005 and Glowka et al. in 2008 also confirmed the linear relationship between Treo dose and AUC (Beelen et al., 2005; Główka et al., 2008).

Główka et al. reported the results of pharmacokinetic studies in 7 pediatric patients aged 2 to 15 years. Among them, 5 received a dose of $36g/m^2$, 1 received $30g/m^2$, and 1 received $42g/m^2$. The study demonstrated that AUC and Cmax increased in a dose-dependent manner; however, there was considerable variability (70%) in these

parameters, suggesting that conducting pharmacokinetic evaluations in pediatric patients undergoing Treo-based conditioning may be necessary. It is important to note that the study had a small sample size and did not establish a correlation between the outcomes and the administered doses (Główka et al., 2008).

Ten Brink et al. successfully developed and validated a bioanalytical method to measure treosulfan concentrations in serum and established a PK model to describe the concentration-time profile of treosulfan in children. They conducted a study involving 20 children with various malignant and non-malignant diseases, with a median age of 6.2 years at transplantation. All the children received a dose of $42g/m^2$ of Treo. In contrast to Glowka's findings, they observed limited interpatient variability (14%) and no correlation with treatment outcomes (Ten Brink et al., 2014).

Główka et al. published PK results of treosulfan and its monoepoxide S, S-EBDM in 16 children aged 0.4 to 18 with different malignant and non-malignant hematological disorders. They discovered a linear correlation between the area under the curve (AUC) of S, S-EBDM, and Treo, indicating that the active epoxy-transformer of treosulfan does not accumulate in the body beyond the parent drug. This finding is crucial for clinical application and determining the timing of transplant infusion (Główka et al., 2015).

In 2018 Danielak et al. conducted PK studies in 14 children, investigating both Treo and its mono-epoxy transformer S, S-EBDM. They found that most Treo (approximately 68% of total Treo Clearance) is transformed into S, S-EBDM within the bloodstream. The PK of S, S-EBDM was highly variable. Unlike Glowka's study in 2015, a weak correlation between Treo and S, S-EBDM exposure was reported,

suggesting the need for separate monitoring of this active epoxide in addition to the parent compound (Danielak, Kasprzyk, et al., 2018).

2.11.1.3.3. Treo Dose Exposure Response Relationship Studies

Our center conducted a study on 87 patients with β-TM undergoing HCT to assess the PK of Treo and its relationship with various outcomes. The study included primarily children [median age 9.0 years (range 1.5–25)]. Treo was administered with Flu and ThioT, with a total dose of 42 g/m^2. We evaluated the influence of Treo PK on rejection, toxicities, OS, EFS, and TRM, but no significant association was found between Treo PK and these outcome parameters. However, a trend was observed towards improved OS with high Treo clearance (>7.97 L/h/m2) and low day 1 AUC (<1828 mg*h/L). In a posthoc analysis, we found that low Treo clearance (<7.97 L/h/m^2) was significantly linked with poor OS and EFS (Mohanan et al., 2018).

Van der Stoep et al. examined Treo PK in 77 pediatric patients who underwent transplantation for both malignant (15.6%) and non-malignant (84.4%) conditions. The patients, with a median age of 4.8 years (ranging from 0.2 to 18.3 years), were given Treo either alone (35.5%) or in combination with Flu (67.5%) and ThioT (67.5%). The dose of Treo given varied based on the patient's age, with 12 patients under one year receiving a total dose of 30 g/m^2 and 65 patients over one year receiving 42 g/m^2. The patients were then categorized into three exposure groups based on their levels of exposure to the drug on day 1: low (<1,350 mg*h/L), medium (1,350-1,650 mg*h/L), and high (>1,650 mg*h/L). The authors found that patients in the high-exposure group were at a higher risk for mucosal and skin toxicity than those in the low-exposure group. Additionally, the risk of experiencing two or more toxicities was

more significant in the high-exposure group compared to the low-exposure group. However, no significant association was found between exposure and aGVHD, engraftment, chimerism, or survival (van der Stoep et al., 2017).

Chiesa and colleagues examined the correlation between Treo pharmacokinetics (PK), overall survival (OS), and donor engraftment in a cohort of 87 children [median age 1.6 years (range 0.2–16.7)] who underwent HSCT mainly for Primary immunodeficiency (91%). The patients received Treo in combination with fludarabine with a total dose of 42 g/m^2 for children >12 months, 36 g/m^2 for children aged 3–12 months, and 30 g/m^2 for children ≤3 months. The study showed that a higher cumulative Treo AUC (cAUC) over three days was associated with increased mortality risk in multivariable analysis. In addition, children with cAUC >6,000 mg*h/L had higher treatment-related mortality (TRM) compared to children with cAUC <6,000 mg*h/L (39% vs. 3%). Although a trend was observed for low AUC to be linked with poor donor engraftment (≤20%), this was observed only in univariable analysis. The authors proposed a target cAUC of 4,800 mg*h/L, corresponding to 1,600 mg*h/L daily (Chiesa et al., 2020).

These results suggest a moderate relationship between Tre exposure and toxicity but no consistent relationship with survival. While TDM may be helpful in specific cases and subgroups, the evidence does not currently support routine use in patient care. Further research is needed to clarify these findings, especially in a non-malignant context.

2.12 Endothelial Dysfunction and Role of EASIX in HCT

The endothelium, composed of endothelial cells (ECs), forms a complex biological interface that acts as a semipermeable monolayer, separating all tissues from the bloodstream. This vascular endothelium is a dynamic organ actively regulating vascular tone, cellular adhesion and movement, coagulation, vessel wall permeability, and various inflammatory processes (Deanfield et al., 2007; Luft et al., 2021; Pober and Sessa, 2007). During AlloHCT, the host ECs may contribute to adaptive immune responses (Biedermann, 2008).

During AlloHCT, ECs face a plethora of challenges, including the toxic effects of the conditioning regimen and immunosuppressive drugs, inflammatory molecules released from damaged cells and tissues, endotoxins due to impaired mucosal barriers, the

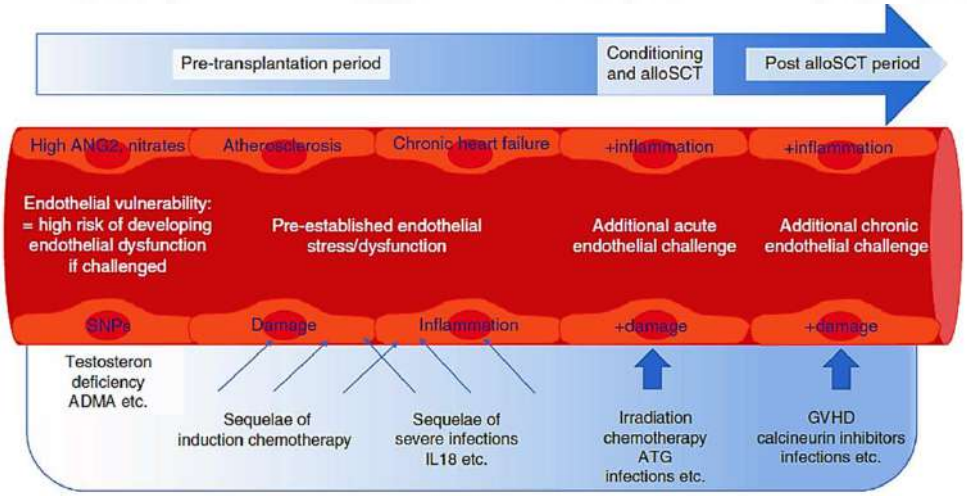


Fig 2.16 Endothelial challenges during allogeneic stem cell transplantation. Endothelial cells experience stressing influences before, during, and after HCT. In the pre-transplantation period, patient-specific endothelial vulnerability and pre-established endothelial damage set the stage for subsequent challenges during conditioning therapy, immune suppression, and post-transplant complications. (Luft et al., 2021)

engraftment of donor leukocytes, and alloreactive immune responses (Biedermann, 2008). The response of patients' ECs to these challenges may be influenced by acquired endothelial distress resulting from comorbidities and pretreatment toxicity, as well as an inherent endothelial vulnerability, which genetic polymorphisms (Rachakonda et al., 2018) can influence, Fig 2.15.

The Endothelial Activation and Stress Index (EASIX) was developed by Luft et al. (Luft et al., 2017) to assess endothelial damage, its association with transplant-associated thrombotic microangiopathy (TMA), and its predictive value for steroid-refractory GVHD. EASIX is based on routine laboratory parameters, including elevated creatinine levels, increased lactate dehydrogenase (LDH) levels, and decreased thrombocyte counts. EASIX measured on the day of transplantation is indicative of SOS (Jiang et al., 2021). Additionally, EASIX measured at the onset of aGVHD predicts NRM (Luft et al., 2020). Moreover, EASIX-measured pre-HCT is associated with endothelial cell dysfunction conditions, such as early fluid retention (Varma et al., 2020) and early hyperbilirubinemia (Dai et al., 2021).

Endothelial damage caused by chronic hemolysis and excessive iron overload in patients with β-TM could lead to severe endothelial damage. Additionally, the hypercoagulability state in β-TM could contribute to endothelial dysfunction suggesting EASIX could be a potent biomarker for patients with β-TM.

However, there are no studies attempted in β-TM. We hypothesized that EASIX could be a predictive biomarker for HCT outcomes in patients with β-TM, as most patients undergoing HCT at our center are at high-risk.

2.13 Metabolomics in HCT

Metabolomics analyses give us an unprecedented view of the biochemical changes caused by disease or therapeutic intervention and aid in identifying biomarker signatures corresponding to specific biochemical changes that occur in our system that increase the sensitivity and specificity required for clinical diagnosis (Johnson et al., 2016). Growing evidence suggests metabolomics plays a role in different aspects of graft vs. host disease (GVHD). Allogeneic transplant recipients' pre-transplant cytokine profiles and metabolic status provided a metabolomic signature for acute GVHD (Reikvam et al., 2016). Recent metabolomics studies also suggest that T-cells show increased glycolytic activity. Effector T (Teff) cells depend on oxidative phosphorylation and fatty acid oxidation during GVHD (Byersdorfer et al., 2013). Serum metabolomic profiling pre-HCT in patients with and without acute GVHD revealed branched-chain amino acid and fatty acid metabolites as predictors of GVHD (Michonneau et al., 2019). Another study showed that chronic GVHD patients showed reduced branched-chain amino acids and increased sulfur-containing metabolites post-HCT. Altered pretransplant serum levels of homocitrulline, adenosine, and altered purine/pyrimidine metabolism were associated with endothelial damage in allo-HCT (Alborghetti et al., 2019). Pharmacometabolomic analysis in plasma samples of HCT patients pre-conditioning predicted busulfan clearance (Lin et al., 2016). Another study highlighted that increased deferoxamine in the urine metabolome of patients was associated with reduced Bu clearance (Kim et al., 2017; Navarro et al., 2016). A recent study also showed that pre-conditioning plasma metabolites in the cysteine and methionine metabolism pathways and the glycine, serine, and threonine metabolism pathways are associated with relapse.

In contrast, metabolites in amino acid metabolism (D-arginine and D-ornithine, arginine, and proline) were associated with acute GVHD (McCune et al., 2021). Another recent study revealed that plasma a-ketoglutaric acid was consistently elevated both before and at the onset of cGVHD in children and adolescents (Subburaj et al., 2022). A more recent study identified dysregulated amino acid metabolism in patients with extensive fluid retention, PreHCT inflammation, and the development of systemic steroid-requiring aGVHD affecting TRM in AML/MDS (Reikvam et al., 2023). These studies highlight that the metabolic changes post-conditioning can alter the plasma metabolome, thus impacting HCT outcomes.

Although there is growing evidence of the role of metabolites as biomarkers for early complications such as GVHD, no studies have attempted to identify markers for predicting SOS. Also, there is an absolute lack of data on plasma metabolomics in patients with β-TM post-TFT conditioning.

3. PATIENTS AND METHODS

3.1 Patients

Patients with TM receiving a Treo-based conditioning (TFT) regimen before HCT between March 2016 and April 2021 were included in the study after obtaining written informed consent from the patient/parents respectively. A retrospective cohort of patients receiving a TFT regimen enrolled in a previous research study was also included to analyze Flu PK. The Institutional review board approved both these studies (IRB No: 9411, dated 29-04-2015, and IRB No: 7437, dated 16-03-2011). The patients were risk-stratified based on the Vellore risk classification as published previously (Mathews et al., 2007). Only patients with an HLA identical related donor or a ≥9 of 10 high-resolution HLA-matched unrelated donors were included in this analysis. Patients with prior transplantation history and non-Indian ethnicity were excluded from the study.

The conditioning regimen consisted of ThioT 8 mg/kg on day -6, Flu 30 mg/m^2/day x 4 days from day-5 to -2, and Treo 14 gm/m^2 x 3 days from day-5 to -3 before HCT. GVHD prophylaxis included administration of cyclosporine alongside a brief course of methotrexate. Cyclosporine was administered at a dose of 2.5 mg/kg intravenously over four hours twice daily starting day 3 and changed to oral administration at 5 mg/kg twice daily when mucositis resolved. Cyclosporine levels were monitored biweekly, and the dose was adjusted to achieve a target level of 200–300 ng/ml. The methotrexate dose was 10 mg/m^2 on day +1 and 7 mg/m^2 on day 3, 6 and 11. Patients received bone marrow or peripheral blood stem cells from matched siblings or matched unrelated donors.

All patients were de-identified by assigning a unique patient number ID (UPN) to protect confidentiality. All patient data were stored as Electronic Medical Records (EMR), which only could be accessed by authorized clinicians and staff within the hospital.

3.2 Blood sample collection and Processing

3.2.1 Blood Sampling for Pharmacokinetic Analysis

Peripheral blood samples were collected in heparin tubes at predetermined time points just before (0hr: Pre), at the end of infusion (EOI) & 2, 4, and 24hrs after Flu/Treo infusion separately based on the Limited sampling model (LSM) reported previously by us (Mohanan et al., 2017, 2018). For Treo level measurements, blood samples were immediately adjusted to a pH of 5.5 by adding 50µL of 1M citric acid/mL of blood to avoid artificial ex vivo conversion of Treo to S, S-EBDM. Blood samples for measuring Flu levels did not require any stabilization steps. The samples were centrifuged at 13000 r.p.m. for 5 mins to obtain plasma and stored at –80^{o}C until further analysis.

3.2.2 Blood Sampling for Pharmacogenomic Analysis

Peripheral blood (9mL) was collected in EDTA tubes before the start of conditioning from patients before HCT, and DNA was extracted using the Gentra Pure gene kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions. The quality and quantity of DNA extracted were estimated by measuring the absorbance of diluted DNA at 260nm and 280nm using a nanodrop spectrophotometer.

Quality of DNA= Absorbance of DNA at 260nm / Absorbance of DNA at 260nm

DNA was considered good quality if the OD260/OD280 ratio ranged from 1.8-2.2.

3.2.3 Blood Sampling for Metabolomic Studies

Peripheral blood samples (9 mL) were collected in EDTA anticoagulant tubes before the start (Before Thiotepa infusion) and at the end of conditioning (24hr post day-1 Treo confusion). Plasma was immediately separated and stored at –80^{o}C until further analysis.

3.3 Pharmacogenomic Analysis

3.3.1 SNP Profiling using DMET Array

The DMET Plus Premier Pack array (Affymetrix, Santa Clara, California, USA) contains 1936 (1931 SNPs and 5 CNVs) covering drug metabolism markers in 225 genes, including 47 phase I enzymes, 80 phase II enzymes, 52 transporters, and 46 other genes. The molecular inversion probe (MIP) technology was employed to perform multiplex genotyping of these genetic variants. Genotypes were determined with DMET console version 1.3 (Affymetrix, Santa Clara, California, USA) using the Dynamic Genotype Boundaries algorithm version 2. Quality control (QC) was performed after excluding copy number variants, X-chromosomal variants, and tri-allelic variants on the array. Variants with unreliable cluster plots, i.e., plots without distinct cluster boundaries, were excluded. Further QC consisted of the exclusion of variants with a call rate below 0.90, a minor allele frequency (MAF) below 0.05, and

variants that deviated from the Hardy-Weinberg equilibrium (HWE $p<0.0001$), and the exclusion of samples with a call rate below 0.90.

3.3.2 Discovery cohort

We screened 51 samples with available Flu/Treo PK for SNPs in an exploratory analysis using a DMET array. The DNA samples were assessed for quality using a spectrophotometer (Qubit, ThermoFisher Scientific, Waltham, MA USA), and genotyping was performed subsequently (MedGenome Pvt Ltd, Bangalore, India). Genotypes were determined for each SNP site and reported as homozygous wild-type, heterozygous, homozygous variant, and 'no call' where no genotype was called. Genotype call rates ranged from 99% to 100%. SNPs with a call rate of less than 99% were excluded from subsequent analyses. The genotype and allele frequencies were calculated and tested for deviations from Hardy-Weinberg equilibrium using the chi-square ($\chi 2$) test ($p>0.05$). The association, if any, between SNPs (at an allele frequency of >.05%) on Flu and Treo PK was then evaluated, and the significant genetic polymorphisms (those associated with Flu/Treo PK) were further validated in the validation cohort.

3.3.3 Validation Studies

Four polymorphisms showed significant association with Treo PK: 3'UTR variants in *GSTA4* (Glutathione S-transferase; rs7496) & *NQO1* (NAD(P)H dehydrogenase 1; rs10517), a missense variant in *GSTZ1* (rs1046428), and an intronic variant in *CES2* (Carboxylesterase 2; rs4783745) in the discovery analysis. In addition, *GSTA1*B* [Glutathione S-transferase haplotype comprising promoter polymorphisms -

rs3957356 and rs3957357] was also screened due to its relevance on Bu PK as reported previously (Abbasi et al., 2011; Ansari et al., 2013, 2013, 2016, 2017; Elhasid et al., 2010; Gaziev et al., 2010; Kim et al., 2011), using a Dynamic array Integrated Fluidic Circuit (IFC) - based customized EP1 system (Standard BioTools Inc. California, USA). The overall study design is summarized in **Figure 3.1**. We screened for these 5 SNPs in pre-HCT samples from patients receiving the TFT regimen.

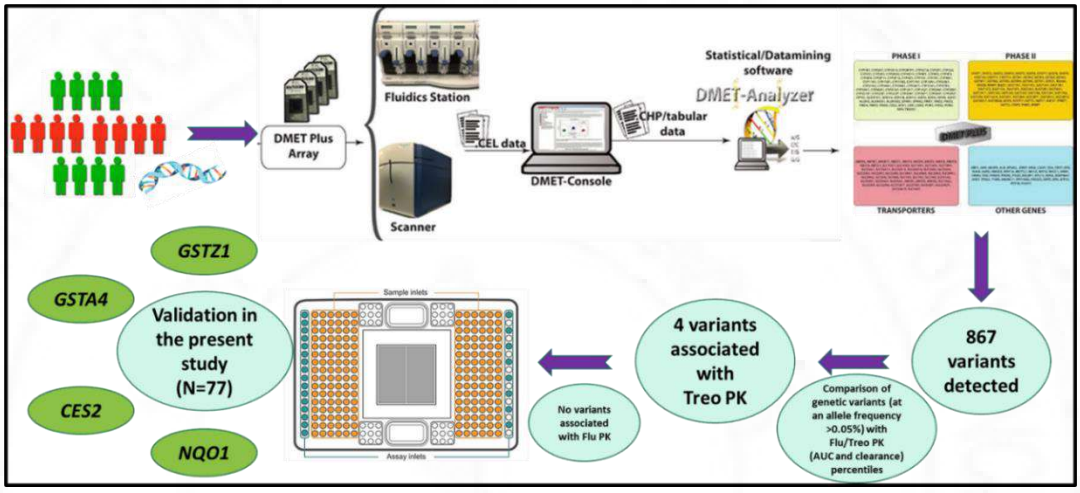


Figure 3.1 Workflow employed in Flu/Treo Pharmacogenetic studies.
The study design can be split into two phases: 1) The Discovery phase, where 51 patients were genotyped using DMET plus array, and 2) the selected variants were then validated in an independent cohort of 77 patients using a Dynamic array Integrated Fluidic Circuit (IFC) - based customized EP1 system.

Additionally, selected polymorphisms in the *NT5E* (Ecto-5'-nucleotidase; rs2295890) and *DCK* (Deoxycytidine kinase; rs11544786) genes (with an allele frequency of >0.1 based on 1000 genome database or with clinical significance) encoding the rate-limiting enzymes in the Flu metabolic pathway were screened using the pre-HCT genomic DNA by PCR followed by Sanger sequencing.

3.3.4 Screening of Selected SNPs in prospective samples

We developed in-house assays for detecting the selected five polymorphisms in prospective samples. *GSTA1*B*, *NQO1,* and *GSTZ1* polymorphisms were screened using PCR followed by Restriction Fragment Length Polymorphism (RFLP) analysis, while *CES2* and *GSTA4* polymorphisms were detected by bidirectional sanger sequencing. **Table 3.1** lists primer sequences, annealing temperatures, and restriction enzyme details for the selected genetic polymorphisms.

Gene	Forward Sequence	Reverse Sequence	Product Size (kb)	Annealing Temperature	Restriction Enzyme	SNP(s) rs.ID
GSTA1	AAGCCAGTTTCTGCTGACTTGCAC	GTTAAACGCTGTCACCGTCT	297	60°C	EarI	Rs3957356, rs3957357
GSTZ1	GGGGAAGAGGTGTAGTGATGG	AAGAGTGTGTCAGGTGTGCAA	288	63°C	MluI	rs1046428
GSTA4	ACTTTTTTGATTTTTGAACCGTGCA	TATTAAGTTGGGTCCTAAGTTGGGT	501	65°C	NA*	rs7496
CES2	CTGCCCTTGACCACATTCTA	CCAGGACTGGTTGGGTAAAA	482	60°C	NA*	rs4783745
NQO1	ACCTGGCCCTTGCAATCTT	GCACCACAAGAGGGCAGT	379	63°C	NcoI	rs10517

Table 3.1 Primer Sequences and Conditions.
The primer sequences, expected PCR product size, annealing temperature, and restriction enzymes used genotyping five selected polymorphisms involved in Treo metabolism are described.

3.3.5 Hardy-Weinberg Equilibrium

The Hardy-Weinberg equilibrium is a fundamental concept that states that the genetic diversity within a population will remain unchanged across generations without disruptive factors. When mating occurs randomly within a large population without disruptive influences, this principle predicts that both genotype and allele frequencies will remain stable and constant as they are in equilibrium.

Various factors, including mutations, natural selection, non-random mating, genetic drift, and gene flow, can disrupt the Hardy-Weinberg equilibrium.

Due to the prevalence of these disruptive forces in nature, the Hardy-Weinberg equilibrium is rarely applicable in reality. Thus, the equilibrium described by Hardy-Weinberg serves as an idealized state, and deviations from this equilibrium can be used to measure genetic variations in natural populations.

Allele Frequencies Equation: $p + q = 1$

Genotype Frequencies Equation: $p^2 + 2pq + q^2 = 1$

Hardy-Weinberg equilibrium can calculate the expected common homozygotes, expected heterozygotes, expected rare homozygotes, and the frequency range of the 2 (p and q) alleles from the observed genotypes.

Court Lab Hardy Weinberg calculator was used to determine whether the SNPs were in HWE. Chi-squared p-values were determined with 1 degree of freedom under HWE, and alleles segregate randomly in the population, allowing expected genotype frequencies to be calculated from allele frequencies (https://www.scribd.com/doc/246388807/Court-Lab-HW-Calculator). Comparing the predicted and observed genotype frequencies provides a test of HWE (e.g., using a chi-square statistic).

3.4 Assay of Flu & Treo-S, S-EBDM levels using LC-MS/MS

Flu, Treo, and S, S-EBDM levels in plasma samples were measured using Shimadzu-Nexera X2 ultra HPLC consisting of binary gradient pumps (LC-30AD), autosampler (SIL-30AC), mobile phase degasser (DGU20ASR), and a column oven (CTO-20AC) combined to an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto,

Japan). The chromatograms were analyzed using LC Solutions software (Shimadzu, Kyoto, Japan).

3.4.1 Treo & S, S-EBDM level measurement

3.4.1.1 Reagents and Chemicals

Treosulfan used for preparing both Treo & S, S-EBDM standards was a kind gift from Medac (Hamburg, Germany). 4′-Aminoacetophenone (Internal Standard), formic acid, Ammonium Formate, and citric acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide was obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile and LC/MS grade methanol were purchased from Fisher Scientific (Thermo Fisher Scientific, Waltham, Mass., USA). HPLC-grade water (Qualigens, India) was used for all experiments. The Treo/S, S-EBDM assay standards were prepared in drug-free blank plasma (obtained from the Christian Medical College hospital blood bank).

3.4.1.2 Sample Processing for Treo-S, S-EBDM Assay

Sample processing was carried out as reported previously with a few modifications (Romański et al., 2014). Briefly, stock solutions (freshly prepared before each run) containing both Treo and S, S-EBDM were prepared by alkalinization with 1N NaOH solution. In a 25 mL volumetric flask, 0.1392 grams of Treo was dissolved in 15 mL water. The resulting solution was titrated using 5 mL of a volumetric solution of 0.1 M NaOH and then topped with water to reach a total volume of 25 mL. The resulting stock solution contained approximately 5 mM Treo, 10 mM S, S-EBDM, and 5 mM

S, S-DEB, with a molar ratio of 1:2:1. The calibration standards (Treo: 23-5720 µM and S, S-EBDM: 17-8723 µM) were prepared by serial dilution in HPLC grade water. Then, 50 µL of the acidified plasma was spiked with 50 µL of each standard solution and 50 µL of IS (4′-Aminoacetophenone), vortexed, precipitated with 150 µL of 100% Acetonitrile, and centrifuged at 13,000 rpm at 40 C for 5 minutes. For patient samples, 50 µL of water was added instead of the standard solution. The resulting clear filtrate (1 µL) was injected via an autosampler for analysis in Liquid Chromatography coupled with a tandem mass spectrometer (LC-MS/MS). All the solutions of Treo and S, S-EBDM were freshly prepared each time before the analysis because of the limited stability of the epoxides.

3.4.1.3 Quantification of Treo and S, S-EBDM using LC-MS/MS

The MS parameters were adjusted to yield maximum multiple reaction monitoring (MRM) signals. The Q1/Q3 for Treo, S, S-EBDM, and IS were set at 296.00>183.00 m/z, 200.00>87.10 m/z, and 136.20>94.20, respectively, in the positive electrospray ionization mode, **Table 3.2**.

Analytes	Retention Time (min)	MRM transition, (m/z)	Dwell Time (msec)	Q1 Pre Bias (V)	Collision energy (CE)	Q3 Pre Bias (V)
Treosulfan	*1.8*	*296.00>183.00*	*100*	*-25.0*	*-12.0*	*-20.0*
S,S-EBDM	*1.3*	*200.00>87.10*	*100*	*-15.0*	*-9.0*	*-18.0*
4′-Aminoacetophenone AAP (IS)	*4.5*	*136.20>94.20*	*50*	*-11.0*	*-15.0*	*-19.0*

Table 3.2 Mass transitions and optimized MS/MS parameters for quantification of Treo and S, S-EBDM with Internal standard.
Optimized MRM transitions used for quantification of Treo and S, S-EBDM by an LC-MS/MS-based assay. MRM, Multiple reaction monitoring; Q, Quadrupole mass filter.

Chromatographic separation of the analytes was done using Zorbax Eclipse Plus C18 (100mm × 2.1 mm, 3.5 µm; Agilent, CA, USA) protected with a C18 guard column from the same source using isocratic elution with a mobile phase 0.01M Ammonium Formate buffer and Acetonitrile (95:5, v/v) at a flow rate of 0.4 mL/min maintained at 40°C. The total run time was 5.5 minutes. The retention time for S, S-EBDM, Treo, and IS were 1.3, 1.8, and 4.5 minutes respectively. The representative chromatogram is shown in **Figure 3.3**. The chromatograms were analyzed using LC Solutions software (Shimadzu, Kyoto, Japan).

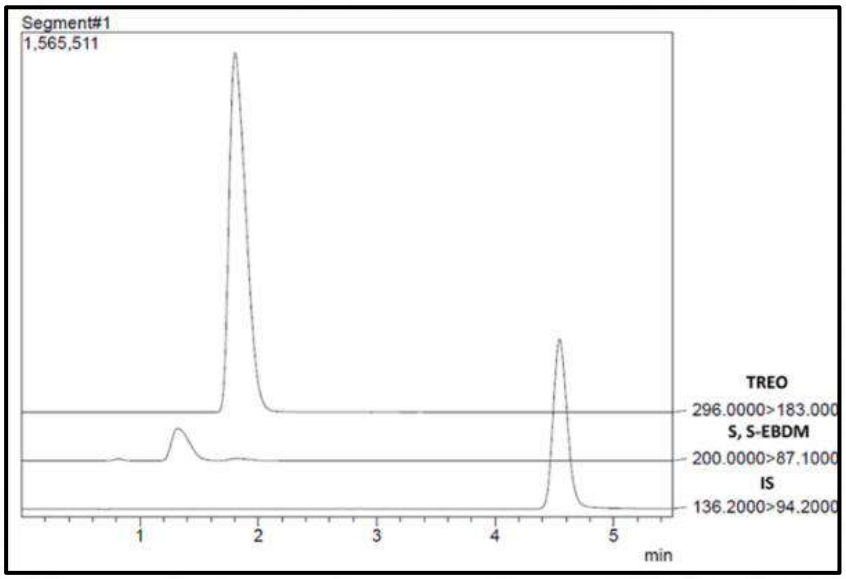


Figure 3.3 Representative Chromatogram showing Treo, metabolite S, S-EBDM, and Internal standard (4'-Aminoacetophenone, AAP).
Representative chromatograms were obtained by analyzing the Mid QC sample of Treo and S, S-EBDM with IS spiked in blank plasma.

3.4.2 Validation of Treo and S, S-EBDM Assay

Treo assay was validated for its specificity, Linearity, precision, accuracy, and recovery before application in patients' samples.

3.4.2.1 Accuracy and Intra-day Precision

Accuracy measures how close the experimental value is to the actual value. It measures the degree of repeatability of an analytical method under regular operation and is expressed as the percent relative standard deviation for a statistically significant number of samples. The inter-day precision was>90%. **Table 3.3** represents the intra-day accuracy and precision of the Treo - S, S-EBDM assay.

Standard concentrations (µM)	Accuracy %		Precision CV%	
	Treo	S, S-EBDM	Treo	S, S-EBDM
Low QC Treo- 12 µM S, S-EBDM- 17 µM	96.65	92.06	2.4	1.3
Low QC Treo- 23 µM S, S-EBDM- 34 µM	104.93	86.62	3.8	2.5
Mid QC Treo- 358 µM S, S-EBDM- 545 µM	103.24	96.34	1.7	1.5
High QC Treo- 2860 µM S, S-EBDM- 4382 µM	100.85	91.25	1.9	1.9
High QC Treo- 5720 µM S, S-EBDM- 8723 µM	99.95	96.50	2.1	2.0

Table 3.3 Representative table showing MS validation parameters – Inter-day Accuracy and Precision.
Inter-day precision and accuracy were calculated based on individual experiments on five days. The inter-day CV% was <5%. CV, coefficient of variation.

3.4.2.2 Linearity

The Linearity of the method is a proportional relationship of response Vs. the analyte concentration over the operating range. The acceptability of linearity data is often judged by examining the correlation coefficient of the Y-intercept of the linear

regression line for the response Vs. the concentration plot. The regression coefficient (R^2) >0.99 generally proves a good data fit for the regression line. The linearity range for evaluation depends on the purpose of an analytical test method. The ICH guidelines recommended analyzing a minimum of five concentration levels and specific minimum selected ranges to test the linearity of the assay. The assay developed was linear for a concentration range of 23-5720 µM and 17-8723 µM for Treo and S, S-EBDM, respectively, with mean $R^2 = 0.99 \pm 0.001$. **Figure 3.4** represents the Linearity and the range of the Treo assay as depicted by the concentration vs. area ratio curve.

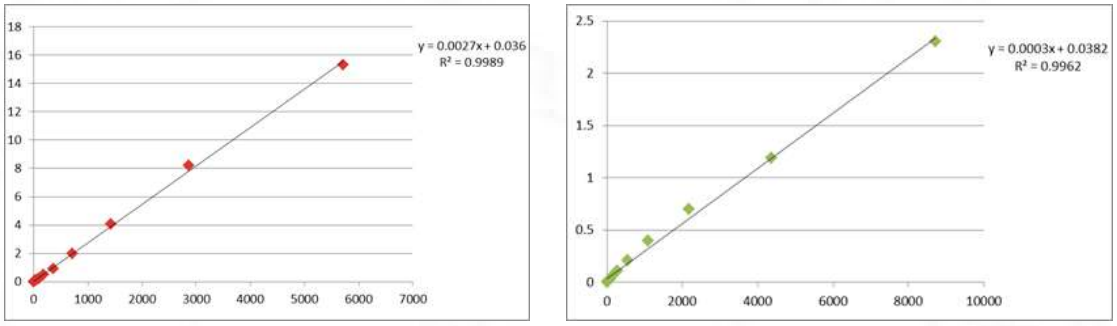


Figure 3.4 Linearity and range of Treo and S, S-EBDM Assay
The assay developed was linear for a concentration range of 23-5720 µM and 17-8723 µM for Treo and S, S-EBDM, respectively, and is depicted by the Concentration (X-axis) Vs.—area ratio (Y-axis) curve.

3.4.2.3 Recovery

Recovery is expressed as the amount/weight of the compound of interest analyzed in the matrix as a percentage of the theoretical amount present in the medium. The mean recovery of Treo and S, S-EBDM from plasma was 95-100 %. There was no matrix interference nor any carryover between the runs.

3.4.2.4 Limit of Detection and Lower Limit of Quantification

LOD (Limit of Detection) is the lowest amount of analyte that can be detected above baseline noise, typically three times the noise level, and the lower limit of quantification (LLOQ) is the lowest concentration of analyte in a sample that can be quantified reliably, with acceptable accuracy and precision. The LLOQ for both Treo and S, S-EBDM were 1.4 µM and 2.1 µM, respectively, and the LOD was 0.7 µM and 1.05 µM. Patient samples were then analyzed using this validated method.

3.4.3 Flu level measurement by LC-MS/MS

Flu levels in plasma samples were measured by LC-MS/MS, as reported previously (Mohanan et al., 2017). Briefly, a series of F-araA standards (25 µL of 1‑1000 µM) and internal standard 5-FC (25 µL of 250 ng/mL) were added to pre-labeled tubes containing 250 µL of drug-free blank plasma and vortexed for 30 seconds. Ice-cold Acetonitrile was added and centrifuged at 13000 r.p.m. for 25 min at 4°C. The supernatants were dried under nitrogen gas at 40 °C. The residue was dissolved in 100 µL of mobile phase (10 mM Ammonium acetate pH 5.0 and 100% Acetonitrile 9:1 v/v), and 1 µL was injected into the analytical column (Syncronis C8, 2.1 × 50 mm, 5 µM, Thermo Scientific, Inc.). Patients' samples were prepared similarly except for adding 25 µL of deionized water instead of standard Flu. The drug-free plasma spiked with three different concentrations (lower, middle, and higher concentration) of pure Flu were prepared and stored as controls/calibrators at −80 °C, used as quality controls during every run. The samples were analyzed using LC-MS/MS. The Q1/Q3 for F-araA was set at 286.2/154.1 and 262.1/130.05 for the internal standard, 5-FC in the positive ESI mode, respectively. The representative chromatogram is shown in **Figure**

3.5. The chromatograms were analyzed using LC Solutions software (Shimadzu, Kyoto, Japan).

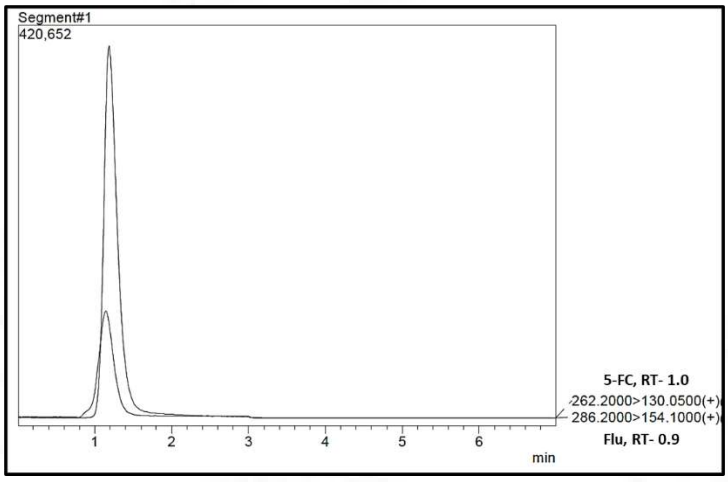


Figure 3.6 Representative Chromatogram showing Flu and 5-FC (IS) obtained by analyzing the Mid QC sample of Flu with 5-FC (IS) spiked in blank plasma.

The overall PK-PD study design is summarized in **Figure 3.6**.

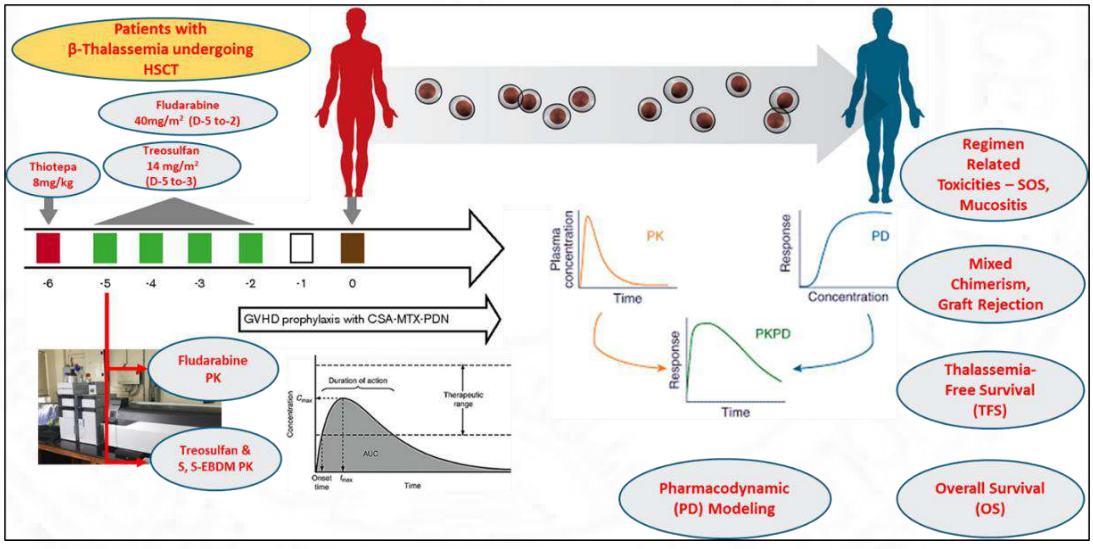


Figure 3.6 Study design of Flu &Treo-S, S-EBDM PK-PD studies
Flu and Treo-S, S-EBDM PK were evaluated in Patients with TM receiving a TFT regimen. Flu/Treo & S, S-EBDM levels were analyzed using LC-MS/MS. PopPK was estimated and subjected to PD modeling.

3.5 Population Pharmacokinetics (PopPK)

PopPK models were used to calculate PK estimates- AUC and Clearance for Flu, Treo, and its metabolite S, S-EBDM.

3.5.1 Flu Pharmacokinetics

Nonlinear mixed effects modeling analysis was performed with Monolix (version 5.1.0) using the Stochastic Approximation Expectation-Maximization (SAEM) method. A two-compartment model best describes Flu PK data. The PK parameters estimated included clearance and volume (CL (L/hr/m^2) and V (L/m^2)) along with the inter-compartmental clearance and peripheral compartment volume (Q (L/hr/m^2) and V_2 (L/m^2)). Also, the individual post hoc parameter values were used to estimate the area under the concentration curve (AUC). A log-normal distribution was assumed for the inter-individual variability of the parameters. A combined additive and proportional residual error model was used with the assumed normal distribution of the residuals. The covariates tested in the POPPK model are demographic and biochemical parameters such as age, weight, sex, BSA, hemoglobin count, WBC count, platelet count, serum bilirubin/creatinine/ferritin, ALT/AST levels.

The relationships between the PK parameters and categorical covariates were described using the following model: $\theta=\theta_{Base}*\exp(\beta*covariate)$ and the relationships between the PK parameters and continuous covariates were described using the following model: $\theta=\theta_{Base}*(covariate/median\ covariate)^{\beta}$. A covariate was considered significant in the Univariate analysis if the addition of the covariate to the model reduced the objective function value (OFV) by at least 3.84 units ($p < 0.05$, based on

the χ^2test for the difference in the -2 log-likelihood between two hierarchical models that differ by 1 degree of freedom).

3.5.2 Treo and S, S-EBDM PK

Pharmacokinetic parameters for both parent and metabolite compounds were estimated using nonlinear mixed effects modeling via nlmixr2 in R(4.3.0) using the Stochastic Approximation Expectation-Maximization (SAEM) method. We tested one-compartment and two-compartment models to explain both the compound's metabolism and appropriate residual errors: additive and proportional were included. Allometric weight scaling for clearance and volume was tested. Covariates such as age and serum creatinine were tested on Treo clearance. The typical body weight of the population was set to 70kg, and the exponential term was set as 0.75 for Treo clearance and 1 for the volume of distribution. The addition of covariates was considered if the -2 log-likelihood ratio significantly improved the fit at the least by $P<0.01$ of the model. Further, the model was evaluated by a series of the goodness of fit predicted vs. observed plots, visual predictive check plots, residual vs. time, and prediction plots.

3.6 Pre-HCT EASIX Scoring

The Endothelial Activation and Stress Index (EASIX) is a simple biomarker calculated using lactate dehydrogenase (LDH), creatinine, and platelet counts. The EASIX score was calculated as serum LDH (U/L) x serum creatinine (mg/dL)/platelet count ($x10^9$/L) using laboratory data recorded during the pre-HCT period. The biochemical test results in the pre-transplantation period (within two weeks pre-HCT) for scoring

EASIX were accessed through EMR records and shared by authorized clinical colleagues.

3.7 Clinical Definitions and Study Endpoints

HCT outcome endpoints such as RRTs, engraftment, graft rejection, GVHD, Post-HCT chimerism, and survival status were collected through a retrospective chart review at the end of the follow-up of 1 year for the analysis. An absolute neutrophil count of $>500x10^6$/L on three consecutive days was noted as neutrophil engraftment. The RRTs, including mucositis, were graded according to NCI-CTCAE criteria (Common Terminology Criteria for Adverse Events (CTCAE) | Protocol Development | CTEP, n.d.). Hepatic Sinusoidal Obstruction Syndrome (SOS) was graded according to Baltimore criteria (Carreras et al., 2019). GVHD was graded using Glucksberg criteria (Carreras et al., 2019). Any deaths occurring within the first 100 days post-HCT were regarded as Transplant Related Mortality (TRM). Early (TRM D+30) and late TRM (TRM+100) are deaths occurring within 30- and 100-days post-HCT, respectively, primarily due to RRTs and infections. The one-year Thalassemia-free survival (TFS) was defined from the time of transplant to an event such as primary graft rejection/failure or death up to one-year post-HCT. The one-year overall survival (OS) was defined as the percentage of patients alive at the last follow-up at one-year post-HCT.

The primary objective of the study was to evaluate the dose-exposure-response relationship of Flu, Treo, and its epoxy metabolite- S, S-EBDM on HCT outcomes. We also attempted to derive a therapeutic range of Flu, Treo, and S, S-EBDM in this uniform cohort of patients with high-risk TM. The primary endpoints included 1-year

Thalassemia-free Survival (TFS) and Overall Survival (OS). Secondary endpoints included Neutrophil engraftment, Chimerism status, RRTs, Graft rejection, GVHD, Early (TRM D+30), and late TRM (TRM+100) Transplant related mortality.

3.7.1 Whole blood Chimerism Analysis

Short tandem repeats (STRs) exhibit significant size polymorphism, resulting in varying-length PCR products. The number of repeats at these polymorphic sites tends to differ among individuals and even between the two alleles of the same individual. Consequently, when targeting multiple STR/VNTR sites, some are expected to provide valuable information. An STR/VNTR marker is considered informative if any of the patient's alleles differ from the donor's. The STRs are amplified using fluorescent-labeled primers, and the PCR products undergo capillary electrophoresis using the ABI 3130/3500 genetic analyzer. Once we identify the informative STR/VNTR markers unique to the patient/donor pairs, we employ only the informative marker (s) for post-BMT chimerism analysis. Our primary STR marker panel comprises VWF, Tho1, F13A1, FGA, FES, and ACTBP2 loci. Generally, one or more of these markers will be informative, except for monozygotic twins. These markers were selected based on their heterozygosity data within our population. When none of these markers provide informative results in a patient/donor pair, a secondary set of markers (TPOX, GT-GpIIb/IIIa, or AMG) will be tested.

3.7.1.1 Procedure

During the initial analysis of the first post-BMT sample, multiplex PCR incorporating all the markers is necessary, along with the pre-BMT and donor samples. Subsequently, only the relevant markers are included for PCR in multiplex or uniplex format. Uniplex

reactions using direct blood PCR can also be employed for this purpose. The PCR products are checked for amplification by Agarose gel electrophoresis (100v for 20-30 minutes). The amplified products are then subjected to fragment analysis (Sellathamby et al., 2006).

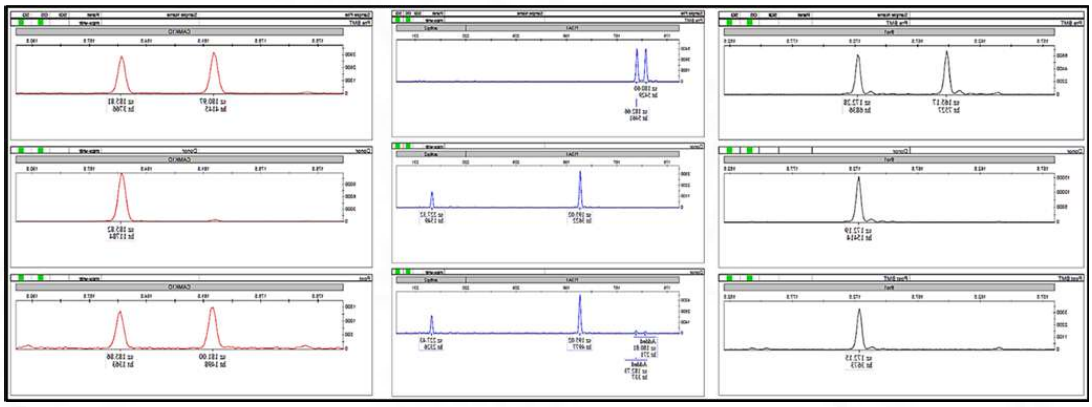


Figure 3.6 Whole blood chimerism analysis examples for (A) Complete chimerism, (B) Mixed chimerism, and (C) Rejection.
Schematic representation of complete chimerism, mixed chimerism, and rejection in different donor-recipient pairs following AlloHCT

Capillary electrophoresis is employed for fragment analysis to examine the fluorescent-labeled fragments following PCR. This process serves a dual purpose: firstly, to determine informative markers, and secondly, to quantitatively calculate the results, specifically the percentage of recipient and donor cells, utilizing these informative markers. A marker is deemed informative if at least one unique, distinctive peak (allele) is present in the patient's sample that is not seen in the donor sample. Capillary electrophoresis was performed in genetic analyzer ABI 3130 / 3500. The percentage of recipient and donor cells is calculated based on informative markers per standard guidelines. Examples of chimerism results are shown in **Figure 3.6**.

Day+28 chimerism analysis showing more than 95% of donor genetic marker patterns were considered as achieving complete chimerism (CC). Mixed Chimerism (MC) was

defined as the presence of >5% residual host chimerism at any time post-HCT, rejection as >90% residual host chimerism in peripheral blood, as described previously (Fouzia et al., 2018).

3.8 Pharmacodynamic (PD) Modelling for Treo & S, S-EBDM

A Cox proportional hazard analysis was performed to determine the factors affecting the outcomes of Post-HCT, especially graft rejection and TFS. The covariates included were Treo AUC, S, S- EBDM AUC, Treo/S, S-EBDM AUC ratio, genetic polymorphisms, in addition to the standard demographic variables such as age, sex, Lucarelli classification, CD34 dose, Donor source, and HLA match on time to rejection and time to death at the end of 1 year. Significant variables ($P<0.05$) from this univariate analysis were taken forward for multivariate analysis. To identify the therapeutic range/target for Treo AUC, a quadratic model of Treo AUC vs. probability of success (defined as being alive at the last follow-up (1-year post-HCT), with <5% probability of graft rejection) was fitted. All the analyses were done using R (version 4.3.0, R Foundation for Statistical Computing, Vienna, Austria).

3.9 Global Plasma Metabolomic Profiling

To identify predictive biomarkers for SOS, we performed Global/Untargeted metabolomics in collaboration with Dr. T.S. Keshava Prasad, Centre for Systems Biology and Molecular Medicine, Yenepoya Research Centre, Yenepoya University, Mangalore, India.

3.9.1 Plasma Metabolite Extraction

Briefly, 50 µL of plasma was mixed with 950 µL of extraction solvent (Triple solvent method (Acetonitrile: Methanol: Water - 2:2:1) for 5 minutes at room temperature, followed by vortexing for 15 minutes and then sonication in an ultrasonic water bath for 15 minutes and kept at -20^{o}C overnight. Following overnight incubation, the samples were centrifuged at 10,000 rpm for 15 minutes at 4°C, and the supernatant was carefully transferred to a new tube while discarding the sediment. Subsequently, the metabolite extract was dried using a SpeedVac concentrator (Thermo Fisher Scientific, USA). The sample was then resuspended in 0.1% formic acid and diluted 1:3 for data acquisition. Epicatechin was spiked in the diluted sample as an internal standard at 100 ng/ml concentration. The diluted sample was loaded for LC-MS/MS analysis.

3.9.2 LC-MS/MS-based Global Metabolomics

Global Plasma Metabolomics was carried out using liquid chromatography (LC) followed by MS/MS analysis using QTRAP 6500 mass spectrometer (ABSciex) coupled with Agilent 1290 infinity II LC system with a C18 RRHD Zorbax column (20 x 150 mm, 1.8 µM particle size). Analyst software version 1.6.3 was used for data acquisition, and the Analyst Device Driver was used for setting the parameters for the analysis. The separation of the metabolites was carried out using a 25-minute LC method. Solvent A was 0.1 % formic acid in MilliQ water, and solvent B was 0.1 % formic acid in 90 % acetonitrile; the flow rate was set to 0.25 mL/ min. The LC method was set to 25 minutes with the following gradient: 2% B for 1-10 mins, 30 % B at 10-14 mins, 60 % B at 14-18 mins, 95 % B for 18-21 mins, and 2 % B for 21-25 mins.

3.9.3 MS Data Acquisition

The mass spectrometry data acquisition was carried out with the IDA method (Information dependent acquisition) in low mass mode. The IDA method was built using the EMS (enhanced mass spectra) to EPI (enhanced product ion) modes. The top five spectra from the EMS mode were used for analysis in the EPI (MS/MS) mode, using high-energy CID (collision-induced dissociation). The metabolite data were acquired in positive polarity at 4500 V, with a probe temperature of 450 °C. The compound parameters were set at a declustering potential (DP) of 100 V and collision energy (CE) of 10 V. Data were collected from three independent biological replicates and three technical replicates for each biological replicate. Blank runs were performed after every triplicate sample run to maintain the system stability and prevent carryover.

3.9.4 Processing of untargeted metabolomics data

The raw mass spectrometry files in .wiff format were converted into the .mzML format using MSConvert (Chambers et al., 2012). The converted files were then employed for data analysis utilizing the MetaboAnalyst version 5.0 tool (Using MetaboAnalyst 5.0 for LC–HRMS spectra processing, multi-omics integration, and covariate adjustment of global metabolomics data).

3.9.5 Database searches for Metabolite assignment

Metabolite identification was carried out using a compound identification tool MS2Compound. The background database utilized for this analysis was HMDB (Human Metabolite Database) (Wishart et al., 2018).

3.9.6 Data Analysis

MetaboAnalyst 5.0 was employed for data analysis. LC-MS spectra processing module was used for data analysis. LC-MS platform was selected as UPLC-Ion_Trap. **Table 3.4** contains the parameters for mass spectrometry raw file processing.

Parameter	Parameter setting
Peak Picking	Method: centWave
	min_peakwidth: 5.0
	max_peakwidth: 30.0
	ppm: 5.0
	mzdiff: 0.05
	snthresh: 10.0
	noise: 1000
	prefilter: 3.0
	value_of_prefilter: 100.0
Peak Alignment	Method: obiwarp
	Bandwidth: 10.0
	minFraction: 0.8
	minSamples: 1
	maxFeatures:100
	integrate: 1
	extra: 1
	span: 0.25
	profStep:1
Peak Annotation	Polarity: negative/positive
	Perc_fwhm: 0.6
	Mz_abs_iso: 0.005
	Max_charge: 2
	Max_iso: 2
	Corr_eic_th: 0.85
	Mz_abs_add: 0.001

Table 3.4 MS Parameters for Metabolite peak identification, alignment, and Annotation
MS raw file processing parameters for peak identification, alignment, and Annotation of metabolites using Global metabolomics are tabulated.

3.9.7 Statistical Analysis for global metabolomic profiling

Metabolites whose intensities had a relative standard deviation of more than 25% were filtered out. Data normalization was done using quantile normalization. Post-normalization data were scaled using the option of Autoscaling. The significantly

different metabolites (p-value ≤ 0.05) with a fold change of >1.3 and <0.76 were considered as upregulated and downregulated, respectively.

3.10 Statistical analyses

The patient's pre-HCT characteristics and PK parameters were summarized by standard descriptive statistics. Individual Flu, Treo, S, S-EBDM exposure, and parent-to-metabolite exposure ratio (Treo AUC/S, S-EBDM AUC) were continuous variables for the outcome analysis. Fisher's exact test and Pearson's chi-square test were used for testing individual variables influencing HCT outcome. Correlation between Flu, Treo, and S, S-EBDM exposure with continuous variables was estimated using Pearson's coefficient correlation. Associations between Flu, Treo, & S, S-EBDM exposure, genotype groups, and clinical outcomes were done using Mann–Whitney U-test or ANOVA. We also did a quartile analysis on Flu/Treo/S, S-EBDM AUC, and CL on HCT outcomes. The relative risk of variables on the HCT outcomes was performed by logistic regression. The receiver operating characteristic (ROC) curve was used to assess the utility of the EASIX score in predicting TRM+100. Log-rank Cox regression was used for the survival analysis, and the Kaplan–Meier curves were generated for 1-year OS and TFS. All statistical analyses were performed by R Statistical software (version 4.3.0; R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism software (version 8.4.3; GraphPad Software Inc, San Diego, CA, USA).

4. RESULTS

4.1 Evaluation of dose-exposure response relationship to Treo and S, S-EBDM in patients with β-TM undergoing HCT

This Chapter describes the dose-exposure response relationship to Treo and its active metabolite S, S-EBDM in patients with β-TM undergoing HCT. We also attempted to derive a therapeutic range of Treo and S, S-EBDM to explore the feasibility of therapeutic dose management (TDM) of Treo.

4.1.1 Patient Demographics

Of 116 patients who underwent HCT with a TFT regimen in our center between March 2017 and April 2021, seventy-seven patients gave consent, were included in the study, and followed up for at least 1-year post-HCT. The median age of the patients was 8 years (2-21 years), and 42 (55%) were males. All patients received PBSCT grafts. Nine patients (12%) received HLA-matched unrelated donor sources. Most patients were Class III (Class III High Risk: 25%; Class III Low Risk: 53%), and 19% belonged to the Class II risk category. Sixty-eight of 77 (88%) patients had a matched sibling donor (MSD), while nine had HLA-matched unrelated donors (MUD, 12%). Detailed demographics of these patients are summarized in **Table 4.1.**

Table 4.1.1 Patient Demographics

Patient Characteristics	N / Median (range)	%
Age (years)	8 (2-21)	Na
Sex (Male/Female)	42/35	55/45
Body Weight (Kg)	21 (11-67)	Na
Lucarelli Classification Class I/ II/ III	02/15/60	03/19/78
Vellore Risk Classification Class III High Risk/Low Risk	19/41	25/53

HLA Disparity Full Match Not full match	 75 02	 97 03
Donor Source Matched sibling donor (MSD) Matched unrelated donor (MUD)	 68 09	 88 12
Cell dose ($CD34^+$ x 10^6/kg)	10 (03-19)	Na
Serum Ferritin (ng/mL)	2654 (100-9812)	Na
Serum Creatinine (mg/dL)	0.26 (0.13-0.74)	Na
Treo formulation Generic Innovator	 45 32	 58 42

Categorical variables are presented as n (%). Continuous variables are expressed as median (median, range).

4.1.2 Treo & S, S-EBDM levels

A total of 385 plasma samples from 77 patients were analyzed to develop the PopPK model for Treo and its metabolite. The median Cmax (peak plasma concentration at the end of conditioning) of plasma Treo and S, S-EBDM levels were 560 (188-1665) and 33 (8-163) mg/L, respectively. The 24h samples had no detected Treo or S, S-EBDM levels in all patients. The time-concentration profiles for Treo and S, S-EBDM are presented in **Figure 4.1.1**. We observed 41% and 63% inter-individual variability in Cmax of Treo and S, S-EBDM, respectively.

4.1.3 Assessment of systemic Treo & S, S-EBDM exposure using PopPK model

PopPK analysis was done using nlmxir2 in R (4.3.0). We built a base model with one compartment with an allometric weight scale on clearance and volume, which best described the PK of Treo. Postmenstrual calculated age and serum creatinine were tested as covariates on the model, which did not significantly improve the model.

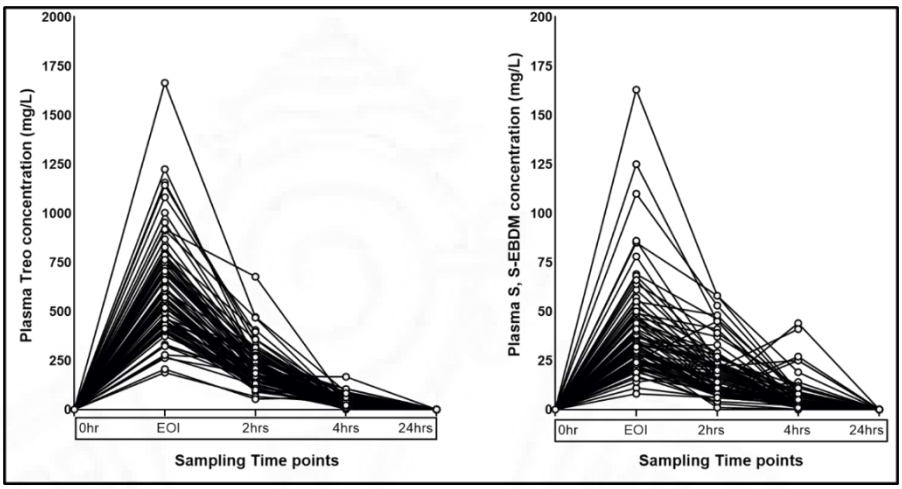


Fig 4.1.1 Plots showing Treo (left) and S, S-EBDM (right) concentrations vs. time points in the study cohort.
Spaghetti plots representing Treo and S, S-EBDM concentrations vs. time points acquired for patients included in the study. Plasma Treo and S, S-EBDM levels (mg/L) were plotted against 5 study time points (0hr- Pre Treo infusion, EOI- End of Treo infusion and 2hrs, 4hrs, and 24hrs post Treo infusion).

Finally, one compartment model with allometric scaling on clearance and volume of distribution and combined additive-proportional residual error for both parent and the metabolite was developed and validated using the goodness of fit (GOF) predicted vs. observed plots, visual predictive check plots, residual vs. time, and prediction plots. The GOF plots show that one compartment model best described the observed plasma concentrations. The plot of observed vs. population predicted concentration shows uniformly distributed points on either side of the lines of identity. The conditional weighted residuals (CWRES) were within the acceptable range and were uniformly spread when CWRES were plotted against population predictions and also against time, **Figure 4.1.2**

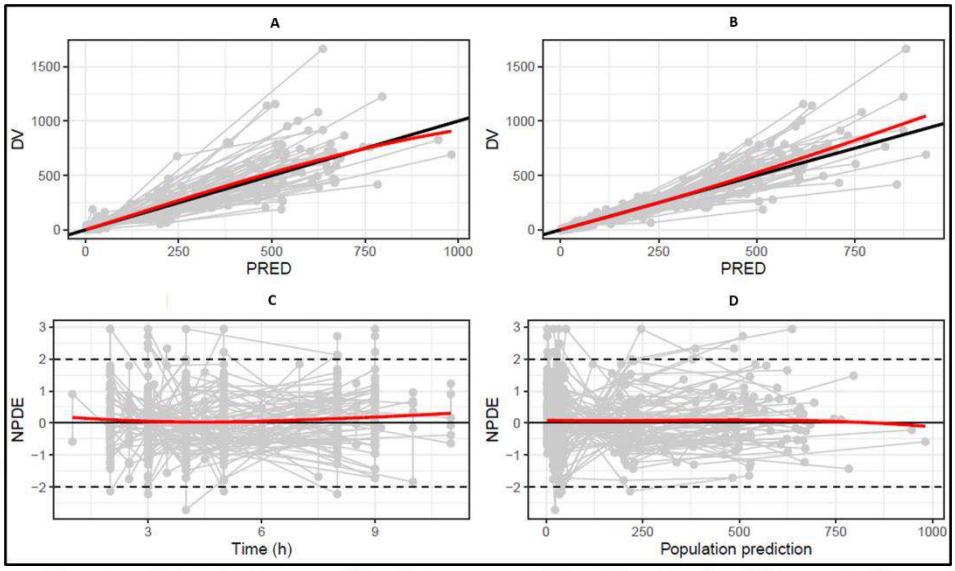
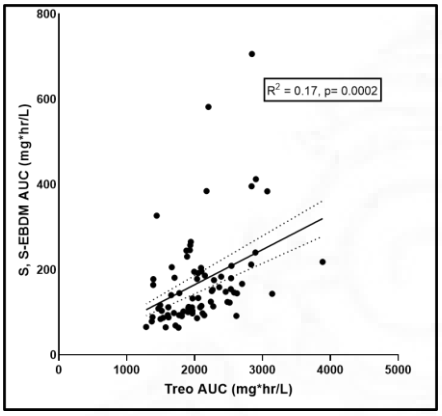


Fig 4.1.2 Basic goodness-of-fit plots of the final model. (a) observations versus population predictions; (b) observations versus individual predictions; (c) conditional weighted residuals versus time; (d) conditional weighted residuals (CWRES) versus population predictions. The solid black line represents the line of identity. Points are individual data. Red dashed lines represent regression. Black dashed lines represent CWRES.

PopPK analysis revealed a two-fold variability in Treo exposure [1993 (1286-3886 mg*h/L)] and ten-fold variability in S, S-EBDM exposure [143 (64-706 mg*h/L)]. We also observed a moderate positive correlation between Treo and S, S -EBDM exposure (R^2 = 0.17, p= 0.0002). However, we did not observe a correlation between Age and Treo Clearance, **Figure 4.1.3**. No other demographic or biochemical covariate explained variability in Treo or S, S-EBDM PK.



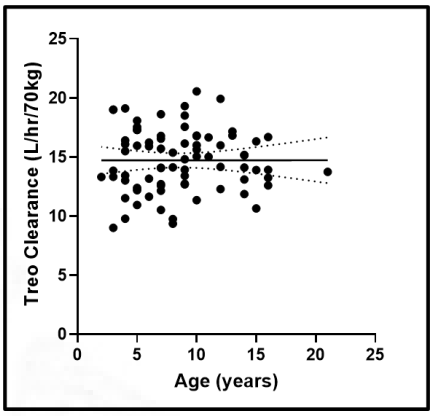


Figure 4.1.3 Correlation between Treo with S, S-EBDM exposure (Left), and Treo Clearance with age (right)
Left- Correlation analysis between the Treo (X-axis) and S, S-EBDM (Y-axis) exposure in each patient showing a moderate positive linear relationship (R^2 = 0.17, p= 0.0002 & Right- Correlation analysis between the Age (X-axis) and Treo Clearance (Y-axis) in each patient showing absence of correlation

4.1.4 Role of Genetic Polymorphisms in explaining variability in Treo and S, S-EBDM Exposure.

We had previously screened for all genetic variants using DMET Plus Premier Pack (Affymetrix, USA) in 51 patients with TM who received the TFT regimen. The effect of genetic polymorphisms (at an allele frequency of >0.05%) on Treo PK was evaluated (S, S-EBDM PK was not assessed then). We identified four polymorphisms that were significantly associated with Treo PK- 3'UTR variants in GSTA4 (Glutathione S-transferase; rs7496) & NQO1 (NAD(P)H dehydrogenase 1; rs10517), a missense variant in GSTZ1 (rs1046428), and an intronic variant in CES2 (Carboxylesterase 2; rs4783745) genes. **Table 4.1.2** describes the distribution of wild-type and variant genotypes of the selected polymorphisms in this patient cohort.

Genetic Polymorphisms	Homozygous Reference N(%)	Heterozygous N (%)	Homozygous N (%)
GSTA1*B haplotype	36 (47%)	35 (45%)	06 (8%)
***NQO1* (rs10517)**	51 (66%)	26 (33%)	-
GSTA4 (rs7496)	55 (69%)	20 (26%)	02 (3%)
GSTZ1 (rs1046428)	53 (69%)	20 (26%)	04 (5%)
CES2 (rs4783745)	38 (50%)	33 (43%)	05 (7%)

Table 4.1.2 Frequency of genetic variants with potential association with Treo and metabolite S, S-EBDM exposure
GST, Glutathione S- transferase; NQO1, NAD(P)H Quinone Dehydrogenase 1; CES2, Carboxylesterase 2; na, not applicable. For further comparison with Treo PK, heterozygous and homozygous variants were combined.

We then tested if these SNPs explain the variability in Treo/S, S-EBDM PK by comparing Treo and S, S-EBDM exposure in patients carrying wild type vs. variant alleles of each of these 5 SNPs/Haplotypes. Two of the five polymorphisms screened (*GSTA1*B* and *NQO1)* were significantly associated with Treo and S, S-EBDM exposure. Treo AUC was considerably lower in patients with variant genotype (n=26) for *NQO1* rs10517 polymorphism compared to those with wild type (n=51) genotype [1890 (1441-2537) vs. 2100 (1286-388), p=0.02)]**.** S, S-EBDM exposure was significantly higher in patients with variant genotype for *GSTA1*B* polymorphism (n=41) compared to those with wild type genotype (n=36) [164 (65-706) vs. 115 (64-385), p=0.03)]. S, S-EBDM exposure was significantly lower in patients with variant genotype (n=26) for *NQO1* rs10517 polymorphism compared to those with wild type genotype (n=51) [110 (64-327) Vs. 154 (65-706), p=0.01)] **Figure 4.1.4**. No

associations were observed with the other three polymorphisms screened (*CES2*, *GSTA4*, and *GSTZ1)*.

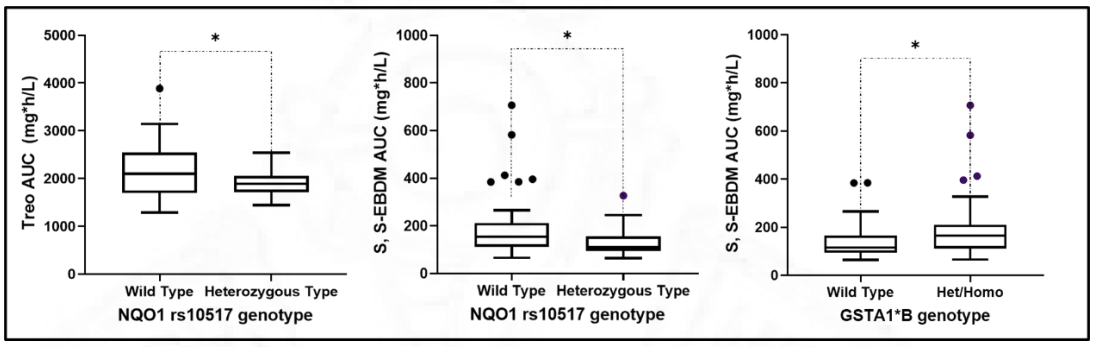


Fig 4.1.4 NQO1/ GSTA1*B genotypes influence Treo and S, S-EBDM Exposure. Association between NQO1 and GSTA1*B genotypes with Treo and S, S-EBDM exposure *Wild type- Homozygous reference genotype, Het/Homo- Heterozygous and homozygous genotype, the p-value was calculated by Mann Whitney U Test. *Asterisks indicate the significance level (p-value); *Means p< 0.05.

4.1.5 HCT outcomes

We then assessed the HCT outcomes in this patient cohort. Seventy-five patients (97.4%) engrafted at a median of 16 days post-HCT (range: 12–43 days), while two did not engraft (2.6%). Post-HCT chimerism was evaluated in all patients alive beyond day +28 (n = 75). Eight patients (10.4%) had mixed chimerism (4% - 84%) on day +28. Five patients (6.5%) had graft rejection within one year.

We then assessed the incidence of RRTs such as mucositis and SOS in these patients: 19 (25%) developed Hepatic SOS per Baltimore criteria. Mucositis GradesII–IV was observed in 40 (52%) patients; 3 (4%) developed grade I mucositis, while the remaining 44% did not develop mucositis. Seventeen of the 75 evaluable patients developed acute GVHD (23%). The early and late TRM were 5.2% and 10.4%, respectively, while the 1-year OS and TFS were 83% and 82%, respectively. The primary cause of death was Graft failure/rejection, GVHD, and infections.

4.1.6 Impact of Treo & S, S-EBDM exposure on HCT outcomes

We then evaluated the influence of Treo & S, S-EBDM exposure on HCT outcome parameters, including engraftment, rejection, RRTs, TRM, OS, and TFS. Treo AUC was lower in patients who had graft rejection vs. those who did not {1655 vs. 2037 mg*h/L (p=0.07), **Figure 4.1.5**}, showing a trend to significance.

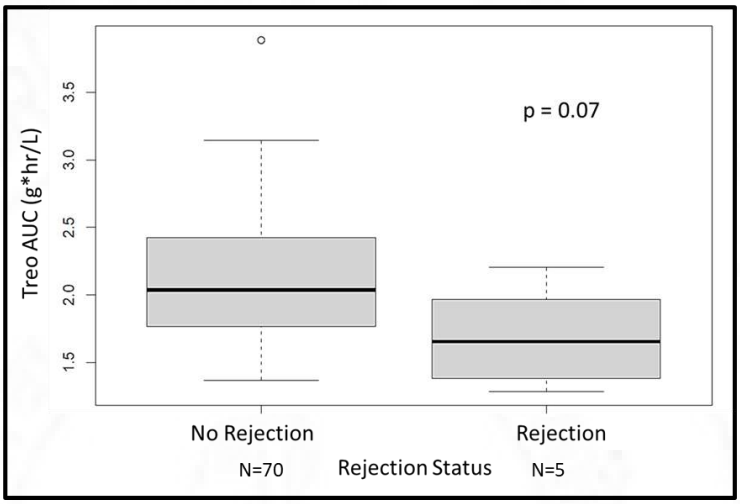


Fig 4.1.5 Association between Treo exposure in patients with and without graft rejection

There were no associations between Treo or S-S-EBDM AUC and other HCT outcomes, including engraftment or RRTs. Patients who developed SOS had had significantly low Treo/ S, S-EBDM AUC ratio compared to patients who did not develop SOS {13 (4.4- 20.2) vs. 17 (3.8-28.6), p=0.04, **Figure 4.1.6**}.

Upon quartile analysis, we observed that patients with high Treo/ S, S-EBDM AUC >18.5 had decreased incidence of SOS (5% vs. 31%, p=0.03), suggesting that increased conversion of Treo to S, S-EBDM could increase the incidence of RRTs. None of the other HCT outcomes, including rejection or survival, were influenced by the Treo/S, S-EBDM exposure ratio.

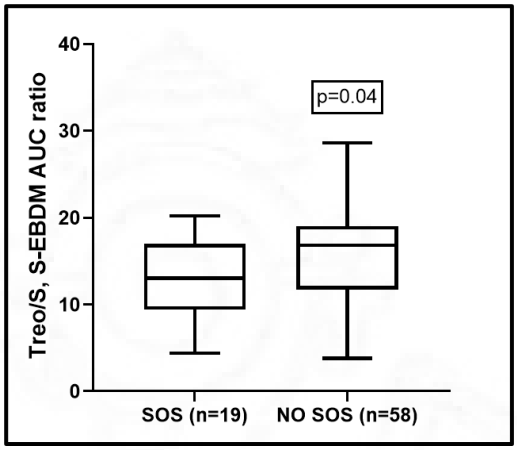


Fig 4.1.6 Association between Treo/S, S-EBDM exposure ratio in patients with and without hepatic SOS
Comparison of Treo AUC between patients who had graft rejection with those who did not

4.1.7 Influence of Genetic Polymorphisms on HCT Outcomes

We then assess the impact of the screened genetic polymorphisms on early HCT outcomes. While there were no associations between genotypes and engraftment, chimerism status, or RRTs, we observed that patients with variant genotype for *NQO1* (rs10517) polymorphism had significantly inferior 1-year OS and TFS {92.2% Vs. 69.2%, p= 0.008, and 69.2% Vs. 88.2%, p=0.04 respectively; **Figure 4.1.7**}. We hypothesize that patients with variant genotype for *NQO1* (rs10517) polymorphism have decreased systemic exposure to both Treo and S, S-EBDM impacting TFS and early mortality. However, validation in a larger cohort is warranted.

We also observed that patients with variant genotypes for *GSTA1*B* polymorphism had significantly inferior 1-year OS and TFS {94.4% vs. 75.6%, p= 0.02 for *GSTA1*B* and 91.7% Vs. 73.2%, p=0.04 respectively, **Figure 4.1.8**} compared to the patients who carried wild-type genotypes.

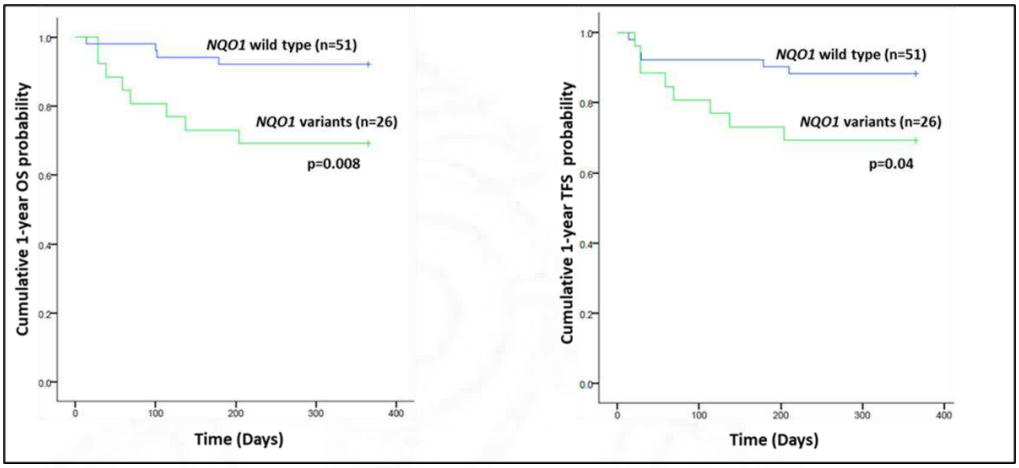


Fig 4.1.7 Impact of NQO1 polymorphism on 1-year OS and TFS
Kaplan-Meier survival curves showing associations between NQO1 '3'UTR variant (rs10517) genotype with 1-year OS (left) and TFS (right).

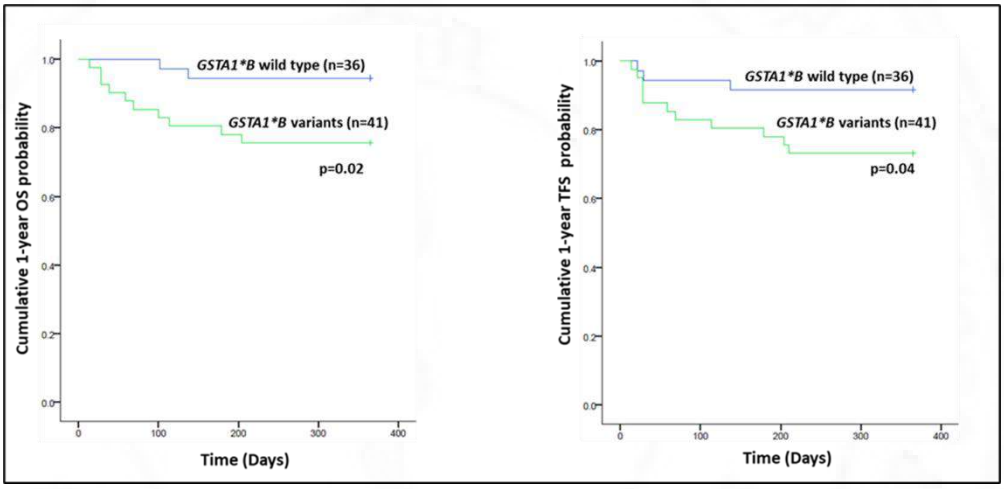


Fig 4.1.8 Impact of GSTA1*B polymorphism on 1-year OS and TFS
Kaplan-Meier survival curves showing associations between GSTA1*B promoter polymorphism variant genotype with 1-year OS (left) and TFS (right).

4.1.8 Pharmacodynamic Modeling and Optimal Treo exposure prediction

Since we found low Treo AUC (0-∞) to be associated with graft rejection (trend to significance), we then used the PD model to find the Treo exposure measure that could predict 1-year OS and TFS. The 1-year OS and TFS were modeled stepwise using Cox proportional hazards. There was a trend for low Treo AUC (0-∞) to be associated with rejection [HR (95%CI) =0.08 (0,1.37); p=0.08). Four covariates significantly

predicted 1-year OS. *GSTA1*B* and *NQO1 (rs10517)* variant genotype, the incidence of Acute GVHD, and hepatic SOS greatly influenced mortality, **Table 4.1.3**.

Table 4.1.3 Cox proportional hazards model for 1-year Mortality and Graft Rejection

Covariates	1-year OS hazard ratio	1-year OS p-value	Rejection hazard ratio	Rejection p-value
Treosulfan AUC g*hr/L	0.46 (0.12, 1.84)	0.27	**0.08 (0, 1.37)**	**0.081**
S, S-EDBM AUC g*hr/L	0.07 (0, 95.5)	0.47	5.5 (0.01, 2928.23)	0.59
Treosulfan/S, S-EDBM AUC ratio	1.01 (0.91, 1.12)	0.84	0.97 (0.83, 1.14)	0.75
Age (years)	1.1 (0.97, 1.24)	0.15	1.07 (0.88, 1.3)	0.48
Sex	1.64 (0.52, 5.18)	0.4	4.82 (0.54, 43.15)	0.16
Lucarelli score	2.12 (0.9, 5)	0.087	2.53 (0.64, 10.06)	0.19
$CD34^+$ Dose ($x10^6$/kg)	1.14 (0.93, 1.4)	0.21	1.09 (0.78, 1.53)	0.61
Donor source	2.66 (0.72, 9.82)	0.14	0 (0, Inf)	1
CES2	1.35 (0.43, 4.26)	0.61	1.46 (0.24, 8.76)	0.68
GSTA1	**5.05 (1.11, 23.06)**	**0.037**	1.53 (0.26, 9.16)	0.64
GSTA4	0.49 (0.11, 2.26)	0.36	1.67 (0.28, 10)	0.57
GSTZ1	0.69 (0.19, 2.57)	0.58	0 (0, Inf)	1
NQO1	**4.43 (1.33, 14.74)**	**0.015**	0.56 (0.06, 4.99)	0.6
aGvHD	**0.16 (0.05, 0.58)**	**0.0051**	0 (0, Inf)	1
Hepatic SOS	**0.19 (0.06, 0.6)**	**0.0045**	1.05 (0.12, 9.42)	0.96

1-year Mortality and Graft rejection were modeled stepwise using Cox proportional hazards using R statistics. AUC, Area under the curve; GST, Glutathione S-transferase; NQO1, NAD(P)H dehydrogenase 1; CES2, Carboxylesterase 2; SOS, Sinusoidal Obstruction Syndrome

As low Treo exposure showed a trend to increased graft rejection, we then attempted to find the optimal cutoff for Treo exposure that could give the maximum probability of better 1-year TFS and OS. Treo exposure ≥1660 mg*hr/L was significantly associated with better 1-year TFS (88.5% Vs. 62.5%, p=0.029) and trend to better 1-year OS (90.2% Vs. 68.8%, p=0.07); **Figure 4.1.9**.

We then performed multivariate analysis to identify if the therapeutic target cutoff of Treo exposure >1660 mg*hr/L could independently predict better 1-year TFS. Therefore, we included all the pre-HCT risk factors like age, Lucarelli risk score,

The Vellore risk score, $CD34^+$ cell dose, Donor source, *GSTA1*B*, and *NQO1* rs10517 polymorphisms in the analysis. Upon multivariate Cox regression, we identified Treo exposure >1660 mg*hr/L and *GSTA1*B* to independently predict better 1-year TFS and *GSTA1*B* variant genotype to predict inferior 1-year TFS, **Table 4.1.4**.

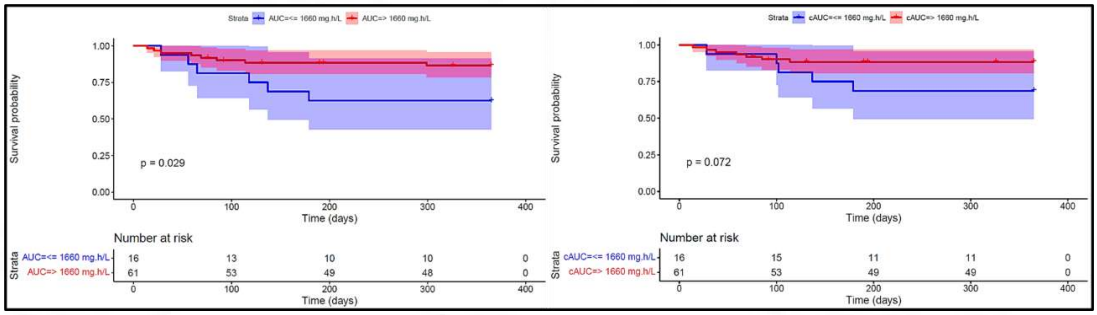


Figure 4.1.9 Treo AUC and the probability of 1-year TFS and OS
Kaplan–Meier curve for 1-year TFS (left) and OS (right) in patients above and below the upper success probability Treo AUC cutoff of 1660 mg*hr/L.

Table 4.1.4 Univariate and Multivariate Cox regression analysis for Predictors of 1-year TFS

Variable (s)	Univariate			Multivariate		
	Risk	95% CI	P value	Risk	95% CI	P value
Higher Age	1.09	0.97-1.23	0.13	-	-	-
Lucarelli Risk Classification	1.00	1.00-1.00	0.96	-	-	-
Vellore Risk Classification	1.00	1.00-1.00	0.94	-	-	-
CD34+ cell dose	1.13	0.93-1.37	0.21	-	-	-
Donor source	2.16	0.60-7.75	0.23	-	-	-
PreHCT serum ferritin levels	**1.00**	**1.00-1.00**	**0.06**	-	-	-
*GSTA*B* variant genotype	**3.65**	**1.02-13.10**	**0.05**	**3.75**	**1.04-13.47**	**0.04**
NQO1 rs10517 variant genotype	**2.98**	**1.03-8.61**	**0.04**	**-**	**-**	**-**
Treo AUC ≥1660 mg*hr/L	**0.32**	**0.11-0.92**	**0.04**	**3.23**	**1.12-9.34**	**0.03**

1-year TFS was modeled stepwise using Cox proportional hazards using SPSS. AUC, Area under the curve; GST, Glutathione S-transferase; NQO1, NAD(P)H dehydrogenase 1.; HR, Hazard ratio; CI, Confidence interval.

4.2 Impact of Fludarabine exposure on HCT outcomes

This Chapter describes the dose-exposure response relationship to Flu in patients with β-TM undergoing HCT.

4.2.1 Patient Demographics

Of 144 patients who underwent HCT with a TFT regimen in our center between March 2016 and November 2019, ninety-six patients who gave consent were included in the study. The patients were followed up for at least 1 year post-HCT. The demographics are summarized in **Table 4.2.1**. We also had a retrospective cohort of seventy-three patients to analyze Flu PK. The median age of the patients was 9 years (1-25 years), and 95 (56%) were males. The majority of patients (98%) received PBSC grafts. Most patients belonged to Class III (High Risk-44.9%; Low risk-39.7%), and 14.2% belonged to Class II β-TM.

Table 4.2.1: Patient Demographics

Parameters	N=169 median (range)	%
Age (years)	9 (1-25)	NA
Sex (Male/Female)	95/74	56/44
BSA (m^2)	0.87 (0.19-1.75)	NA
Stem Cell Source		
Bone marrow	03	2
Peripheral Blood	166	98
HLA Match		
Identical	156	92
Mismatch	13	8
CD34 Cell dose (x10^6cells/kg)	10 (3-18)	NA
Lucarelli Classification		
Class I	2	1
Class II	24	14
Class III	143	85
Vellore Risk Classification		
Class III High Risk	76	45
Class III Low Risk	67	40

Donor type		
Matched sibling donor (MSD)	134	79
Matched-related donor (MRD)	9	5
Matched unrelated donor (MUD)	26	16
Polymorphisms		
***NT5E* (rs2295890)[a]**		
Homozygous reference	122	74
Heterozygous variant	39	24
Homozygous variant	03	2
***DCK* (rs1154478)[b]**		
Homozygous reference	151	89
Heterozygous variant	17	10
Homozygous variant	01	1

Categorical variables are presented as n (%). Continuous variables are expressed as median (median, range).

[a]*NT5E* genotyping was performed only for 164 patients: NA- Not applicable.

4.2.2 Flu levels

Plasma Flu levels were measured using the validated LC-MS/MS method as reported previously (REF). The median Cmax (peak plasma concentration at the end of conditioning) of plasma Flu levels was 560 (188-1665) µM/mL. We observed 44% inter-individual variability in the Cmax of Flu, **Figure 4.2.1**.

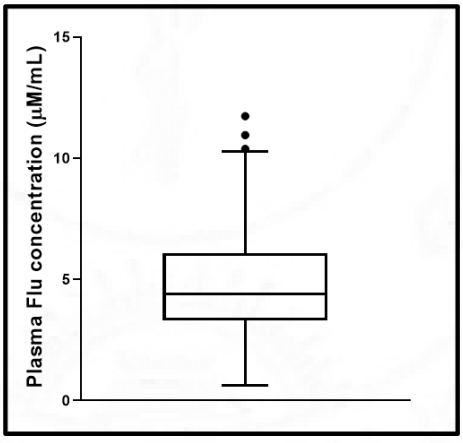


Fig 4.2.1 Interindividual variation in Flu Cmax. The range of fludarabine Cmax in the study cohort showed significant interindividual variation (p< 0.0001) as analyzed by a one-sample t-test.

4.2.3 Assessment of Flu systemic exposure using PopPK Model

PopPK analysis was done using Monolix (version 5.1.0). A 2-compartment model best described the data. The dose was normalized to BSA for the following analysis and was considered the base model. The population pharmacokinetic parameters for the base model are shown in **Table 4.2.2**.

Table 4.2.2 PopPK of Flu in Patients with β-TM Undergoing HCT

Parameter	Base	RSE (%)	Age	RSE (%)	p-value
CL (L/h/m^2)	6.7	5.1	6.9	4.9	
β: CL*			-0.19	47.0	0.034
V (L/m^2)	18.6	4.9	16.7	5.5	
Q (L/h/m^2)	13.9	9.1	17.8	10.2	
V_2 (L/m^2)	29.2	5.0	28.2	4.4	
σ additive (μM)	0.064	13.9	0.061	14.2	
σ prop (CV%)	8.3	8.1	8.6	7.7	
-2 Log-likelihood**	1693.2		1689.0		0.04
IIV	(CV%)	RSE (%)	(CV%)	RSE (%)	
CL	60	6.8	58	6.6	
V	36	16.8	34	22.1	
Q	57	17.3	66	16.3	
V_2	24	47.7	26	25.1	

**** Covariate model: CL=CLpop*(age/8)^b.***

***** p-value represents the significance of the change in the -2 log-likelihood (based on the c^2test) relative to the base model.***

The Goodness of Fit (GOF) plots for the final model were evaluated and showed no apparent visual bias for the predictions. The plot of observed concentration vs. population predicted concentration shows uniformly distributed points on either side of lines of identity. The conditional weighted residuals (CWRES) are within the acceptable range and are uniformly spread when CWRES are plotted against population predictions and also against time, **Figure 4.2.2**.

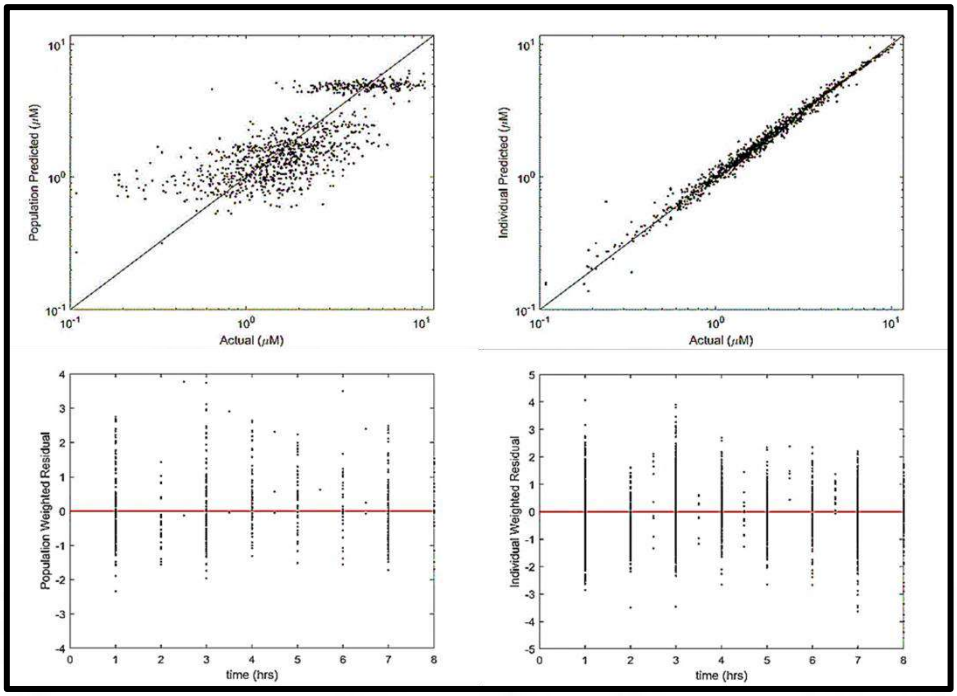


Figure 4.2.2 PopPK diagnostic plots. Plots from Top Left to Bottom Right are as follows: actual vs. population predicted concentration; actual vs. individual predicted concentration; population-weighted residuals vs. time; and individual weighted residuals vs. time. The solid black line in each plot is the line of identity. Points are individual data. Red dashed lines represent regression. Black dashed lines represent |CWRES|.

The median post hoc estimated F-araA AUC and CL for the first dose was 19 (3-81) μmol*h/mL and 7 (2-38) L/h/m^2. There was significant inter-individual variation (IIV) in F-araA PK (27 and 19-fold in AUC and CL). F-araA CL decreased with increasing age (p=0.04), explaining 4.6% of the IIV on the F-araA CL. None of the other demographic/biochemical covariates, including *NT5E/DCK* polymorphisms, significantly affected F-araA PK, **Figure 4.2.3**. There was a trend of increasing CL with increasing Glomerular Filtration rate (p=0.16).

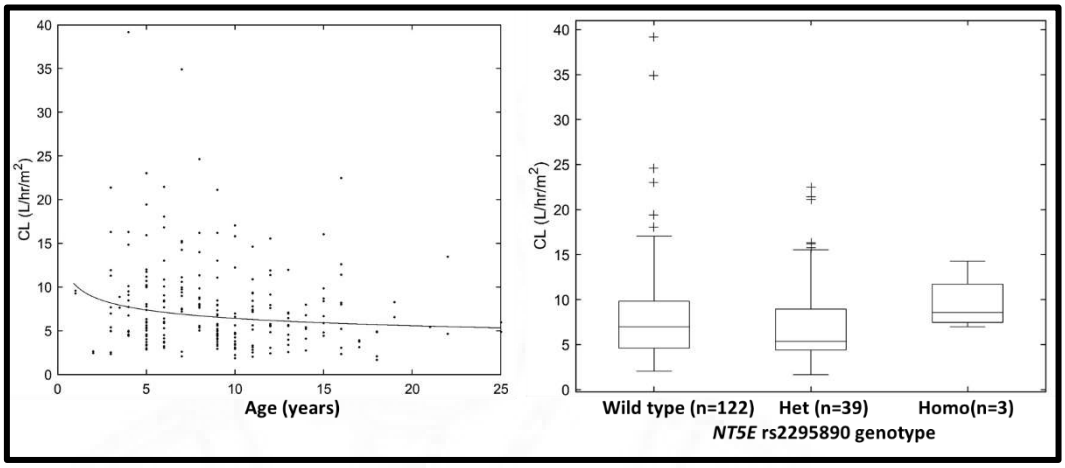


Figure 4.2.3 Associations between Flu Clearance and Age (left) and NT5E Polymorphism (right)
Left- Correlation analysis between the Age (X-axis) and Flu Clearance (Y-axis) in each patient showing a linear correlation & Right- Correlation analysis between the NT5E genotype (X-axis) and Flu Clearance (Y-axis) in each patient showing absence of correlation.

4.2.4 HCT outcomes

HCT outcome endpoints are listed in **Table 4.2.3**. All patients were followed up for at least one year post-HCT. Seven patients (4%) died early due to RRT and other transplantation-related complications, while 162 patients had documented engraftment (median day of engraftment was 16 days (range: 12–43 days). Post-transplant hematopoietic chimerism evaluated in all patients who were alive beyond day +28 post-HCT (n=160) showed complete chimerism (CC) in 148 (92%) and mixed chimerism (MC) in 12 (8%) on day +28 post-HCT. Eleven of the 160 evaluable patients (7%) rejected their graft, with the median time of rejection of 1.5 months (0.7-13 months). Hepatic SOS and mucositis were documented in 31 (18%) and 87 patients (I-3%, II- 24%, III- 24%, IV-0.6%), respectively. Forty-four (27%) patients developed acute GVHD, while eighteen (12%) had chronic GVHD. Overall, 135 (80%) patients were alive at the end of one year, and the 1-year TFS was 78%.

Table 4.2.3 HCT outcomes

Parameters	N=169	%
Engraftment		
Yes	162	96
No	07	4
Days for engraftment	16 (12-43)	NA
Day+28 Chimerism		
CC	148	92
MC	12	8
NE	09	-
Rejection		
Yes	11	7
No	149	93
NE	09	-
Mucositis		
Yes	87	51
No	82	49
Hepatic SOS		
Yes	31	18
No	138	82
Acute GVHD		
Yes	44	27
No	118	73
NE	07	-
Chronic GVHD		
Yes	18	12
No	128	88
NE	23	-
TRM		
D+30		
Yes	15	9
No	154	91
D+100		
Yes	22	13
No	147	87
1-year OS		
Alive	135	80
Dead	33	20
Lost to Follow-up	01	-
1-year TFS		
Event	37	22
No event	132	78
Lost to Follow-up	01	-

4.2.5 Role of Flu Exposure and genetic variants on HCT Outcomes

We then tested the relationships between systemic Flu exposure and various clinical outcomes. No significant associations were found between Flu exposure and any individual clinical outcome endpoints, **Figure 4.2.4**. Flu exposure did not impact long-term HCT outcomes such as 1-year TFS and OS. No association was observed between genetic polymorphisms (in *NT5E* & *DCK*) and any HCT outcomes.

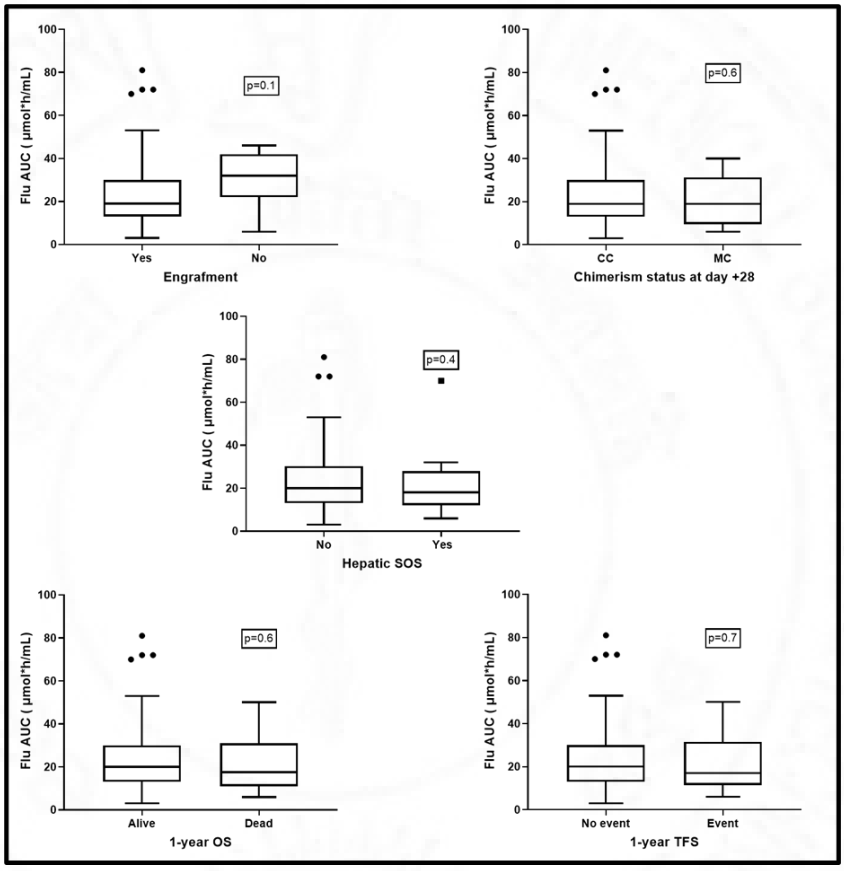


Figure 4.2.4 No significant association between Flu exposure and clinical outcomes.

4.3 Endothelial Activation and Stress IndeX (EASIX) – measured pre-HCT predict transplantation-related mortality in patients with β-TM

This Chapter describes the utility of EASIX as a prognostic biomarker for predicting early TRM in patients with β-TM who underwent HCT with a uniform TFT conditioning regimen at our center.

4.3.1 Patient Demographics

Our center performed HCT on 281 individuals diagnosed with β-TM between January 2012 and December 2019, receiving a TFT conditioning regimen. There were 172 males and 109 females, with a median age of 9 (1 to 25 years). Most patients (39.1% class III high risk; 47.7% class III low risk) had class III β-TM, while 12.1% were classified as class II β-TM. The patient characteristics are summarised in **Table 4.3.1**.

Table 4.3.1 Patient Characteristics of the entire cohort

Parameters	**N=281, Median (range)/N(%)**
Age (years)	9 (1-25)
Sex (Male/Female)	172/109 (61.2/38.8)
BSA (m^2)	0.87(0.19-1.75)
<u>Stem Cell Source</u> **Bone marrow** **Peripheral Blood**	 5 (1.8) 276 (98.2)
<u>HLA Match</u> **Identical** **One antigen mismatch**	 260 (92.5) 21 (7.5)
CD34 Cell dose (x10^6cells/kg)	10 (2.2-18)
Pesaro Classification **Class I** **Class II** **Class III** **Vellore Risk Classification** **Class III High Risk** **Class III Low Risk**	 3 (1) 34 (12.1) 244 (86.8) 110 (39.1) 134 (47.7)

Donor type	
Sibling donor	218 (77.5)
Non-sibling family donor	23 (8.2)
Unrelated donor	40 (14.3)

4.3.2 EASIX-PreHCT Scoring

Due to the absence of LDH measurements during the PreHCT period, the EASIX-PreHCT score could only be calculated for 184 out of 281 patients (65.5%). Among these 184 patients, the EASIX-PreHCT scores ranged from 0.11 to 24.42, with a median of 0.78. Comparing patients with available EASIX-PreHCT scores to those without, there was no significant difference in the rate of TRM+100 (14.7% versus 11.3%; P = 0.47). Additionally, there was no significant disparity in the overall mortality rate between these two groups (21.2% versus 17.5%; P = 0.53).

4.3.3 HCT Outcomes

The patients were monitored for a median duration of 30 months, ranging from 0.3 to 108 months. HCT outcomes are summarised in **Table 4.3.2**.

Table 4.3.2 HCT outcomes of the entire cohort

Parameters	**N=281 N(%)**
Engraftment	
Yes	267 (95.1)
No	14 (4.9)
Days for engraftment	16 (10-43)
Day+28 Chimerism	
Complete chimerism	239 (90.8)
Mixed Chimerism	24 (9.2)
Rejection	
Yes	17 (6.4)
No	247 (93.6)
Mucositis	
Yes	146 (51.9)
No	135 (48.1)

Hepatic VOD	
Yes	54 (19.2)
No	227 (80.8)
Acute GVHD	
Yes	72 (26.9)
No	195 (73.1)
Chronic GVHD	
Yes	31 (12.8)
No	211 (87.2)
TRM	
D+30	
Yes	27 (9.6)
No	254 (90.4)
D+100	
Yes	38 (13.5)
No	243 (86.5)
OS Status	
Alive	225 (80.0)
Dead	56 (20.0)
EFS status	
Event	60 (21.4)
No event	221 (78.6)

4.3.4 ROC Analysis Using EASIX- PreHCT for Predicting TRM+100.

The median EASIX-PreHCT score was significantly higher in patients who experienced TRM+100 than those without TRM+100 (1.09 versus 0.75; P = 0.008). The area under the ROC curve, indicating the predictive value of the EASIX-PreHCT score for TRM+100, was 0.661. By setting a cutoff of 0.85 for the EASIX-PreHCT score, the prediction of TRM+100 achieved a sensitivity of 70.4% and specificity of 62%, **Figure 4.3.1**. Patients with an EASIX-PreHCT score greater than 0.85 had a significantly higher TRM+100 rate compared to those with a score below 0.85 (24.4% versus 7.5%; P = 0.003)

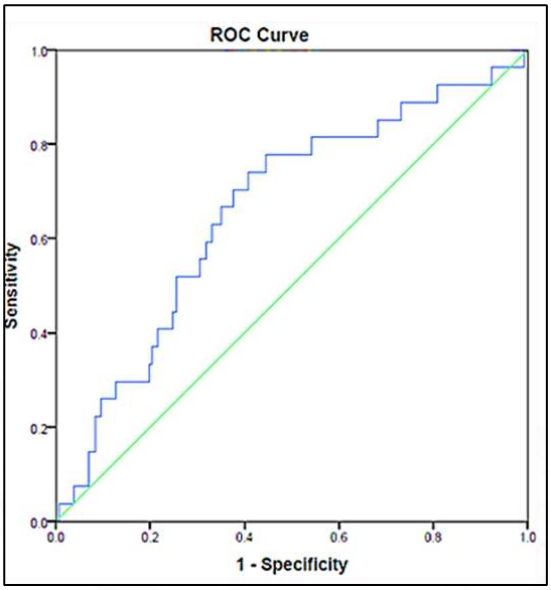


Figure 4.3.1 ROC curve for EASIX Pre-Tx as a predictor of TRM at day 100

4.3.5 Analysis of Predictors of TRM+100 in Class III β-TM cohort

We then focused on class III β-TM patients, consisting of 156 individuals, after excluding those with class I and II thalassemia. **Table 4.3.3** provides demographic information and HCT outcomes for this uniform cohort of class III patients.

Table 4.3.3 Patient characteristics for all class III patients with thalassemia undergoing HCT where EASIX-PreTx was available (N=156)

Parameters	N=156 Median (range)/ N(%)
Age (years)	9 (1-25)
Sex (Male/Female)	97/59 (62.2/37.8)
Stem Cell Source **Bone marrow** **Peripheral Blood**	 3 (1.9) 153 (98.1)
HLA **Identical** **One antigen/allele mismatch**	 147 (94.2) 9 (5.8)
CD34 Cell dose (x10^{6}cells/kg)	10 (2.2-18)
Vellore Risk Classification **Class III Low Risk** **Class III High Risk**	 83 (53.2) 73 (46.8)
Engraftment	146 (93.6)

Day+28 Chimerism **Complete chimerism** **Mixed Chimerism**	 129 (88.4) 17 (11.6)
Day+60 Chimerism **Complete chimerism** **Mixed Chimerism**	 107 (83.6) 21 (16.4)
Day+100 Chimerism **Complete chimerism** **Mixed Chimerism** **Rejection**	 98 (81.0) 22 (18.2) 1 (0.8)
Rejection **Yes** **No**	 10 (6.8) 136 (93.2)
Mucositis	84 (53.8)
Hepatic VOD	35 (22.4)
Acute GVHD **Yes** **No**	 43 (29.5) 103 (70.5)
Chronic GVHD **Yes** **No**	 19 (14.6) 111 (85.4)
TRM **D+30** **D+100**	 17 (10.1) 25 (16)
OS Status: Dead	35 (22.4)
EFS status: Event	37 (23.7)

We then compared baseline characteristics and patient outcomes based on their EASIX- PreHCT scores using the cutoff of 0.85, **Table 4.3.4**. No significant differences were observed between the EASIX >0.85 and EASIX <0.85 groups concerning age, sex, proportions of class III high-risk patients, mixed chimerism, SOS, or GVHD. However, the rate of TRM+100 was significantly higher in the EASIX >0.85 group (25% versus 8.3%; P = 0.008, **Figure 4.3.2**).

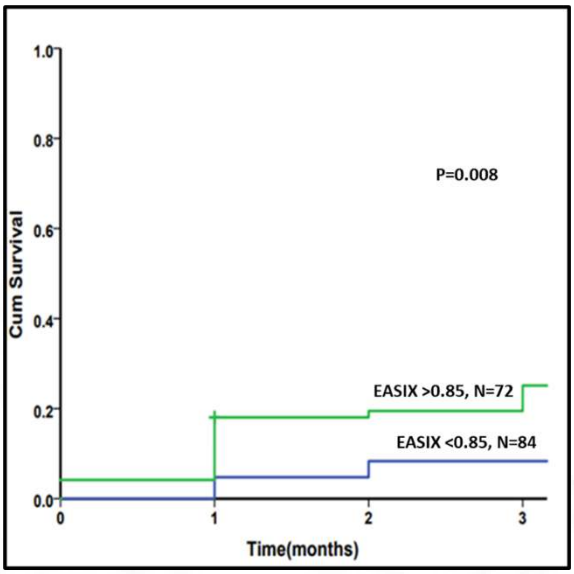


Fig 4.3.2 TRM+100 curve for class III patients TM using the proposed EASIX-PreTx cut-off of 0.85 (N=156) (green line = EASIX >0.85, blue line = EASIX <0.85)

Among the 156 class III β-TM patients with available EASIX scores, 25 died within 100 days of transplantation, including 18 with EASIX >0.85 and 7 with EASIX <0.85. The causes of TRM+100 in the EASIX >0.85 group included primary graft failure, GVHD, RRT (SOS and intracranial bleeding), and infection. In the EASIX <0.85 group, causes of TRM+100 were primary graft failure, GVHD, RRT (SOS and intracranial bleeding), and infections.

Table 4.3.4 HCT outcomes for all class III patients with β-TM undergoing HCT where EASIX-PreTx was available.

Parameters	EASIX >0.85 (N=72)	EASIX<0.85 (N=84)	P value
Age	10.5 (1-21)	8.5 (2-25)	**0.010**
Male Sex	43 (59.7)	54 (64.3)	0.620
CD34 cell dose	10 (2-18)	10 (3-14)	0.188

Donor Source **Matched Sibling** **Matched Non-Sibling** **Matched unrelated**	 57 (79.2) 6 (8.3) 9 (12.5)	 66 (78.6) 8 (9.5) 10 (11.9)	0.964
Proportion of one HLA antigen/allele mismatch donors	6 (8.3)	3 (3.6)	0.303
The proportion of class III High risk	38 (52.8)	35 (41.7)	0.199
The proportion of patients having neutrophil engraftment	65 (90.3)	81 (96.4)	0.189
Mixed chimerism at day 28 (N=146)	7 (10.8)	10 (12.3)	0.802
Mixed chimerism at day 60 (N=128)	12 (22.6)	9 (12.0)	0.146
Mixed chimerism at Day 100	12 (25.0)	10 (13.7)	0.123
Rejection	6 (9.2)	4 (4.9)	0.341
Hepatic VOD	17 (23.6)	18 (21.4)	0.848
Acute GVHD	22 (33.8)	21 (25.9)	0.362
Chronic GVHD	7 (13.0)	12 (15.8)	0.802
TRM+30	13 (18.1)	4 (4.8)	**0.010**
TRM+100	18 (25.0)	7 (8.3)	**0.008**

We also compared patients who experienced TRM+100 and those who did not. Patients with TRM+100 were older, received a higher CD34 cell dose, had higher rates of non-sibling and HLA-mismatched donors, mixed chimerism, graft rejection, SOS, acute GVHD, and higher PreHCT EASIX scores, **Table 4.3.5**.

Table 4.3.5 Comparison of patients who had TRM at day 100 versus those who did not among the class III β-TM where EASIX was available.

Parameters	**TRM+100 N(%)**		**P value**
	Yes (N=25)	**No (N=131)**	
Age	12 (4-20)	9 (1-25)	**0.001**

Male Sex	17 (68)	80 (61.1)	0.654
CD34 cell dose (x 10^6/kg)	10.3 (2-18)	9.3 (3-16)	**0.045**
Donor source **Matched sibling** **Matched related** **Matched unrelated**	 10 (40) 5 (20) 10 (40)	 113 (86.3) 9 (6.9) 9 (6.9)	**0.001**
Proportion of one antigen/allele mismatch donors	7 (28)	2 (1.5)	**0.001**
Proportion of class III High risk	14 (56)	59 (45)	0.384
Patients having neutrophil engraftment	16 (64)	130 (99.2)	**0.001**
Mixed chimerism at day 28 (N=146)	7 (46.7)	10 (7.6)	**0.001**
Mixed chimerism at day 60 (N=128)	5 (62.5)	16 (13.3)	**0.003**
EASIX-Pre-Tx >0.85	18 (72)	54 (41.2)	**0.008**
Rejection (N=146)	7 (46.7)	3 (2.3)	**0.001**
Hepatic VOD	13 (52)	22 (16.8)	**0.001**
Acute GVHD (N=146)	12 (75)	31 (23.8)	**0.001**

Table 4.3.6 presents the results of univariate and multivariate analyses, identifying predictors of TRM+100 in this uniform subgroup of class III β-TM patients. The factors independently predicting TRM+100 were EASIX >0.85, Pre-HCT serum ferritin >3210 ng/mL, and the use of unrelated donors. Mixed chimerism was excluded from the multivariable analysis as it applied only to surviving patients at the time of chimerism testing.

Table 4.3.6 Univariate and multivariate analysis for predictors of TRM+100 amongst class III patients (N=156)

Variable	**Univariate**			**Multivariate**		
	Risk	**95%CI**	**P value**	**Risk**	**95%CI**	**P value**
Age > 9.5 years	3.0	1.19-7.78	**0.020**	1.4	0.46-4.45	0.538
Sex: Female	1.3	0.55-3.37	0.514			

Class III Vellore risk	1.5	0.66-3.68	0.316			
CD34 cell dose	1.2	0.98-1.39	0.078	1.1	0.86-1.31	0.559
HLA mismatch	25.1	4.83-130.22	**0.001**	18.4	0.98-345.51	0.051
Donor source – Matched related non-sibling donor v/s others	6.3	1.76-22.36	**0.005**	3.9	0.79-19.55	0.096
Donor source – Matched unrelated donor v/s others	12.6	4.14-38.0	**0.001**	14.6	2.96-71.56	**0.001**
Pre-transplant ferritin > 3210 ng/mL	5.0	1.78-14.2	**0.002**	6.1	1.63-23.05	**0.007**
Mixed chimerism at day 28 (N=146)	10.6	3.18-35.2	**0.001**			
EASIX-Pre-Tx >0.85	3.7	1.43-9.39	**0.007**	3.9	1.19-13.05	**0.025**

On survival analysis, we also observed that patients with an EASIX <0.85 demonstrated significantly better OS (mean 2-year OS: 82.7% ± 4.2% versus 70.6% ± 5.4%; log-rank P = 0.05, **Figure 4.3.3**).

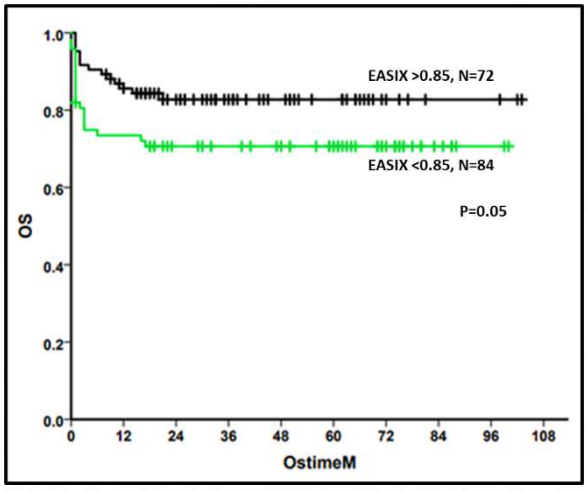


Fig 4.3.3 Figure 1. Kaplan-Meier survival curves for OS based on the proposed EASIX Pre-Tx cutoff (0.85) (n = 156) show significantly better OS in patients with EASIX-PreTx score <0.85 (mean 2-year OS, 82.7% ± 4.2 % versus 70.6% ± 5.4%; log-rank P =0.05). The black line represents EASIX-PreTx <0.85; the green line, EASIX-PreTx >0.85, time on the X axis is expressed in months.

4.4 A pilot study to identify biomarkers of sinusoidal obstruction syndrome using global plasma metabolomic profiling

This Chapter describes the results of a pilot study to identify the plasma metabolome changes due to TFT conditioning in patients with β-TM and to identify metabolomic biomarkers unique to hepatic SOS.

4.4.1 Patient Demographics

Global plasma metabolomics was performed in paired plasma samples before and at the end of conditioning from patients receiving the TFT regimen. We included four patients with very severe SOS and 3 with no hepatotoxicity. The patient demographics are summarized in **Table 4.4.1**. The demographics were comparable between the two groups.

Table 4.4.1 Patient Demographics

Patient Characteristics	SOS Group (N=4)	No SOS Group (N=3)
Age (years)	10 (8-14)	4 (4-9)
Sex (Male/Female)	3/1	2/1
Lucarelli Classification **Class I/ II/ III**	III-100%	III-100%
Vellore Risk Classification **Class III Low Risk/** **High Risk**	- LR-100%	LR-67% HR-33%
HLA Disparity **Full Match** **Not full match**	100% -	100% -
Donor Source **Matched sibling donor** **Matched-related donor**	100% -	100% -
Cell dose ($CD34^+x10^6$/kg)	9 (7-12)	6 (5-11)
Serum Ferritin (ng/mL)	2710 (726-3975)	2121 (1659-2892)

Categorical variables are displayed as n (%). Continuous variables are expressed as median (median, range).

4.4.2 Treo-based regimen alters plasma metabolome postconditioning.

To understand the plasma metabolomic changes due to the conditioning regime, we compared the metabolome changes before and at the end of the conditioning. We identified 654 metabolites after QC filtering using a pooled QC sample (a pooled matrix sample generated by taking equal volumes of each experimental sample). The results were evaluated by PLS-DA analysis, and the score graph clearly illustrates the distinct separation observed between the plasma samples of both groups, **Figure 4.4.1**.

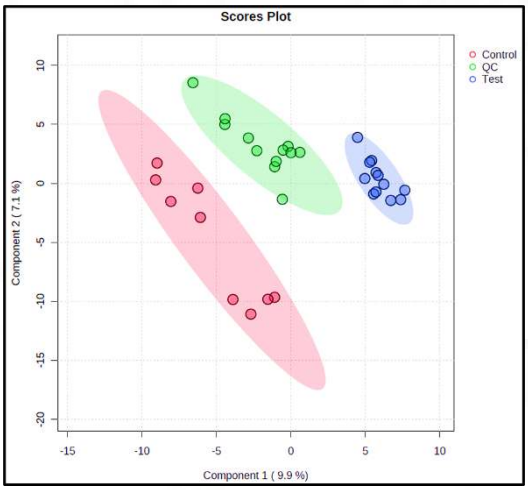


Figure 4.4.1 Partial least squares discriminant analysis (PLS-DA) indicating effective group discrimination. PLS-DA model comparing metabolomic profiles among pre-conditioning samples (red), QC sample (green), and end-of-conditioning samples(blue). The amount of variance explained is shown in parentheses on each axis.

We further analyzed the differentially regulated metabolites between pre and end-of-conditioning groups. Fold change analysis ((p-value ≤ 0.05) with a fold change of >1.3 and <0.76 for up and downregulated, respectively) revealed 14 upregulated and 18 downregulated metabolites, **Figure 4.4.2**

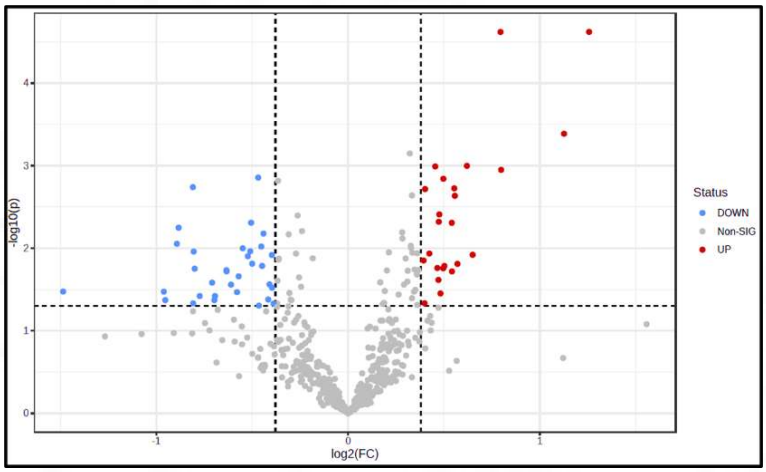


Figure 4.4.2 Volcano plot of differential metabolites between pre and end of conditioning groups. Red and blue represented up and down-regulated metabolites, respectively; Gray represented metabolites that had no significant change.

Some of the upregulated metabolites of biological relevance included Glycerophospholipids (Phosphatidylethanolamine), Oligopeptide (Tetragastrin), phenolic glycosides (3'-(2",3"-Digalloylglucosyl)-phloroacetophenone) and gallates (Theaflavin-3-gallate). We also identified down-regulated metabolites such as Acyl carnitines (L-Palmitoylcarnitine), Glycerophospholipids (Phosphatidic acid), and xenobiotics (cefazolin). The complete list of dysregulated metabolites is tabulated in Appendix A1.

4.4.3 Pre-conditioning plasma metabolomic profile predicts SOS.

We then compared metabolomic profiles in pre-conditioning plasma samples between patients with and without SOS to identify predictive biomarkers for developing SOS.

We identified 476 metabolites after QC filtering using a pooled QC sample. The PLS-DA score graph clearly illustrates the distinct separation observed between the plasma samples of both groups without any clustering, **Figure 4.4.3**.

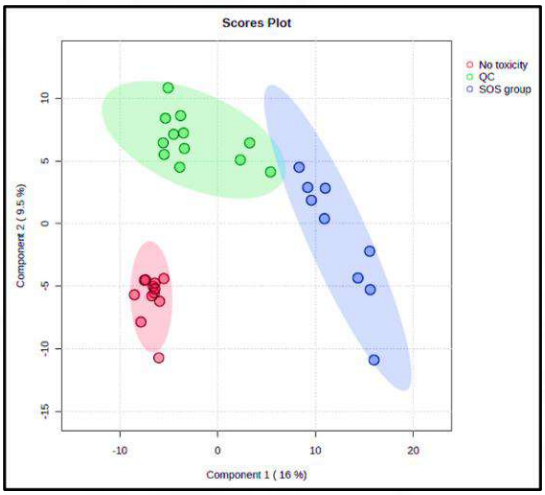


Figure 4.4.3 PLS-DA model comparing metabolomic profiles among patient samples without SOS (red), QC sample (green), and with SOS (blue) collected before conditioning. The amount of variance explained is shown in parentheses on each axis.

We further analyzed the differentially regulated metabolites between the groups. Additionally, fold change analysis revealed 86 upregulated and 65 downregulated metabolites, **Figure 4.4.4**. Further, the over-representation/pathway enrichment analysis yielded no enriched pathways from the dysregulated metabolites.

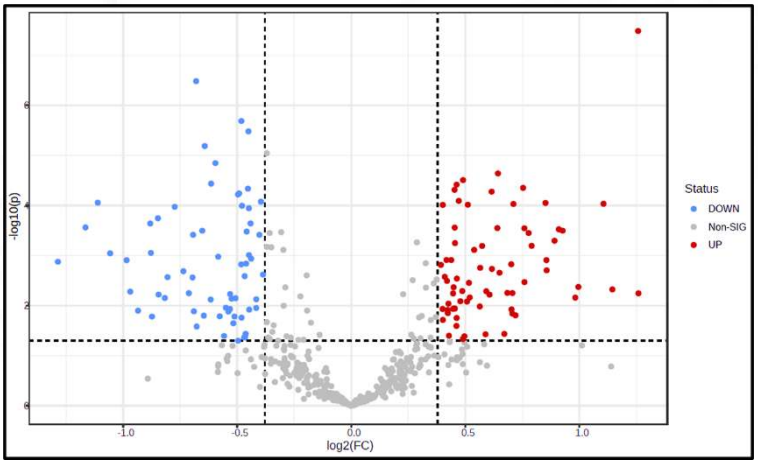


Figure 4.4.4 Volcano plot of differential metabolites in patients with SOS (Pre-conditioning). Red and blue represented up and down-regulated metabolites, respectively; Gray represented metabolites that had no significant change.

Some of the upregulated metabolites of biological relevance included Purine nucleosides (Succinyladenosine), Glycerolipids (Diacylglycerols), Glycerophospholipids (Phosphatidic acid, Lysophosphatidic acid), and Pyrimidines (Thymidine 5'-triphosphate). We also identified down-regulated metabolites such as Glycerophospholipids (Phosphatidylglycerol, Phosphatidylcholine, Phosphatidylethanolamine) and FattyAcyls (Leukotriene D4). The complete list of dysregulated metabolites is tabulated in Appendix A2.

Pathway enrichment analysis revealed the top 20 pathways represented in **Figure 4.4.5**. Some of the key metabolic pathways, such as Glycerolipid metabolism, phospholipid biosynthesis, citric acid cycle, aspartate metabolism, gluconeogenesis, steroid biosynthesis, pyruvate metabolism, Warburg effect, pyrimidine, and purine metabolism, were dysregulated in patients with SOS.

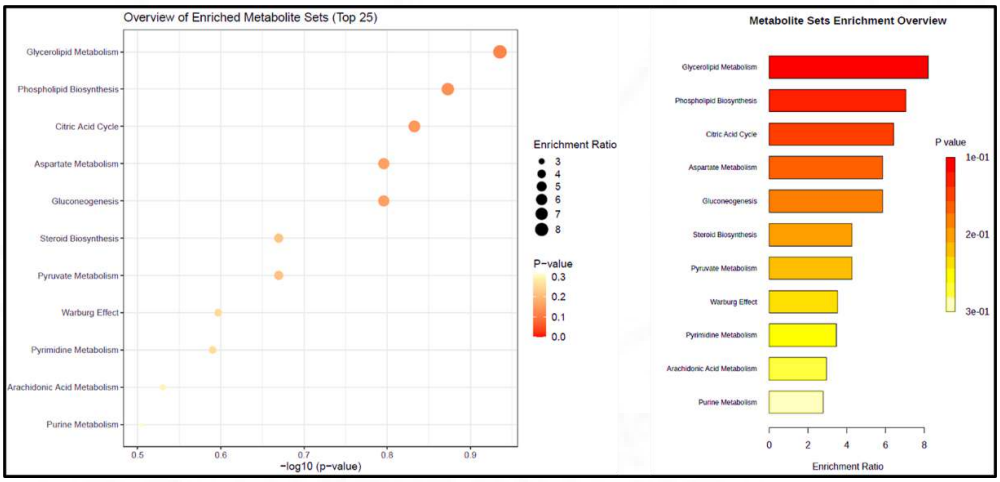


Figure 4.4.5 Metabolite pathway enrichment analysis to identify pathways enriched in the patient group with SOS compared to no SOS.

4.4.4 Post-conditioning plasma metabolomic profile predicts SOS.

We then compared metabolomic profiles in the plasma samples between patients with and without SOS at the end of conditioning to identify the regimen-associated dysregulated metabolites that lead to SOS, which also could be used as plausible prognostic biomarkers for developing SOS. We identified 528 dysregulated metabolites after QC filtering using a pooled QC sample.

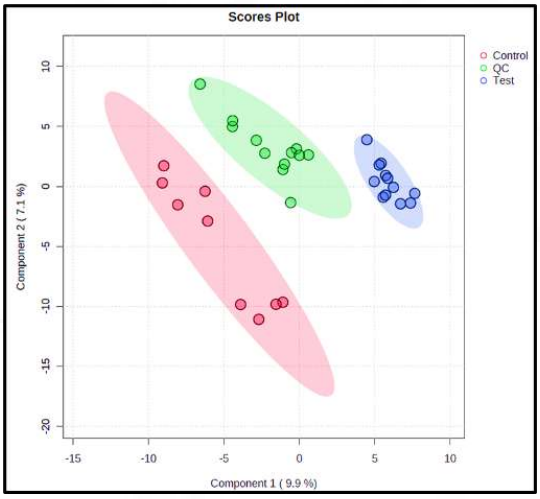


Figure 4.4.6 PLS-DA model comparing metabolomic profiles among patient samples without SOS (red), QC sample (green), and with SOS (blue) collected before conditioning. The amount of variance explained is shown in parentheses on each axis.

The PLS-DA score graph clearly illustrates the distinct separation observed between the plasma samples of both groups without any clustering, **Figure 4.4.6**. We further analyzed the differentially regulated metabolites between the groups.

Further, fold change analysis revealed 27 upregulated and 32 downregulated metabolites, **Figure 4.4.7**.

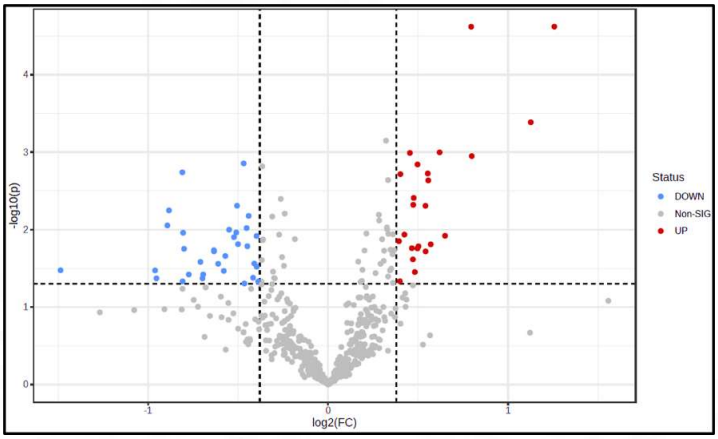


Figure 4.4.7 Volcano plot of differential metabolites in patients with SOS (End of TFT conditioning). Red and blue represented up and down-regulated metabolites, respectively; Gray represented metabolites that had no significant change.

Some of the upregulated metabolites of biological relevance identified included Fatty acyls (13-Hydroxyoctadecadienoic acid (13-HODE); alpha-Dimorphecolic acid; 9,10-Epoxyoctadecenoic acid; 12,13-EpOME; (Z)-13-Oxo-9-octadecenoic acid) and down-regulated metabolites such as Carboxylic acids (Cysteinylhydroxyproline, Hydroxyprolyl-Cysteine), Purines (Hypoxanthine) and Organooxygen compounds (Erythronic acid, Threonic acid). The complete list of dysregulated metabolites is tabulated in Appendix A3. The over-representation analysis did not yield any enriched pathways from the dysregulated metabolites.

5. DISCUSSION

The use of a TFT conditioning regimen has significantly reduced RRTs and improved AlloHCT outcomes, especially in patients with high-risk β-TM (George et al., 2019b; Mathews et al., 2013). Graft rejection/primary graft failure that could lead to early mortality post-HCT still limits its success (George et al., 2015). While the role of conditioning regimen drug exposure on HCT outcome has been extensively evaluated in patients receiving Bu/Cy, resulting in targeted dose adjustment of Bu (Palmer et al., 2016) to improve outcomes, no such effort has been made for a toxicity-reduced conditioning regimen containing TFT.

In this largest single-center study, we have evaluated the dose-exposure-response relationship to Flu & Treo and its active metabolite S, S-EBDM in a uniform cohort of patients with high-risk β-TM undergoing HCT. We have also assessed selected genetic variants in genes directly or indirectly involved in Flu/Treo metabolism. We then evaluated the role of EASIX as a predictive marker of HCT outcomes. Finally, we compared the plasma metabolomic profile between the pre and end of the conditioning and its role in predicting biomarkers of SOS.

5.1 Evaluation of dose-exposure response relationship to Treo and S, S-EBDM in patients with β-TM undergoing HCT

We established and validated a robust, rapid, and cost-effective LC-MS/MS-based method to quantify Treo and S, S-EBDM concentrations simultaneously in patients' plasma samples. Previously High-Performance Liquid Chromatography with Refractive Index Detection (HPLC-RID) was used to measure Treo and its metabolites

(Główka et al., 2013; Mohanan et al., 2018). However, this method is limited by its low sensitivity and specificity compared to LC-MS/MS. This method also requires a large volume (>250µL) of patients' plasma to quantify drug levels, whereas LC-MS/MS requires very minimal amounts of plasma samples to quantify drug levels. HPLC with ultraviolet detection (HPLC-UV) methods have also been described to measure Treo and its metabolites (Koyyalamudi et al., 2016; Ten Brink et al., 2014). Due to the time-consuming and laborious derivatization for HPLC-UV assays, LC-MS/MS assays are superior and feasible for routine analysis. Romański *et al.* established an LC-MS/MS assay to determine Treo and S, S-EBDM in plasma and tissue samples (Romański et al., 2014). We adopted the same methodology with minor modifications (use of a different internal standard and change in elution protocol). The advantages of the assay, such as the short run-time and the need for minimal plasma samples, make it an ideal methodology for TDM purposes.

Treo PK has been reported previously in patients undergoing HCT for malignant and benign conditions and in various combinations (Beelen et al., 2005; Danielak, Twardosz, et al., 2018; Główka et al., 2008; Koyyalamudi et al., 2016; Mohanan et al., 2018; Nemecek et al., 2011; Ten Brink et al., 2014). PopPK analysis in the present study revealed a two and 10-fold variability in Treo and S, S-EBDM exposure, respectively. The Treo PK estimates were comparable to the previous studies, **Table 5.1**. Except for a pilot study (Główka et al., 2015), there are no extensive reports on S, S-EBDM PK to date. The authors observed large IIV (14-fold) in S, S-EBDM exposure similar to ours, but they did not address the impact of the metabolite exposure on HCT outcomes. Despite a similar half-life, S, S-EBDM levels were 2-magnitude lower than Treo levels suggesting rapid elimination as reported previously (Danielak,

Kasprzyk, et al., 2018; Romański et al., 2016). Also, there was no strong correlation between Treo and S, S-EBDM exposure, suggesting the lack of a linear relationship between Treo and S, S-EBDM exposure. Genetic and other physiological factors could explain non-linearity and high IIV in S, S-EBDM exposure.

Recent dose-exposure response studies (Chiesa et al., 2020; Stoep et al., 2022) have attempted to derive a therapeutic range for Treo exposure to personalize Treo dosing for improved HCT outcomes in patients with non-uniform diagnoses with varying Treo-based conditioning regimens. Our previous study (Mohanan et al., 2018) evaluated Treo PK in 87 patients with TM, but we failed to observe clear-cut clinical associations with Treo exposure. In the present study, we elucidated the dose-exposure response relationship of Treo and its metabolite S, S-EBDM. We identified higher Treo exposure to be associated with better HCT outcomes. Specifically, lower Treo exposure was associated with graft rejection. Also, low Treo exposure showed a trend of significance to increased mortality. Though S, S-EBDM exposure alone did not influence HCT outcomes, higher S, S-EBDM to Treo exposure was associated with an increased incidence of SOS. We then arrived at a therapeutic cut-off of 1660mg*hr/L for better TFS and OS.

Unlike for Bu, where under and overexposure results in poor HCT outcomes, a therapeutic window could not be assigned for Treo. However, we can propose that higher Treo exposure (>1660mg*hr/L) benefits patients with TM undergoing HCT. A a recent study by Chiesa et al. demonstrated that target cumulative exposure of 4800 mg*h/L for Treo provided the highest likelihood of survival and sustained engraftment in 87 children, the majority with Primary immunodeficiency (Chiesa et al., 2020)

S.No	Diagnosis	N	Age Median (Range)	Conditioning Regimen	Treo Dose	Day 1 Treo Exposure (AUC) mg*h/L	Day 1 Treo Clearance L/h	Significant Findings	Refs
1	HM- 15 (28.3%) IEI- 32 (60.4%) GD (11.3%)	53	3.5 (0.9 – 12)	Treo/Flu/ThioT- 26 (49.1%) Treo/Flu- 22 (41.5%) Treo/Flu/Mel- 4 (7.5%) Treo/Mel- 1 (1.9%)	30 g/m^2- 6 (11.3%) 36 g/m^2- 11 (20.8%) 42 g/m^2- 36 (67.9%)	HM- 1649 (1419 – 1889) IEI- 1648 (1457 – 1896) GD- 1634 (1407 – 1876)	16.4 L/h/70 kg	Model-informed recommended for patients <2 years; No outcomes were evaluated.	Rosser et al., 2023
2	IEI- 38 (35%) HBP- 55 (50%) BMF- 17 (15%)	110	5.2 (0.2-18.8)	Treo/Flu- 37 (32%) Treo/Flu/ThioT- 77 (68%)	30 g/m^2- 18 (16%) 42 g/m^2- 92 (84%)	30 g/m^2-1776 (IQR, 1129 to 1977) 42 g/m^2- 1562 (IQR, 1140 to 1860)	-	Mucositis was associated with high Treo AUC; No associations with other clinical outcomes including survival or toxicities.	Stoep et al., 2022
3	IEI- 79 (91%) IBD- 5 (6%) JMML- 2 (2%) IEM- 1 (1%)	87	1.6 (0.2-16.7)	Treo/Flu (100%)	30 g/m^2- 4 (5%) 36 g/m^2- 23 (26%) 42 g/m^2- 60 (69%)	30 g/m^2-4,521 (4,352–4,740), 36 g/m^2- 5,204 (2,321–9,023), 42 g/m^2- 4,590 (2,880–14,647)	17.31 L/h/70 kg	A cumulative Treo AUC of 4,800 mg*h/L maximized the probability of success (> 20% engraftment & no mortality) at 82%.	Chiesa et al., 2020
4	HBP- 31(40%) HM- 12 (16%) IEI- 22 (29%) BMF- 11 (14%) Other- 1 (1%)	77	4.8 (0.2-18.3)	Treo/Flu- 25 (36%) Treo/Flu/ThioT- 52 (64%)	30 g/m^2- 12 (16%) 42 g/m^2- 65 (84%)	30 g/m^2- 1561 (511–3250) 42 g/m^2- 1744 (732-3,544)	6.98 L/h/20 kg	High IIV in Treo PK and high Treo AUC was associated with early toxicities including skin and mucositis.	Van der Stoep et al., 2017)
5	β-TM	87	9.0 (1.5-25)	Treo/Flu/ThioT- 77 (100%)	42 g/m^2- 100%	1,326 (126–4,484)	11.2 L/h/m^2	High IIC in Treo PK; no clearcut associations with HCT outcomes	Mohanan et al., 2018
6	NBL – 2; ALL – 5 ; ES - 2; BDA – 1; SCN – 1; ALD – 2; CML - 1; AML – 1; WAS - 1	16	7.5 (0.4–18)	Treo based	30 g/m^2- 1 (6%) 36 g/m^2- 8 (50%) 42 g/m^2- 7 (44%)	735 1309 ± 921 1960	23.1 13.9 ± 7.1 3.6	Linear association with Treo AUC with dose; No outcomes were evaluated.	Główka et al., 2015

Table 5.1.1 Comparison of Treo PK with previous reports

However, a more recent study by Van der Stoep et al. failed to observe any association between Treo exposure and early/late HCT outcomes in 110 pediatric patients with non-uniform malignant disorders (50% with hemoglobinopathies). Although higher Treo exposure increases the risk of skin toxicity, the authors claim that there is no benefit of Treo TDM as it is not limiting the success of HCT (Stoep et al., 2022). More recently, Sebastian et al. performed a multicenter dosing simulation PK study in 53 children with malignant and non-malignant hematological disorders and demonstrated that model-based dosing was more accurate than BSA-based conventional dosing, especially for young children. The authors chose literature-based target cumulative exposure of 4800 mg*h/L for dosing simulations that may not reflect a mixed patient cohort (Rosser et al., 2023).

We propose a therapeutic cut-off (>1660mg*hr/L) for patients with TM undergoing HCT with Treo-based conditioning. We also suggest that the proposed therapeutic cut-off should be cautiously evaluated in other diagnoses before implementation as clinical outcomes differ between the underlying diagnosis. Strikingly, we also observed that in the patients in the high Treo exposure group (>2400 mg*hr/L), there were no graft rejections, lesser incidence of RRTs (One patient developed SOS), and one death (patient died due to dengue), **Figure 5.1**. It is worth noting that patients with higher Treo exposure were not associated with regimen-related toxicities or any other transplant-related complications, as reported previously (Stoep et al., 2022, 2023). Our results indicate that TDM-based dose-escalation would benefit patients, especially in the underexposure group, minimizing graft rejection and early TRM without resulting in treatment-related toxicities.

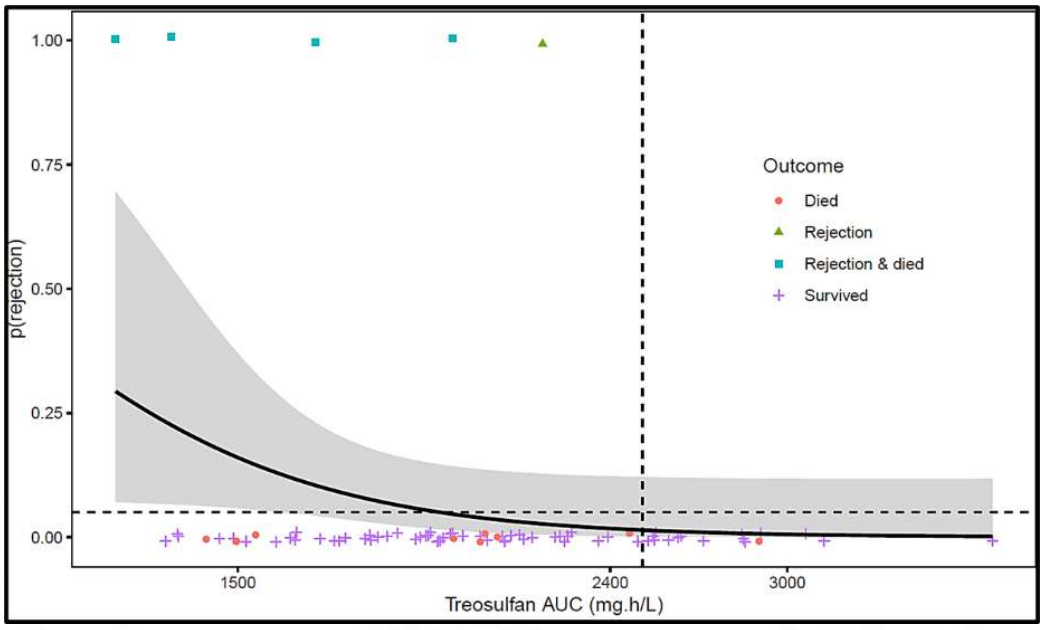


Figure 5.1.1 PD model fit of the quadratic expression describing the change in probability of success with increasing Treo Exposure

Pharmacogenetics (PG) can explain the variability in the dose-exposure-response relationship among patients to any drug and treatment outcome. Since previous studies showed (Feit et al., 1970) that Treo undergoes spontaneous non-enzymatic conversion to its metabolite, Treo PG has not been explored so far.

GSTs are a family of enzymes that catalyzes the conjugation of glutathione to specific electrophilic substances, such as carcinogens and therapeutic drugs. Bu is metabolized in the liver by GSTs, predominantly GSTA1 (Johnson et al., 2008; Kusama et al., 2006). Functional polymorphisms in the promoter region of the *GSTA1* influence enzyme activity affecting Bu PK, thereby influencing HCT outcomes. Patients with *GSTA1*B* are poor metabolizers of Bu, thereby exhibiting high system Bu exposure, leading to organ toxicities and adverse HCT outcomes (Ansari et al., 2013: 201, 2016, 2017; Kim et al., 2007).

Romanski et al. performed a kinetic analysis to examine Treo–GSH conjugation *in vitro* and showed that Treo does not undergo spontaneous or GST-mediated conjugation with GSH (Romański and Główka, 2019). We screened for GSTA1*B haplotype (comprising promoter polymorphisms – rs3957356 and rs3957357), which was shown to explain variability in busulfan PK in the present study and assessed the effect of the genotype on Treo/EBDM exposure. We observed that the patients carrying variant genotypes for *GSTA1*B* polymorphism had increased S, S-EBDM exposure. It is possible that patients with a variant genotype for *GSTA1*B* polymorphism have reduced capacity to clear/detoxify the epoxy metabolite resulting in increased S, S-EBDM exposure.

We also performed an exploratory analysis to study Treo metabolism using the DMET array. Interestingly, we identified a novel marker, 3'UTR polymorphism, in the *NQO1* gene (rs10517) associated with Treo PK. We then validated the effect of this polymorphism in the present study. Concordant with our previous findings, this polymorphism was associated with decreased Treo and S, S-EBDM exposure. It is possible that patients with variant genotypes for *NQO1* rs10517 polymorphism are good metabolizers of Treo as they exhibited increased Treo and S, S-EBDM clearance. Ours is the first study to explore the role of genetic variants in explaining the inter-patient variability in Treo/metabolite exposure.

Since phase II detoxification enzymes *GSTA1*B* and *NQO1* rs10517 polymorphisms explained Treo & S, S-EBDM PK variability, we tested if these SNPs could influence HCT outcomes. We assessed the impact of *GSTA1*B* and *NQO1* rs10517 polymorphisms on overall and thalassemia-free survival post-HCT. We observed that patients with *NQO1* and *GSTA1*B* variant genotypes had significantly inferior 1-year

OS and TFS. To validate our findings, we further evaluated the impact of these polymorphisms in a large extended retrospective cohort of 314 patients with TM. Strikingly, only the *GSTA1*B* variant genotype significantly impacted poor survival post-HCT (Pai et al., 2023). Previous studies have demonstrated that *GSTA1*B* polymorphism is a predictive biomarker for Survival and treatment-related toxicities in patients receiving Bu-containing conditioning regimens (Ansari et al., 2017; Yuan et al., 2021). GSTA1*B polymorphism may affect Treo metabolism, increasing S, S-EBDM exposure and causing early toxicity and GVHD, resulting in inferior survival. We demonstrated that *GSTA1*B* could be a plausible prognostic biomarker in HCT after Treo-based conditioning. Our results suggest that *NQO1* and *GSTA1* polymorphisms could aid in establishing pre-emptive genotype-based Treo dosing. However, further studies evaluating genotype-based dosing are warranted.

Limitations of the present study include its non-randomized nature and lack of validation cohort to confirm the proposed therapeutic cut-off. We, therefore, hypothesize that higher Treo exposure decreases the risk of graft rejection, thereby preventing early mortality. However, a prospective trial is warranted to validate the present findings. Our study suggests that lower Treo exposure predicts rejection and survival after HCT, and it is possible to make targeted dose adjustments to achieve optimum Treo exposure in patients with β-TM.

5.2. Impact of Fludarabine exposure on HCT outcomes

Several studies have reported Flu exposure on HCT outcomes in patients undergoing HCT for both malignant and benign conditions and in various combinations of Flu-based regimens (Chung et al., 2019; Ivaturi et al., 2017; Jurgen B. Langenhorst et al., 2019; Long-Boyle et al., 2011; McCune et al., 2012, 2015; Mohanan et al., 2017; Sanghavi et al., 2016; Takahashi et al., 2021). Ours is the first study to address the exposure-response relationship to Flu in a uniform cohort of patients with β-TM receiving a fixed dose of 40 mg/m^2/day of Flu.

Our study demonstrated a wide IIV in Flu exposure (27-fold). Age was the only covariate explaining Flu Clearance, probably due to decreasing renal function with increasing age. However, similar to previous reports, we did not observe any correlation between Flu Clearance and GFR or Creatinine clearance (Jurgen B. Langenhorst et al., 2019; Lichtman et al., 2002). It is to be noted that all the patients in the present study had normal renal function, and none received a reduced Flu dose. None of the other biochemical or demographic parameters examined could account for this variability. We also did not identify any genetic polymorphisms that could explain variability in Flu PK.

Although previous studies have explored the dose-exposure response relationship for drugs like Bu (Chandy et al., 2005b; Chiesa et al., 2010; Gaziev et al., 2010; Balasubramanian Poonkuzhali et al., 2001; Poonkuzhali et al., 1999), Cy (Balasubramanian et al., 2012; McCune et al., 2007), and Treo (Mohanan et al., 2018) in patients with β-TM, no such attempt was made for Flu in this population. In the present study, despite observing significant IIV in Flu PK, none of the parameters correlated with HCT outcomes. This lack of association could be attributed to the

lower incidence of events such as rejection or TRM in this cohort. A recent study (Takahashi et al., 2021) also revealed that Flu exposure alone was not strongly associated with NRM or OS. Still, increased exposure to both Flu and Cy was associated with a >16-fold higher NRM. Langenhost et al. predicted optimal cumulative exposure of 20 mg*h/L for better EFS, lower TRM, and lower rejection. However, the study cohort was heterogeneous, and the optimal exposure range was not confirmed in an independent cohort (J. B. Langenhorst et al., 2019). A recent study in a mixed cohort of patients with malignant and non-malignant conditions did not identify any relationship between Flu PK (total dose 240 mg/m^2) and HCT outcomes (Chung et al., 2019). These findings suggest no clear-cut evidence of a dose-exposure-response relationship for Flu in HCT, and the role of TDM in Flu is still unclear in a non-malignant setting.

We propose a more accurate understanding of the relationship between Flu dosage, and exposure could be achieved by comparing pharmacodynamic endpoints, such as lymphosuppression or immune recovery, in patients with β-TM receiving a TFT regimen. We also plan to explore the effects of combined Flu and Treo exposures to create a composite exposure measure on HCT outcomes.

5.3 Endothelial Activation and Stress IndeX (EASIX) – measured pre-HCT predict transplantation-related mortality in patients with β-TM

To optimize the TFT regimen, we investigated the role of a new biomarker EASIX in stratifying TRM in patients with class III β-TM undergoing HCT. Our study focused on AlloHCT recipients who received a TFT conditioning regimen. We observed a TRM rate of 13.5% in our cohort. The primary causes of death were GVHD, graft failure, RRTs, and infection. In our pursuit of improving clinical outcomes for this group, we examined the predictive value of the EASIX score calculated before transplantation in relation to TRM. Notably, we found a higher TRM rate among patients with high EASIX-PreHCT scores who underwent HCT for class III β-TM, and the associated remained significant on multivariate analysis.

These findings prompt inquiries into whether pretransplant endothelial dysfunction is linked to graft failure or GVHD and whether strategies to improve endothelial function could mitigate these complications. No significant differences were observed between the two EASIX groups in terms of other outcomes, such as RRT, GVHD, mixed chimerism rate, and overall graft rejection (primary and secondary).

Originally proposed as a predictor of survival following GVHD in reduced-intensity transplantation for malignancies in adults, the EASIX score has demonstrated predictive value in various contexts, including NRM after HCT (Luft et al., 2020; Shouval et al., 2019), SOS (Jiang et al., 2021), intensive care requirements post-transplantation (Peña et al., 2021), Survival in multiple myeloma (Song et al., 2020), fluid overload after HCT (Varma et al., 2020), toxicity following chimeric antigen receptor T cell therapy (Greenbaum et al., 2021; Peña et al., 2021), and even mortality

following COVID-19 infection (Kalicińska et al., 2021). As the parameters used to calculate the EASIX score are not specific to any particular condition, the underlying biological basis for its broad predictive utility remains partially understood.

In our routine clinical practice, we currently measure the necessary parameters to calculate the EASIX score immediately before and on the day of transplantation. Our plans involve prospective validation of this biomarker in HCT for β-TM and other predictors, such as PK data for the conditioning drugs. Upon successful prospective validation, this biomarker can potentially serve as a tool for predicting outcomes in these patients. Additionally, it can aid in selecting patients who should be assessed for therapeutic interventions such as defibrotide or statins (Jiang et al., 2021), which aim to mitigate TRM.

The limitations of this analysis include its retrospective nature and the use of different time points to measure EASIX before HCT. We did not evaluate additional endothelial markers, such as C-X-C Motif Chemokine Ligand 8 (CXCL8), Interleukin 18 (IL18), and insulin-like growth factor 1 (IGF1) levels. Additionally, these findings may not apply to other methods of LDH detection and alternative strategies for class III β-TM HCT recipients, such as those involving pre-HCT immunosuppression (Gaziev et al., 2016). Measuring EASIX before transplantation may not account for the effects of various drugs used in HCT, such as calcineurin inhibitors, sirolimus, calcium channel blockers, and angiotensin II inhibitors, which can also impact endothelial integrity.

In conclusion, EASIX shows promise as a biomarker associated with TRM in patients undergoing HCT for class III β-TM.

5.4 A pilot study to identify biomarkers of sinusoidal obstruction syndrome using global plasma metabolomic profiling

TFT-based reduced toxicity regimen has significantly improved transplant outcomes, especially in patients with class III high-risk β-TM. However, hepatic sinusoidal obstruction syndrome (SOS) is a potentially life-threatening complication after HCT, causing damage to sinusoidal endothelial cells and hepatocytes in zone 3 of the hepatic acinus. It can be triggered by various factors, including the toxic effects of the conditioning regimen, the production of cytokines as a result of inflammation and engraftment, and GVHD prophylaxis. The risk factors for developing SOS include a diagnosis of high-risk β-TM, older age, elevated ferritin levels, a previous history of liver disease, myeloablative conditioning (Bu/Cy), and the use of calcineurin inhibitors (CNIs) in GVHD prophylaxis (Mohty et al., 2023).

Treo, unlike Bu, has a moderate toxicity profile and excellent efficacy. However, in our center, hepatic SOS following TFT conditioning is around 25% in patients with β-TM. There are no reports on the mechanism of pathogenesis of SOS associated with Treo-based conditioning in high-risk TM so far. Higher exposure to Bu is reported to be associated with an increased incidence of liver SOS. Targeted dose adjustment of Bu has been shown to reduce toxicities such as SOS (Palmer et al., 2016). We performed Global metabolomics to identify dysregulated metabolites post-TFT conditioning and to identify biomarkers to predict SOS.

We identified Glycerophospholipid metabolism as the most dysregulated following the conditioning. Phosphatidic acids (PAs) are low in the cells and rapidly converted to Phosphatidyl ethanolamine (PEs) in stress conditions due to alteration of lipid

metabolism, which could be caused by conditioning in this scenario. The downregulation of L-Palmitoylcarnitine also suggests that lipid metabolism is dysregulated, affecting fatty acid metabolism and energetics. These findings indicate that TFT conditioning could affect glycerophospholipid metabolism.

Pre-conditioning Succinyl Adenosine (SA) levels were high in patients with SOS. Previously, SA was reported to be elevated in liver conditions such as HCC (Han et al., 2019). Although the mechanism remains elucidated, it could be used as a plausible prognostic biomarker to predict SOS. The precursors of Glycerophospholipids like Diacylglycerols, Phosphatidic acid, and Lysophosphatidic acid were elevated in patients with SOS, suggesting that Glycerophospholipids metabolism may be impaired even before HCT due to excessive iron load and inadequate chelation thereby increasing propensity towards developing SOS. On the other hand, larger glycerophospholipids like Phosphatidylglycerol, Phosphatidylcholine, and Phosphatidylethanolamine were downregulated, suggesting an impaired Glycerophospholipid metabolism similar to their precursors. Therefore, Glycerophospholipids and Succinyl adenosine could be potent biomarkers for predicting SOS before HCT.

Fatty Acyls, notably 13-Hydroxyoctadecadienoic acid (13-HODE); alpha-Dimorphecolic acid, Epoxyoctadecamonoenoic acids (9,10-EpOME & 12,13-EpOME) were upregulated in patients with SOS post-conditioning. Interestingly, all these metabolites are endogenously synthesized from Arachidonic and linoleic acid. We speculate that TFT conditioning disrupts lipid homeostasis, thereby increasing the production of fatty acid metabolites such as alpha-dimorphecolic acid, 9,10- EpOME, 12,13-EpOME, and 13-HODE. Alpha-dimorphecolic acid, derived from linoleic acid,

is a naturally occurring fatty acid that acts as an agonist for peroxisomal proliferator-activated receptor-gamma (PPAR-γ) in human endothelial cells, leads to an increase in the expression of plasminogen activator inhibitor type-1 (PAI-1) elevating the risk of SOS (Marx et al., 1999). It is also reported that 13-HODE is a PPAR-γ agonist, involved in mitochondrial regulation, and stimulates blood leukocytes (Umeno et al., 2020). It is also reportedly elevated in disease conditions such as atherosclerosis, asthma (Mabalirajan et al., 2013), and cancers (O'Flaherty et al., 2013; Yuan et al., 2010). Reportedly, 13-HODE was elevated in patients with nonalcoholic fatty liver disease (Maciejewska et al., 2020), alcoholic liver cirrhosis (Zhang et al., 2017), ethanol-induced liver injury (Anton et al., 2023; Warner et al., 2017; Zhang et al., 2017), steatosis (Maciejewska et al., 2015), and inflammation (Warner et al., 2017; Zhang et al., 2017). However, the role of the aforementioned metabolites in the pathogenesis of SOS remains unclear.

The limitation of this study is the small sample size. Using an untargeted metabolomics approach, we identified a set of plausible biomarkers to predict SOS both at the pre-conditioning stage and at the end of conditioning. Targeted metabolomics for validating these biomarkers is ongoing in the laboratory. Once validated, these biomarkers could help predict patients at risk of developing SOS and help design therapeutic interventions to initiate prophylactic measures for patients at increased risk of developing SOS.

5.5 Study Limitations and Future Directions

- This study is of a retrospective, observational nature.
- A prospective therapeutic drug management (TDM) trial is warranted to validate the therapeutic target exposure cut-off for Treo identified in the present study.
- Although Flu does not predict HCT outcomes, we speculate that pharmacodynamic endpoints, such as lymphosuppression or immune recovery, could be influenced by Flu exposure, which could impact HCT outcomes. Currently, our laboratory is prospectively evaluating immune recovery post-TFT conditioning.
- We did not assess the impact of ThioT exposure on HCT outcome as it was administered one day during conditioning (Day-6 HCT).
- We identified PG markers- *GSTA1*B* and *NQO1* rs10517 genetic polymorphisms. The mechanistic basis of these variants on Treo metabolism remains to be explored.
- We identified EASIX, a biochemical marker for predicting HCT outcomes. Our laboratory is also prospectively assessing this biochemical marker's utility at different HCT time points to be included as a standard of care.
- We also identified a set of predictive biomarkers for SOS from a pilot plasma metabolomic study. However, validation is essential before using it as a biomarker in the clinic. We are now performing targeted metabolomics to validate these biomarkers for routine prognosis.

6. SUMMARY AND CONCLUSIONS

AlloHCT has been used to treat patients with β-TM for over 40 years, demonstrating its capacity to cure an inherited globin disorder. Significant advancements in HCT, such as risk stratification, donor sources, conditioning regimens, and GVHD prophylaxis, have improved HCT outcomes in patients with β-TM. Therefore, AlloHCT remains the only viable curative treatment for patients with β-TM until gene therapy is proven safe. However, the success of HCT is limited by transplant-related complications such as regimen-related toxicities, infections, rejection, and GVHD, to name a few. The introduction of TFT conditioning in 2009 at our center improved HCT outcomes, especially in patients with high-risk β-TM. However, some patients (~15-20%) fail to have successful outcomes, and graft failure/rejection, mixed chimerism, regimen-related toxicities, and GVHD limit the success.

In this doctoral study, we attempted to personalize the TFT regimen by evaluating Flu and Treo PK, PG, and PD, to identify the target exposure range for Flu and Treo as well as tailoring the dose of the drugs with respect to the genotype for better HCT outcomes. We also attempted to identify biomarkers that could help prognosticate patients before HCT. Our study has identified genetic, biochemical, and metabolic biomarkers which could aid in optimizing the TFT regimen.

We evaluated the dose-exposure relationship of Flu, Treo, and its metabolite S, S-EBDM in a large, uniform cohort of patients with β-TM, possibly the largest single cohort study. We also identified a biochemical marker - EASIX, which could predict early Transplantation-Related Mortality in a uniform cohort of high-risk β-TM. We

also conducted a pilot global metabolomic study to identify plausible predictive biomarkers for SOS.

Major Findings

- A simple, rapid, cost-effective MS-based assay was established to quantify Treo and S, S-EBDM in patient plasma samples for routine TDM.
- Lower Treo systemic exposure was associated with increased graft rejection and a trend to increased mortality. Conversely, higher Treo exposure was not significantly associated with RRTs - SOS, mucositis, or TRM.
- Higher Treo to S, S-EBDM exposure ratio was associated with increased incidence of SOS.
- The current proof-of-concept study successfully demonstrated the viability of optimal Treo dosing through TDM.
- PG markers, specifically *NQO1* and *GSTA1*B,* explained the variability in Treo PK and could aid in genotype-based Treo dosing.
- Systemic exposure to Flu did not predict HCT outcomes. Although not statistically significant, higher Flu exposure was associated with decreased engraftment. We speculate that a more comprehensive understanding of the dose-exposure relationship to Flu can be attained by comparing PD endpoints, such as lymphosuppression or immune recovery.
- Patients with variant *GSTA1*B* genotype had higher TRM and inferior TFS, suggesting its utility as a prognostic biomarker for HCT with a TFT regimen.

- Higher EASIX PreHCT scores were associated with a higher rate of early TRM, indicating that EASIX may be used as a biomarker for predicting TRM in high-risk β-TM.
- A pilot metabolomic study highlighted that Glycerophospholipid metabolism was dysregulated following TFT conditioning. Notably, Phosphatidylethanolamine (PEs) was upregulated, while Phosphatidic acid (PAs) was downregulated in patients following TFT conditioning.
- We identified higher levels of Succinyl adenosine, Diacylglycerols, Phosphatidic acid, and Lysophosphatidic acid and lower levels of Phosphatidylglycerol, Phosphatidylcholine, Phosphatidylethanolamine, and Leukotriene D4 in patients who developed SOS before TFT conditioning. These could be plausible biomarkers for SOS.
- We also observed that Fatty Acyls, notably 13-HODE; alpha-Dimorphecolic acid; Epoxyoctadecenoic acid (9,10-EpOME, and 12,13-EpOME), were higher in patients with SOS postconditioning. These metabolites could help as predictive biomarkers.
- The metabolites identified in the present study could serve as predictive biomarkers for SOS if targeted metabolomics validates these findings.
- In conclusion, prognostication of β-TM patients with genetic, biochemical, and metabolomic markers followed by Treo TDM could personalize TFT conditioning for optimal HCT outcomes. A prospective trial is warranted.

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8. LIST OF PUBLICATIONS

1. Kulkarni UP*, **Pai AA***, Kavitha ML, Selvarajan S, Lionel S, Devasia AJ, Korula A, Fouzia NA, Sindhuvi E, Abraham A, Srivastava A, Mathews V, George B, Balasubramanian P. Endothelial Activation and Stress Index-Measured Pretransplantation Predicts Transplantation-Related Mortality in Patients with Thalassemia Major Undergoing Transplantation with Thiotepa, Treosulfan, and Fludarabine Conditioning. Transplant Cell Ther. 2022 Jul;28(7):356.e1-356.e6. doi: 10.1016/j.jtct.2022.05.001. Epub 2022 May 9. PMID: 35550442. **(*- Equal contribution)**
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APPENDICES

A1 – Complete List of Dysregulated Metabolites post TFT conditioning in patients with β-TM

mz_RT	FC	log2(FC)	raw. pvalue	Log (p-value)	Compound (s)	HMDB ID
597.24__652.34	1.8631	0.89772	0.024375	1.6131	Tetragastrin;	HMDB0005775;
717.12__716.14	1.3927	0.47784	0.004711	2.3268	Theaflavin-3-gallate; Isoneotheaflavin 3-O-gallate; Isotheaflavin 3'-gallate; Neotheaflavin 3-gallate;	HMDB0005786; HMDB0029254; HMDB0032904; HMDB0032905;
635.16__810.08	1.3922	0.47733	0.002315	2.6355	3'-(2",3"-Digalloylglucosyl)-phloroacetophenone; 3'-(2",6"-Digalloylglucosyl)-phloroacetophenone;	HMDB0040624; HMDB0040625;
540.24__642.51	1.3431	0.42556	0.035157	1.454	5,9,11-trihydroxyprosta-6E,14Z-dien-1-oate;	HMDB0062413;
679.08__815.66	1.3202	0.40071	0.024667	1.6079	Norbadione A;	HMDB0034350;
796.44__1176.62	1.3016	0.38026	0.032822	1.4838	PE(DiMe(11,3)/DiMe(9,3)); PE(DiMe(9,3)/DiMe(11,3)); PE(DiMe(9,3)/DiMe(9,5)); PE(DiMe(9,5)/DiMe(9,3)); PE(MonoMe(11,3)/MonoMe(11,3));	HMDB0061474; HMDB0061498; HMDB0061502; HMDB0061510; HMDB0061521;
455.04__1045.29	0.76698	-0.38274	0.040028	1.3976	Cefazolin;	HMDB0015422;
715.2__1221.39	0.75898	-0.39787	0.007685	2.1143	Scleroglucan;	HMDB0029948;
487.32__1145.68	0.7582	-0.39935	0.004674	2.3303	Hovenine A; Bassic acid; Glabric acid; 28-	HMDB0030200;

					Hydroxyglycyrrhetic acid; 24-Hydroxyglycyrrhetic acid;	HMDB0034526; HMDB0034689; HMDB0035259; HMDB0035261;
400.32__1237.4	0.72405	-0.46583	0.000454	3.3428	L-Palmitoylcarnitine; 3-Epidemissidine;	HMDB0000222; HMDB0032023;
349.32__914.1	0.71093	-0.49223	0.018358	1.7362	5-Heptadecyl-1,3-benzenediol;	HMDB0038530;
645.48__923.66	0.6888	-0.53785	0.000458	3.339	PA(14:0/18:2(9Z,12Z)); PA(18:1(11Z)/14:1(9Z)); PA(18:1(9Z)/14:1(9Z)); PA(18:2(9Z,12Z)/14:0); DG(9D3/9D3/0:0);	HMDB0114779; HMDB0114897; HMDB0114922; HMDB0114946; HMDB0116428;
504.12__1111.79	0.68056	-0.55521	0.026714	1.5733	Pyranodelphinin B;	HMDB0035419;
455.4__1062.04	0.57169	-0.80668	0.000531	3.2747	(25S)-26-Hydroxy-24-methylenecycloartan-3-one; Nb-Arachidoyltryptamine;	HMDB0034555; HMDB0040817;

A2 – Complete List of Dysregulated Metabolites before TFT conditioning in patients with β-TM who developed SOS

mz_RT	**FC**	**log2(FC)**	**raw.pval**	**logP**	**Compound**	**HMDB ID**
102.12__1185.69	0.73171	-0.45067	3.28E-06	5.4837	Hexylamine; Triethylamine;	HMDB0032323; HMDB0032539;
401.16__1193.59	1.4043	0.48984	3.09E-05	4.5096	Zuclopenthixol; Hemiariensin; Isoyatein; 3b-Hydroxy-6b-(3-chloro-2-hydroxy-2-methylbutanoyloxy)-7(11)-eremophilen-12,8b-	HMDB0015561; HMDB0029869; HMDB0033258; HMDB0041278;

					olide; Italipyrone;	HMDB0041307;
402.24__1194.01	1.3771	0.4616	3.82E-05	4.4183	Buspirone N-oxide; 3'-Hydroxybuspirone; 5-Hydroxybuspirone; 6'-Hydroxybuspirone;	HMDB0061107; HMDB0061108; HMDB0061109; HMDB0061110;
395.4__1193.76	1.5318	0.61522	5.26E-05	4.2787	Ximenic acid; 11,13-Hexacosanedione; 10,12-Hexacosanedione; 7,9-Hexacosanedione; 6,8-Hexacosanedione;	HMDB0035215; HMDB0035549; HMDB0035550; HMDB0035551; HMDB0035552;
945.24__1255.13	1.8041	0.85124	8.86E-05	4.0525	Apigenin 4'-[p-coumaroyl-(->2)-glucuronyl-(1->2)-glucuronide] 7-glucuronide; Apigenin 7-[p-coumaroyl-(->2)-[glucuronyl-(1->3)]-glucuronyl-(1->2)-glucuronide];	HMDB0038296; HMDB0038297;
562.08__1182.12	1.4249	0.5109	9.61E-05	4.0174	Vitisin A;	HMDB0036348;
706.56__1177.09	0.55573	-0.84753	0.00017915	3.7468	PC(14:0/16:0); PC(15:0/15:0); PC(16:0/14:0); PE(15:0/18:0); PE(18:0/15:0);	HMDB0007869; HMDB0007934; HMDB0007965; HMDB0008892; HMDB0008988;
614.76__671.22	0.44592	-1.1652	0.00027281	3.5641	Diatrizoate;	HMDB0014416;
487.32__1120.17	1.6918	0.75855	0.00028382	3.547	Hovenine A; Bassic acid; Glabric acid; 28-Hydroxyglycyrrhetic acid; 24-Hydroxyglycyrrhetic acid;	HMDB0030200; HMDB0034526; HMDB0034689; HMDB0035259; HMDB0035261;

797.52__1170.22	0.63584	-0.65326	0.00031743	3.4984	PG(16:0/22:5(4Z,7Z,10Z,13Z,16Z)); PG(16:0/22:5(7Z,10Z,13Z,16Z,19Z)); PG(16:1(9Z)/22:4(7Z,10Z,13Z,16Z)); PG(18:1(11Z)/20:4(5Z,8Z,11Z,14Z)); PG(18:1(9Z)/20:4(5Z,8Z,11Z,14Z));	HMDB0010582; HMDB0010583; HMDB0010596; HMDB0010625; HMDB0010640;
411.24__1198.88	1.3703	0.45447	0.00056131	3.2508	Risperidone; LysoPA(0:0/16:0); LysoPA(16:0/0:0);	HMDB0005020; HMDB0007849; HMDB0007853;
411.36__1000.63	1.7296	0.79046	0.00063648	3.1962	4,4-Dimethylcholesta-8,14,24-trienol; 5-Dehydroavenasterol; delta8,14-Sterol; MG(0:0/22:2(13Z,16Z)/0:0); MG(22:2(13Z,16Z)/0:0/0:0);	HMDB0001023; HMDB0006852; HMDB0006928; HMDB0011553; HMDB0011583;
393.24__1193.76	1.4525	0.53852	0.00076695	3.1152	CPA(16:0/0:0); Trospium; Methyl (9Z)-6'-oxo-6,5'-diapo-6-carotenoate; Dihydrofukinolide; 10-Hydroperoxy-H4-neuroprostane;	HMDB0007003; HMDB0014354; HMDB0031977; HMDB0034662; HMDB0062274;
431.04__531.1	0.48063	-1.057	0.00089685	3.0473	Bicalutamide; CMP-2-aminoethylphosphonate;	HMDB0015260; HMDB0060067;
591.48__1278.85	1.8092	0.85534	0.0012285	2.9106	DG(14:0/20:3(5Z,8Z,11Z)/0:0); DG(14:0/20:3(8Z,11Z,14Z)/0:0); DG(14:1(9Z)/20:2(11Z,14Z)/0:0); DG(16:0/18:3(6Z,9Z,12Z)/0:0); DG(16:0/18:3(9Z,12Z,15Z)/0:0);	HMDB0007023; HMDB0007024; HMDB0007051; HMDB0007104; HMDB0007105;
581.16__561.65	0.50529	-0.9848	0.0012307	2.9098	Naringin; Lapatinib; 5'-Methoxycastavinol;	HMDB0002927; HMDB0015388;

					Albafuran C; Isocarlinoside;	HMDB0029814; HMDB0030073; HMDB0030721;
667.08__1259.31	0.41014	-1.2858	0.0013229	2.8785	6-{6-carboxy-2-[3,4-dihydroxy-5-(3,4,5-trihydroxybenzoyloxy)benzoyloxy]-3,4-dihydroxyphenoxy}-3,4,5-trihydroxyoxane-2-carboxylic acid; 6-[5-({5-[(3-carboxy-2,5,6-trihydroxyphenoxy)carbonyl]-2,3-dihydroxyphenoxy}carbonyl)-2,3-dihydroxyphenoxy]-3,4,5-trihydroxyoxane-2-carboxylic acid; 6-[4-({5-[(3-carboxy-2,5,6-trihydroxyphenoxy)carbonyl]-2,3-dihydroxyphenoxy}carbonyl)-2,6-dihydroxyphenoxy]-3,4,5-trihydroxyoxane-2-carboxylic acid; 6-{4-[(3-carboxy-2,5,6-trihydroxyphenoxy)carbonyl]-2-hydroxy-6-(3,4,5-trihydroxybenzoyloxy)phenoxy}-3,4,5-trihydroxyoxane-2-carboxylic acid; 6-{5-[(3-carboxy-2,5,6-trihydroxyphenoxy)carbonyl]-2-hydroxy-3-(3,4,5-trihydroxybenzoyloxy)phenoxy}-3,4,5-trihydroxyoxane-2-carboxylic acid;	HMDB0128335; HMDB0128336; HMDB0128337; HMDB0128338; HMDB0128339;
334.32__1030.81	1.8105	0.85636	0.0019603	2.7077	2,4,12-Octadecatrienoic acid isobutylamide;	HMDB0032033;
411__1201.99	1.3284	0.40973	0.0026415	2.5782	Butoconazole; (Acetyloxy)triphenylstannane;	HMDB0014777; HMDB0031789;
415.08__1179.47	1.3781	0.46266	0.0028693	2.5422	(7R)-7-(5-Carboxy-5-oxopentanoyl)aminocephalosporinate; [2-(methoxymethyl)-5-(3,5,6,7-tetrahydroxy-3,4-dihydro-2H-1-benzopyran-2-yl)phenyl]oxidanesulfonic acid; [2-hydroxy-3-	HMDB0060316; HMDB0127859; HMDB0127863; HMDB0127864; HMDB0128725;

					(methoxymethyl)-6-(3,5,7-trihydroxy-3,4-dihydro-2H-1-benzopyran-2-yl)phenyl]oxidanesulfonic acid; [2-hydroxy-6-(methoxymethyl)-3-(3,5,7-trihydroxy-3,4-dihydro-2H-1-benzopyran-2-yl)phenyl]oxidanesulfonic acid; 6-{[6-(2-carboxyeth-1-en-1-yl)-4-methoxy-2H-1,3-benzodioxol-5-yl]oxy}-3,4,5-trihydroxyoxane-2-carboxylic acid;	
483__1094.96	1.3646	0.44853	0.004273	2.3693	Thymidine 5'-triphosphate; Lentinic acid;	HMDB0001342; HMDB0038385;
336.36__1002.94	2.2105	1.1444	0.0047227	2.3258	Pipericine;	HMDB0031678;
689.4__900.65	1.5072	0.59183	0.0051385	2.2892	PA(18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z));	HMDB0115038;
384.12__1057.53	2.3926	1.2586	0.0056523	2.2478	Succinyladenosine;	HMDB0000912;
563.52__1279.02	1.3623	0.44609	0.0056781	2.2458	DG(14:0/18:3(6Z,9Z,12Z)/0:0); DG(14:0/18:3(9Z,12Z,15Z)/0:0); DG(14:1(9Z)/18:2(9Z,12Z)/0:0); DG(18:2(9Z,12Z)/14:1(9Z)/0:0); DG(18:3(6Z,9Z,12Z)/14:0/0:0);	HMDB0007017; HMDB0007018; HMDB0007045; HMDB0007241; HMDB0007269;
497.28__599.39	0.55687	-0.8446	0.0059918	2.2224	Leukotriene D4; APGPR Enterostatin; Absinthin; Anabsinthin;	HMDB0003080; HMDB0006117; HMDB0035742; HMDB0036415;
430.2__545.21	0.56749	-0.81733	0.007011	2.1542	Abiraterone sulfate;	HMDB0060584;

705.12__1179.89	0.70344	-0.5075	0.0070781	2.1501	(5-{5,7-dihydroxy-4-oxo-3,8-bis[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-4H-chromen-2-yl}-2-methoxyphenyl)oxidanesulfonic acid; ({6-[5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-4H-chromen-8-yl]-3,4,5-trihydroxyoxan-2-yl}methoxy)sulfonic acid; ({6-[5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-8-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-4H-chromen-3-yl]-3,4,5-trihydroxyoxan-2-yl}methoxy)sulfonic acid; {2-[5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-4H-chromen-8-yl]-4,5-dihydroxy-6-(hydroxymethyl)oxan-3-yl}oxidanesulfonic acid; {2-[5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-4H-chromen-8-yl]-3,5-dihydroxy-6-(hydroxymethyl)oxan-4-yl}oxidanesulfonic acid;	HMDB0126925; HMDB0126926; HMDB0126927; HMDB0126928; HMDB0126929;
409.2__556.34	0.65237	-0.61623	0.0075293	2.1232	Tamsulosin; 3-Hydroxyglabrol; (E)-2',4,4',6'-Tetrahydroxy-3',5'-diprenylchalcone; 3'-Geranyl-2',4,4',6'-tetrahydroxychalcone; 1-(2,4-Dihydroxyphenyl)-3-[8-hydroxy-2-methyl-2-(4-methyl-3-pentenyl)-2H-1-benzopyran-5-yl]-1-propanone;	HMDB0014844; HMDB0029532; HMDB0031235; HMDB0031236; HMDB0031922;
444.12__1152.57	1.4213	0.50721	0.0082641	2.0828	3'-Azido-3'-deoxy-5'- O-beta-D-glucopyranuronosylthymidine; N-debutylhalofantrine;	HMDB0060752; HMDB0061032;

455.16__1051.89	1.4776	0.56329	0.010355	1.9848	Methotrexate; epsilon-Viniferin; trans-delta-Viniferin; KB 2; Ampelopsin D;	HMDB0014703; HMDB0030604; HMDB0032657; HMDB0033666; HMDB0040894;
638.52__1060.08	0.68324	-0.54953	0.010978	1.9595	Hexabromodiphenyl ethers; 2,2',4,4',5,5'-Hexabromodiphenyl ether; 2,2',4,4',5,6'-Hexabromodiphenyl ether;	HMDB0037515; HMDB0037525; HMDB0037526;
559.2__1189.61	1.3625	0.44628	0.01159	1.9359	Physalin K; Physalin I; Ustiloxin C;	HMDB0034347; HMDB0034402; HMDB0041053;
384.12__1194.15	1.3398	0.42205	0.012108	1.9169	Succinyladenosine;	HMDB0000912;
585__637.68	0.52316	-0.93469	0.012571	1.9006	Cefodizime;	HMDB0041850;
745.44__1040.32	0.61988	-0.68993	0.012945	1.8879	PG(16:0/18:3(6Z,9Z,12Z)); PG(16:0/18:3(9Z,12Z,15Z)); PG(16:1(9Z)/18:2(9Z,12Z)); PG(18:2(9Z,12Z)/16:1(9Z)); PG(18:3(6Z,9Z,12Z)/16:0);	HMDB0010576; HMDB0010577; HMDB0010590; HMDB0010646; HMDB0010660;
541.32__622.74	0.68848	-0.53851	0.013081	1.8834	Ciclesonide;	HMDB0015480;
853.08__1027.18	1.3409	0.42323	0.014024	1.8531	Guanosine tetraphosphate adenosine;	HMDB0001454;
443.4__1149.08	1.6312	0.70594	0.014348	1.8432	Erythrodiol; Uvaol; MG(0:0/24:0/0:0); MG(24:0/0:0/0:0); 4,4-Dimethyl-14alpha-formyl-5alpha-cholesta-8-en-3beta-ol;	HMDB0002360; HMDB0002391; HMDB0011558; HMDB0011588;

						HMDB0012159;
466.2__656.12	0.67054	-0.57661	0.016299	1.7878	Biotripyrrin-a; Biotripyrrin-b; Cisapride; (E)-Squamosamide;	HMDB0003323; HMDB0003324; HMDB0014742; HMDB0041088;
452.46__585.64	0.54579	-0.87357	0.016472	1.7832	Longamide;	HMDB0038842;
382.08__1194.15	1.5921	0.67091	0.03657	1.4369	Celecoxib; dehydrofelodipine;	HMDB0005014; HMDB0061029;
485.28__1183.35	1.5036	0.58841	0.037216	1.4293	LysoPA(22:5(4Z,7Z,10Z,13Z,16Z)/0:0); LysoPA(22:5(7Z,10Z,13Z,16Z,19Z)/0:0); LysoPG(16:0/0:0);	HMDB0114753; HMDB0114754; HMDB0240601;
334.32__1280.99	1.3454	0.42807	0.039773	1.4004	2,4,12-Octadecatrienoic acid isobutylamide;	HMDB0032033;
695.52__1179.07	0.72424	-0.46547	0.042167	1.375	PG(a-13:0/a-17:0); PG(a-13:0/i-17:0); PG(i-12:0/i-18:0); PG(i-13:0/a-17:0); PG(i-13:0/i-17:0);	HMDB0116639; HMDB0116647; HMDB0116666; HMDB0116675; HMDB0116683;
892.2__755.51	1.4036	0.48911	0.046119	1.3361	S-2-Octenoyl CoA; (2E)-Octenoyl-CoA; 6-Hydroxycyclohex-1-ene-1-carboxyl-CoA; 5-Methyl-3-oxo-4-hexenoyl-CoA; 2-ene-Valproic acid CoA;	HMDB0002992; HMDB0003949; HMDB0012179; HMDB0060399; HMDB0060714;
525.12__1167.55	0.70907	-0.496	0.049965	1.3013	Sennidin C; Rheidin A; 6-{3,5-dihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-oxopropanoyl]-6-methoxyphenoxy}-3,4,5-trihydroxyoxane-2-	HMDB0034318; HMDB0038507; HMDB0128783;

					carboxylic acid; 6-{3,5-dihydroxy-4-[3-(4-hydroxy-3-methoxyphenyl)-2-oxopropanoyl]-2-methoxyphenoxy}-3,4,5-trihydroxyoxane-2-carboxylic acid; 6-{3,5-dihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-oxopropanoyl]-4-methoxyphenoxy}-3,4,5-trihydroxyoxane-2-carboxylic acid;	HMDB0128784; HMDB0128785;

A3 – Complete List of Dysregulated Metabolites post TFT conditioning in patients with β-TM who developed SOS

m/z__RT	FC	log2(FC)	raw.pval	logP	Compound	HMDB ID
770.88__1213.71	2.1819	1.1256	0.00041097	3.3862	13-HODE; alpha-Dimorphecolic acid; 9,10-Epoxyoctadecenoic acid; 12,13-EpOME; (Z)-13-Oxo-9-octadecenoic acid;	HMDB0004667; HMDB0004670; HMDB0004701; HMDB0004702; HMDB0029796;
797.04__1177.61	1.7343	0.79437	0.000024	4.6195	(R)-3',7-Dihydroxy-2',4'-dimethoxyisoflavan; Isomucronulatol; 2',7-Dihydroxy-4',6-dimethoxyisoflavan; (Â±)-Sphaerosin; 3'-Hydroxy-3,4,5,4'-tetramethoxystilbene;	HMDB0030717; HMDB0033189; HMDB0033996; HMDB0038128; HMDB0041653;
797.64__1171.61	1.5362	0.61935	0.0010055	2.9976	p-HPEA-EDA; 2'-Deoxymugineic acid; 2-(1,2,3,4-Tetrahydroxybutyl)-6-(2,3,4-trihydroxybutyl)pyrazine; Matricarin; 3'-Deoxyoleacein;	HMDB0029305; HMDB0033909; HMDB0034894; HMDB0035790; HMDB0037494;

321.36__894.77	1.4845	0.56997	0.015482	1.8102	Hexylamine; Triethylamine;	HMDB0032323; HMDB0032539;
607.32__1080.23	1.4706	0.5564	0.0023191	2.6347	Pseudoecgonine; Ecgonine; 2-Hepteneoylglycine; 3-Hepteneoylglycine; 4-Hepteneoylglycine;	HMDB0006348; HMDB0006548; HMDB0094728; HMDB0094729; HMDB0094730;
318.36__1113.73	1.4553	0.54135	0.019104	1.7189	2,3-Dihydro-2-methylthiophene; 2,3-Dihydro-5-methylthiophene;	HMDB0033564; HMDB0033565;
609.24__1072.5	1.4109	0.49661	0.0014418	2.8411	Dodecanol; 2-Butyl-1-octanol;	HMDB0011626; HMDB0041288;
814.92__1273.56	1.386	0.47096	0.024234	1.6156	1-docosene;	HMDB0062602;
445.44__1152.14	0.75925	-0.39735	0.012092	1.9175	Hypoxanthine; Erythronic acid; Threonic acid; Allopurinol; 1-Pentanesulfenothioic acid;	HMDB0000157; HMDB0000613; HMDB0000943; HMDB0014581; HMDB0031160;
391.68__522.45	0.65499	-0.61047	0.027631	1.5586	Trimethylaminoacetone;	HMDB0012296;
668.04__1180.81	0.6446	-0.63353	0.019063	1.7198	Myristic acid; 2,6,10-Trimethylundecanoic acid; 12-Methyltridecanoic acid; Hexanal octane-1,3-diol acetal; Undecanal propyleneglycol acetal;	HMDB0000806; HMDB0002221; HMDB0031072; HMDB0032318; HMDB0032548;

351.24__529.18	0.64435	-0.63408	0.018497	1.7329	S-Methyl methanesulfinothioate;	HMDB0032739;
677.88__806.93	0.61183	-0.7088	0.026136	1.5828	Thorium; 4-Hydroxy-8-methoxy-2H-furo[2,3-h]-1-benzopyran-2-one; Cyclobrassinone; 9-Hydroxy-4-methoxypsoralen; 4,7-Dihydro-5-(4-methyl-3-pentenyl)-1,2,3-trithiepin;	HMDB0029215; HMDB0032659; HMDB0034209; HMDB0036628; HMDB0038182;
678.84__808.07	0.5724	-0.80491	0.010996	1.9588	2,6-Di-tert-butyl-4-ethylphenol;	HMDB0040179;
674.16__680.13	0.57131	-0.80765	0.046568	1.3319	Dodecanal dimethyl acetal; 1,1-Dihexyloxyethane;	HMDB0032246; HMDB0038678;
679.08__799.68	0.57071	-0.80917	0.0018208	2.7397	N,N-Didesmethyltramadol; N,O-Didesmethyltramadol; N,N,O-Tridesmethylvenlafaxine;	HMDB0060849; HMDB0060851; HMDB0061345;
678.36__807.16	0.53865	-0.89258	0.0088373	2.0537	Cysteinylhydroxyproline; Hydroxyprolyl-Cysteine; 1-Isopropyl citrate; (2Z,4'Z)-2-(5-Methylthio-4-penten-2-ynylidene)-1,6-dioxaspiro[4.4]non-3-ene; 2-Isopropyl citrate;	HMDB0028776; HMDB0028860; HMDB0032438; HMDB0032660; HMDB0038083;
666.96__1255.45	0.51378	-0.96077	0.033639	1.4732	6-Propyltridecane; 5-Propyltridecane; Hexadecane; 4-Methylpentadecane;	HMDB0030284; HMDB0030297; HMDB0033792; HMDB0061855;

*m/z- Mass-to-charge ratio, FC- Fold change, HMDB- Human Metabolome Database

PATIENT INFORMATION SHEET

Date :

DEPARTMENT OF HAEMATOLOGY, CHRISTIAN MEDICAL COLLEGE, VELLORE, 632004

TITLE OF RESEARCH PROJECT

Personalizing conditioning regimen in hematopoietic stem cell transplantation

Principal Investigator:
Dr. Poonkuzhali Balasubramanian, Professor, Dept. of Haematology, Christian Medical College, Vellore

Contact Details:

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This study will be carried out in the Department of Haematology, Christian Medical College, Vellore

Purpose of the Research

Hematopoietic stem cell transplantation (HSCT) is the only curative treatment of choice for several blood disorders. However, its usability remains limited by the relatively high risk of serious complications. The level of the drugs can influence the outcome of the transplantation. The individual's genetics may also play a role in determining the concentration of drugs and the patient's response to the treatment. The relationship between the genetic variants, drug levels and treatment outcome is not fully understood. Hence this study would help us to personalize the drugs used in conditioning regimen in HSCT by assessing the relationship for all these drugs with their exposure and response, and by evaluating the role of genetic variants in genes that metabolizes the drugs.

Description of the Research

The current study is designed to evaluate dose, exposure levels and treatment outcome for the drugs used in conditioning regimen prior to HSCT. Genetic associations if any, contributing to their outcomes will also be studied. All patients scheduled to undergo HSCT receiving fludarabine, treosulfan as a part of their conditioning regimen will be included. Peripheral blood sample (2-3mL) will be collected at scheduled time points during after the drug therapy, stored at -80^{o}C for measuring the levels of the drugs. Additionally, 9mL of peripheral blood will be drawn at one time only, to isolate the genetic material (DNA). Simultaneous experiments will be done in the lab using DNA and blood samples.

Study Procedure

Two-3mL blood samples will be collected at pre-dose and at scheduled time points (just before (0hr hour) and at the end of infusion and 2, 4, and 24 hours after Flu/Treo infusion) for two days for measuring drug levels in blood. Nine ml of peripheral blood will be collected during the pre-transplant workup for genetic analysis. No additional blood samples will be collected for study purposes during the entire period of the study.

Potential Harms

None

Potential Discomforts or Inconvenience

Peripheral blood will be taken before and after the doses at scheduled time points. The blood samples will be collected from indwelling venous catheter and hence there is no additional discomfort to the patient.

Potential Benefits

You will not be directly benefited from taking part in this research study. However extended research carried out in the samples collected from you may bring beneficial information which could be used in the future for improving treatment outcome.

Confidentiality

We respect your privacy. Information about you collected for this research study will be stored in the Investigator's research files and will be identified only by a number. No information about you will be given to anyone or be published without your permission, unless required by law.

The people involved in the study may see your health record to check on the study. By signing this consent form, you agree to let these people look at your record. We will put a copy of this research consent form in your patient health record and give you a copy as well.

The data produced from this study will be stored in a secure, locked location. Only members of the research team will have access to the data. This could include external research team members also. Following completion of research study, the data will be kept as long as required and then destroyed as required by the Institution. Published results will not reveal your identity.

Participation

Taking part in this research study is voluntary. You can decide not to take part. You can stop at any time. If you do not take part or if you decide to dropout, there is no penalty nor will you lose any benefits that you are otherwise entitled to. You will not be paid and the investigators will share no commercial benefits.

INFORMED CONSENT FORM TO PARTICIPATE IN A RESEARCH STUDY

DEPARTMENT OF HAEMATOLOGY,
CHRISTIAN MEDICAL COLLEGE, VELLORE, 632004

Study Title:Personalizing conditioning regimen in hematopoietic stem cell transplantation

Study Number :

Subject's Initials :_____

Subject's Name :_____

Date of Birth / Age :_____

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the Sponsor of the study, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s) [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative :_____
Date : _____/_____/_____
Signatory's Name : _____

Signature of the Investigator : _____
Date : _____/_____/_____
Study Investigator's Name : _____

Signature of the Witness : _____
Date :_____/_____/_____
Name of the Witness : _____

ASSENT FORM
Department of Haematology& Department of Clinical Pathology
Christian Medical College
Vellore – 632 004

Title of the Study: Personalizing conditioning regimen in hematopoietic stem cell transplantation

Why are we doing this study?

Hematopoietic stem cell transplantation (HSCT) is the only available option of cure for patients with various hematologic disorders. But the success of this procedure is challenged by some complications including some drug related side effects. So it is necessary to understand what causes one person to do well after therapy but not others; once we identify these factors, we can chose the correct drug for each patient without causing much complications during after transplantation. In this study we are planning to evaluate these factors which will help to personalize the drug type, combination, and dosage in HSCT.

What will happen during the study?

Those who are diagnosed with hematological disorders and scheduled to undergo stem cell transplant and receiving any of the following drug therapy such as Fludarabine, Treosulfan as a part of their conditioning regimen prior to HSCT will be included in the study. Peripheral blood samples will be collected from you at scheduled (7) time points. The liquid part of the blood called plasma and white blood cells will be stored in the laboratory for analysis later.

Are there good things and bad things about the study?

The study might help in exploring the association of drug level and outcome of the transplant thereby predicting a model to choose the right conditioning regimen.

There are no known harms to participating in this study. The discomfort to you would be the pain while taking blood. In case of problems, there will be a doctor to supervise you.

Who will know about what I did in the study?

If you are a part of this study, your name and address will not be given to anyone without your consent unless required by law if we feel your health may be in danger, we may have to report your results to your doctor

Can I decide if I want to be in the study?

Nobody will be angry or upset with you if you do not want to be in this study. We are talking to your parents/ legal guardians about the study, and you should talk to them about it too.

Assent:

I was present when _____ read this form and gave my verbal assent.

Name of the patient	Signature	Date

Name of the person who obtained consent	Signature	Date

SAMPLE COLLECTION PROTOCOL FOR I.V FLUDARABINE/TREOSULFAN PHARMACOKINETICS

DEPARTMENT OF HAEMATOLOGY

(Flu/Treosulfan/Thiotepa Regimen)

Name:	Hospital No:	Age:
Sex:	Height:	Weight:
BSA:	Diagnosis:	
Treosulfan actual dose:	Inj. Treosulfan:	

Fludarabine actual Dose: Inj.Fludarabine:

* Blood sampling:

Time of Infusion start:

Time of Infusion end:

Fludarabine to be infused as a 1hr infusion for the full dose

Treosulfan to be infused as a 5g/hr infusion

*3ml of blood to be collected in ice cold **Heparinized tubes** (Green Top).*

Actual Time taken

Day 1Flu **Day1Treo**

- 1) Pre before infusion
- 2) At the end of infusion (EOI)
- 3) 2 hr post EOI
- 4) 4 hr post EOI
- 5) 24 hr post EOI

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ASTCT American Society for Transplantation and Cellular Therapy

Endothelial Activation and Stress Index-Measured Pretransplantation Predicts Transplantation-Related Mortality in Patients with Thalassemia Major Undergoing Transplantation with Thiotepa, Treosulfan, and Fludarabine Conditioning

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A B S T R A C T

The use of thiotepa-treosulfan-fludarabine conditioning regimen and peripheral blood stem cell grafts is associated with improved outcomes of hematopoietic stem cell transplantation (HCT) in patients with high-risk thalassemia major. However, there remains a need to identify predictors of poor outcomes in this cohort to further optimize outcomes. The Endothelial Activation and Stress Index (EASIX) is a biomarker shown to predict survival in various settings, including graft-versus-host disease, veno-occlusive disease, and nonrelapse mortality following allogeneic HCT. In this retrospective analysis, we evaluated the role of EASIX-PreTx (measured before conditioning therapy) as a biomarker in predicting day +100 transplantation-related mortality (TRM+100) in 281 patients with thalassemia major who underwent HCT with a uniform conditioning regimen using thiotepa-treosulfan-fludarabine at our center between January 2012 and December 2019. The median patient age was 9 years (range, 1 to 25 years), and 109 (38.8%) were females. According to the Pesaro classification (with Vellore modification), 3 patients (1.1%) were class I, 34 (12.1%) were class II, 134 (47.7%) were class III low risk, and 110 (39.1%) were class III high risk. Stem cell donors were matched sibling (n = 218; 77.6%), matched related nonsibling (n = 23; 8.2%), or matched unrelated (n = 40; 14.2%). Five patients (1.8%) received a bone marrow graft, and the others received a peripheral blood stem cell graft. Thirty-eight patients (13.5%) had TRM+100. EASIX-PreTx was available for 184 patients (65.5%). The median EASIX-PreTx was significantly higher in patients with TRM+100 compared with those without TRM+100 (1.09 versus .75; $P = .008$). An EASIX-PreTx cutoff of .85 had 70.4% sensitivity and 62% specificity for predicting TRM+100. The TRM+100 for patients with EASIX-PreTx $>.85$ was significantly higher than those with EASIX $<.85$ (24.4% versus 7.5%; $P = .003$). In a uniform subgroup of class III patients undergoing allogeneic HCT (n = 156), EASIX-PreTx was an independent predictor of TRM+100.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HCT) is a curative treatment for beta-thalassemia major [1]. With the goal of predicting transplantation-related mortality (TRM) and graft rejection after myeloablative conditioning with busulfan and cyclophosphamide, the Pesaro risk stratification scheme classifies patients with thalassemia into 3 groups (class I, class II, and class III) based on liver size (>2 cm), the presence of liver fibrosis, and inadequate chelation [2]. However, this stratification has not been validated in all regions. In developing countries, in which inadequate chelation therapy prior to transplantation is common, most patients fall into the Pesaro class III category. Given the heterogeneity in clinical outcomes in this group, a further refinement in this risk stratification was introduced at our center in which a subset of "class III high-risk" patients was identified as those age ≥ 7 years and with liver size ≥ 5 cm [3]. Subsequently, we showed that use of a conditioning regimen containing thiotepa, treosulfan, and fludarabine and a peripheral blood stem cell graft led to improved outcomes in this subset, with a reduction in early TRM from 46% (with busulfan-cyclophosphamide

Financial disclosure: See Acknowledgments on page XXX.

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2666-6367/

conditioning) to 13% [4]. However, there is a need to identify predictors of poor clinical outcomes to further optimize the clinical outcomes of thalassemia class III patients.

The Endothelial Activation and Stress Index (EASIX) is a simple biomarker calculated using lactate dehydrogenase (LDH), creatinine, and platelet counts. When calculated at the onset of graft-versus-host disease (GVHD) post-transplantation, EASIX-GVHD was found to be predictive of overall survival (OS) and has been validated in other transplantation cohorts [5], primarily in the context of reduced-intensity conditioning HCT for malignancies in adults. Subsequently, EASIX calculated before conditioning was found to be a predictor of individual risk of nonrelapse mortality (NRM) independent of other established criteria and has been validated in different transplantation cohorts [6,7]. The applicability of EASIX appears to be limited in pediatric cohorts, however, owing primarily to the correlation of preconditioning EASIX with age [6]. The same group also identified EASIX measured on day 0 as a validated biomarker for defining a subpopulation of allogeneic HCT recipients with hematologic malignancies at high risk for veno-occlusive disease (VOD) [8]. Consequently, in the present study, we evaluated the usefulness of EASIX as a biomarker for predicting early TRM in patients with thalassemia major who underwent HCT with a uniform conditioning regimen of thiotepa-treosulfan-fludarabine at our center.

METHODS

Patients

On Institutional Review Board approval, we conducted a retrospective analysis including all patients with thalassemia major who underwent allogeneic HCT at our center between January 2012 and December 2019 using a uniform thiotepa-treosulfan-fludarabine conditioning regimen. Donors were HLA-identical or 1 antigen-mismatched sibling/family or unrelated donors.

Baseline demographic information, clinical and laboratory data, and details of transplantation, including donor characteristics, graft characteristics, and clinical outcomes, including graft rejection, TRM, GVHD, and death, were retrieved from the hospital records.

All patients received fludarabine at a dose of 40 mg/m^2/day for 4 days as a 1-hour infusion from day -5 to day -2, treosulfan 14 g/m^2/day for 3 days at the rate of 5 g/hour from day -5 to day -3, and a single dose of thiotepa 8 mg/kg on day -6 prior to HCT. Cyclosporine and short-course methotrexate were used as GVHD prophylaxis. Cyclosporine was administered at a dose of 2.5 mg/kg i.v. over 4 hours twice daily starting on day -3 and changed to oral administration at 5 mg/kg twice daily once mucositis had resolved. Cyclosporine levels were monitored, and the dose was adjusted to achieve a target level of 100 to 300 ng/mL. The methotrexate dose was 10 mg/m^2 on day +1 and 7 mg/m^2 on days +3, +6, and +11.

An absolute neutrophil count of $>500 \times 10^6$/L on 3 consecutive days was defined as neutrophil engraftment. A day +28 chimerism analysis showing >95% of donor genetic marker patterns was considered complete chimerism; mixed chimerism was defined as the presence of >5% residual host chimerism at any time point post-HCT. Graft rejection was defined as >90% residual host chimerism in peripheral blood as described previously [9]. Regimen-related toxicities (RRTs), including mucositis, were graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 5.0 criteria [10] . Hepatic VOD was graded according to the Baltimore criteria [11], and GVHD was graded using the Glucksberg criteria [11]. Any deaths occurring within the first 100-days post HCT were considered transplantation-related mortality (TRM+100).

EASIX-PreTx Scoring

The EASIX score was calculated as serum LDH (U/L) × serum creatinine (mg/dL)/platelet count ($\times 10^9$/L) using laboratory data recorded during the pretransplantation period.

Statistical Analyses

The primary outcome variable was TRM+100. The receiver operating characteristic (ROC) curve was used to evaluate the utility of the EASIX-PreTx score in predicting TRM+100. The median EASIX-PreTx score was compared between patients who survived and those who had TRM+100 using the Mann-Whitney *U* test. All statistical analyses were performed using SPSS version 26 (IBM, Armonk, NY). A *P* value <.05 was considered statistically significant.

RESULTS

Patient Demographics

Between January 2012 and December 2019, 281 patients with thalassemia major underwent HCT at our center using a uniform thiotepa-treosulfan-fludarabine conditioning regimen. The patients included 172 males and 109 females, with a median age of 9 years (range, 1 to 25 years). Most of the patients had class III thalassemia major (39.1% class III high risk; 47.7% class III low risk), and 12.1% were in class II. The demographic data of these patients are summarized in Supplementary Table S1.

EASIX-PreTx Scoring

The EASIX-PreTx score could be calculated for only 184 of the 281 patients (65.5%) owing to the unavailability of LDH measurements in 97 patients in the pretransplantation period. The EASIX-PreTx scores of these 184 patients ranged from .11 to 24.42 (median, .78). There was no significant difference in the rate of TRM+100 in patients with available EASIX-PreTx scores and patients without EASIX-PreTx scores (14.7% versus 11.3%; *P* = .47). Moreover, there was no significant difference in overall mortality rate between these 2 groups (21.2% versus 17.5%; *P* = .53).

HCT Outcome

Patients were followed up for a median of 30 months (range, .3 to 108 months). Fourteen patients (5%) died early due to RRTs and other transplantation-related complications before engraftment, and the 267 patients (95%) had documented engraftment (median time to engraftment; 16 days; range, 10 to 43 days). Other HCT outcome endpoints are listed in Supplementary Table S2. Supplementary Tables S3 and S4 provide data for the 184 patients with available EASIX-PreTx scores.

ROC Analysis Using EASIX-PreTx for Predicting TRM+100

The median EASIX-PreTx score was significantly higher in patients with TRM+100 compared with those without TRM +100 (1.09 versus .75; *P* = .008). The area under the ROC curve for predicting TRM+100 using the EASIX-PreTx score was .661 (Supplementary Figure S1). An EASIX-PreTx score cutoff of .85 had a 70.4% sensitivity and a 62% specificity for predicting TRM+100. The TRM+100 for patients with an EASIX-PreTx score >.85 was significantly higher than that for those with an EASIX-PreTx score <.85 (24.4% versus 7.5%; P =.003).

Analysis of Predictors of TRM+100 in a Uniform Cohort of Class III Thalassemia Patients

After excluding patients with class I and II thalassemia from the study cohort, the analysis for predictors of TRM+100 was performed in a uniform cohort of 156 patients with class III thalassemia. The demographic information and HCT outcomes for this subgroup of class III patients are provided in Table 1.

Table 2 compares demographic details and HCT outcomes in the patients of this subgroup based on EASIX-PreTx score using the cutoff of .85. There were no significant differences between the EASIX >.85 and EASIX <.85 groups in terms of age, sex, and proportions of class III high-risk patients and patients with mixed chimerism, veno-occlusive disease, or GVHD; however, the rate of TRM+100 was significantly higher in the EASIX >.85 group (25% versus 8.3%; *P* = .008) (Supplementary Figure S2). Twenty-five of the 156 class III thalassemia patients with an available EASIX score died within 100 days of transplantation, including 18 with EASIX >.85 and 7 with EASIX <.85. The causes of TRM+100 in the 18 patients with

Table 1
Patient Characteristics for All Class III Patients with Thalassemia Undergoing HCT where EASIX-PreTx was Available (N = 156)

Parameters	Value
Age, yr, median (range)	9 (1-25)
Sex, male/female, n (%)	97 (62.2)/59 (37.8)
Stem cell source, n (%)	
Bone marrow	3 (1.9)
Peripheral blood	153 (98.1)
HLA matching, n (%)	
Identical	147 (94.2)
One antigen/allele mismatch	9 (5.8)
CD34 cell dose, $\times 10^6$ cells/kg, median (range)	10 (2.2-18)
Vellore risk classification, n (%)	
Class III low risk	83 (53.2)
Class III high risk	73 (46.8)
Engraftment, n (%)	146 (93.6)
Day +28 chimerism, n (%)	
Complete chimerism	129 (88.4)
Mixed chimerism	17 (11.6)
Day +60 chimerism, n (%)	
Complete chimerism	107 (83.6)
Mixed chimerism	21 (16.4)
Day +100 chimerism, n (%)	
Complete chimerism	98 (81.0)
Mixed chimerism	22 (18.2)
Rejection	1 (.8)
Rejection, n (%)	
Yes	10 (6.8)
No	136 (93.2)
Mucositis, n (%)	84 (53.8)
Hepatic VOD, n (%)	35 (22.4)
Acute GVHD, n (%)	
Yes	43 (29.5)
No	103 (70.5)
Chronic GVHD, n (%)	
Yes	19 (14.6)
No	111 (85.4)
TRM, n (%)	
D+30	17 (10.1)
D+100	25 (16)
OS status: dead, n (%)	35 (22.4)
EFS status: event, n (%)	37 (23.7)

EASIX >.85 were primary graft failure in 6 (5 of whom died from immediate transplantation-related complications during a second transplant), GVHD in 7, RRT (including veno-occlusive disease and intracranial bleeding) in 4, and infection in 1. In the 7 patients with EASIX <.85, the causes of TRM+100 were primary graft failure in 1, GVHD in 2, RRT (including veno-occlusive disease and intracranial bleeding) in 3, and infection in 1.

Table 3 compares the patients with TRM+100 and those who did not. The patients with TRM+100 were older, received a higher CD34 cell dose, and had higher rates of nonsibling donors, HLA-mismatched donors, mixed chimerism, graft rejection, veno-occlusive disease, and acute GVHD, in addition to higher pretransplantation EASIX scores.

Table 4 shows the results of univariate and multivariate analyses for predictors of TRM+100 in this uniform subgroup of patients with class III thalassemia. On multivariable logistic regression analysis, the factors independently predicting TRM +100 were EASIX >.85, pretransplantation serum ferritin >3210 ng/mL, and use of unrelated donors. Although it was significant in univariate analysis, we omitted mixed chimerism as a variable in the multivariable analysis because this would apply only to those patients who were alive at the time of chimerism testing.

Figure 1 shows the OS of patients according to pretransplantation EASIX score. Patients with an EASIX <.85 had significantly better OS (mean 2-year OS, 82.7% ± 4.2 % versus 70.6% ± 5.4%; log-rank $P = .05$).

DISCUSSION

Allogeneic HCT is a curative modality for thalassemia major [12] and has been associated with higher health-related quality of life compared with transfusion and chelation [13]. In addition, at the willingness-to-pay threshold of Indian per capita gross domestic product, matched related HCT is likely to be more cost-effective compared with transfusion and chelation [14]. Further refinement of risk stratification and evaluation of novel therapeutic approaches in groups with high predicted risk is the way forward to improve transplantation outcomes in patients with thalassemia [3,4,15]. Toward this aim, we evaluated the role of the novel biomarker EASIX in the risk stratification of patients with thalassemia undergoing transplantation, specifically in patients with class III disease undergoing HCT.

We found that TRM+100 in our cohort of allogeneic HCT recipients conditioned with a thiotepa-treosulfan-fludarabine regimen was 13.5%, compared with 16% in a uniform subgroup of class III thalassemia patients. The major causes of death were GVHD, RRT, graft failure, and infection. Toward further optimizing the clinical outcomes in this group, we evaluated the role of EASIX score calculated before transplantation in predicting TRM+100, and found a higher rate of TRM+100 in patients with high EASIX-PreTx scores undergoing HCT for class III thalassemia. This association continued to remain significant even on multivariable analysis.

This finding raises the questions of whether endothelial dysfunction pretransplantation is associated with the occurrence of graft failure or GVHD, and whether novel strategies to improve endothelial function could potentially reduce graft failure or GVHD. We did not find any differences between the 2 EASIX groups in other outcomes, including RRTs, GVHD, mixed chimerism rate, and total (primary and secondary) graft rejection.

The EASIX score was initially proposed for predicting survival following GVHD after reduced-intensity transplantation for malignancies in adults [5]. This biomarker has been subsequently identified as a predictor of NRM following HCT [6,7], VOD [8], the need for intensive care post-transplantation [16], survival in multiple myeloma [17], fluid overload following HCT [18], toxicity following chimeric antigen receptor T cell therapy [19,20], and even mortality following COVID-19 infection [21]. Because the parameters included in EASIX are nonspecific, the biological basis of this widespread predictive utility is not completely clear.

In our routine clinical practice, we currently measure parameters to calculate EASIX immediately pretransplantation and on day 0. We plan to prospectively validate this biomarker in thalassemia transplantations for predicting TRM in combination with other predictors, including pharmacokinetic data of the conditioning drugs. Once prospectively validated, this biomarker potentially can be used to prognosticate in these

Table 2
HCT Outcomes for All Class III Patients with Thalassemia Undergoing HCT where EASIX-PreTx Was Available (N = 156)

Parameter	EASIX >.85 (N = 72)	EASIX <.85 (N = 84)	*P* Value
Age, yr, median (range)	10.5 (1-21)	8.5 (2-25)	**.010**
Male sex, n (%)	43 (59.7)	54 (64.3)	.620
CD34 cell dose× 10^6 cells/kg, median (range)	10 (2-18)	10 (3-14)	.188
Donor source, n (%)			.964
Matched sibling	57 (79.2)	66 (78.6)	
Matched nonsibling	6 (8.3)	8 (9.5)	
Matched unrelated	9 (12.5)	10 (11.9)	
1 HLA antigen/allele mismatch donors, n (%)	6 (8.3)	3 (3.6)	.303
Class III high risk, n (%)	38 (52.8)	35 (41.7)	.199
Neutrophil engraftment, n (%)	65 (90.3)	81 (96.4)	.189
Mixed chimerism at day +28 (N = 146), n (%)	7 (10.8)	10 (12.3)	.802
Mixed chimerism at day +60 (N = 128), n (%)	12 (22.6)	9 (12.0)	.146
Mixed chimerism at day +100, n (%)	12 (25.0)	10 (13.7)	.123
Rejection, n (%)	6 (9.2)	4 (4.9)	.341
Hepatic VOD, n (%)	17 (23.6)	18 (21.4)	.848
Acute GVHD, n (%)	22 (33.8)	21 (25.9)	.362
Chronic GVHD, n (%)	7 (13.0)	12 (15.8)	.802
TRM+30, n (%)	13 (18.1)	4 (4.8)	**.010**
TRM+100, n (%)	18 (25.0)	7 (8.3)	**.008**

Table 3
Comparison of Class III Thalassemia Patients with TRM+100 and Those without TRM+100 where EASIX Was Available (N = 156)

Parameter	TRM+100		*P* Value
	Yes (N = 25)	No (N = 131)	
Age, yr, median (range)	12 (4-20)	9 (1-25)	**.001**
Male sex, n (%)	17 (68)	80 (61.1)	.654
CD34 cell dose, × 10^6/kg, median (range)	10.3 (2-18)	9.3 (3-16)	**.045**
Donor source			**.001**
Matched sibling	10 (40)	113 (86.3)	
Matched related	5 (20)	9 (6.9)	
Matched unrelated	10 (40)	9 (6.9)	
One HLA antigen/allele mismatched donor, n (%)	7 (28)	2 (1.5)	**.001**
Class III high risk, n (%)	14 (56)	59 (45)	.384
Neutrophil engraftment, n (%)	16 (64)	130 (99.2)	**.001**
Mixed chimerism at day +28 (N = 146), n (%)	7 (46.7)	10 (7.6)	**.001**
Mixed chimerism at day +60 (N = 128) , n (%)	5 (62.5)	16 (13.3)	**.003**
EASIX Pre-Tx >.85, n (%)	18 (72)	54 (41.2)	**.008**
Graft rejection (N = 146), n (%)	7 (46.7)	3 (2.3)	**.001**
Hepatic VOD, n (%)	13 (52)	22 (16.8)	**.001**
Acute GVHD (N = 146), n (%)	12 (75)	31 (23.8)	**.001**

patients and select patients for evaluation of such therapeutic strategies as the use of defibrotide or statins [8] to reduce TRM.

Limitations of the present analysis are its retrospective nature and the use of variable time points for measuring EASIX pretransplantation. We did not have information on other markers of endothelial homeostasis, such as CXCL8, IL-18, and insulin-like growth factor 1 serum levels. In addition, these results might not be applicable for other LDH detection methods and other strategies for class III thalassemia HCT recipients, such as those involving pretransplantation immunosuppression [22]. EASIX measured pretransplantation might not account for the effects of various drugs used in HCT, such as calcineurin inhibitors, sirolimus, calcium channel blockers, and angiotensin II inhibitors, which also can affect endothelial integrity.

In conclusion, EASIX is a promising biomarker associated with TRM in patients with class III thalassemia undergoing HCT.

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Table 4
Univariate and Multivariate Analysis for Predictors of TRM+100 among Class III Patients (N = 156)

Variable	Univariate			Multivariate		
	Risk	95%CI	*P* Value	Risk	95%CI	*P* Value
Age >9.5 yr	3.0	1.19-7.78	**.020**	1.4	.46-4.45	.538
Female sex	1.3	.55-3.37	.514			
Class III Vellore risk	1.5	.66-3.68	.316			
CD34 cell dose	1.2	.98-1.39	.078	1.1	.86-1.31	.559
HLA mismatch	25.1	4.83-130.22	**.001**	18.4	.98-345.51	.051
Donor source, matched related nonsibling donor vs others	6.3	1.76-22.36	**.005**	3.9	.79-19.55	.096
Donor source, matched unrelated donor vs others	12.6	4.14-38.0	**.001**	14.6	2.96-71.56	**.001**
Pretransplantation ferritin >3210 ng/mL	5.0	1.78-14.2	**.002**	6.1	1.63-23.05	**.007**
Mixed chimerism at day +28 (N = 146)	10.6	3.18-35.2	**.001**			
EASIX-Pre-Tx >.85	3.7	1.43-9.39	**.007**	3.9	1.19-13.05	**.025**

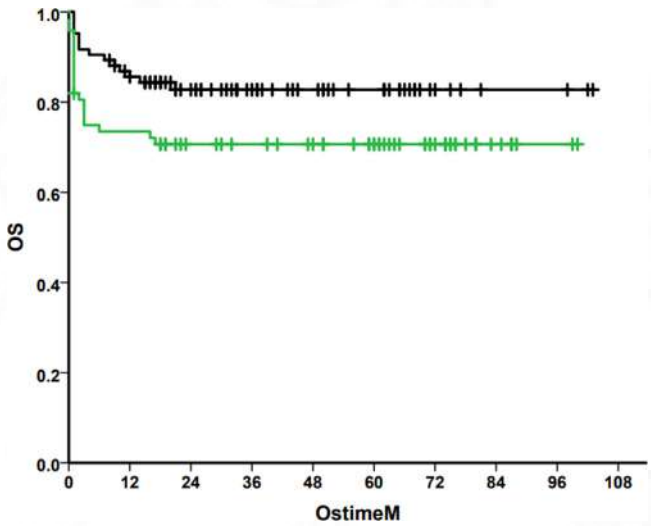

Figure 1. Kaplan-Meier survival curves for OS based on the proposed EASIX Pre-Tx cutoff (.85) (n = 156) showing significantly better OS in patients with EASIX-PreTx score <.85 (mean 2-year OS, 82.7% ± 4.2 % versus 70.6% ± 5.4%; log rank *P* = .05). The black line represents EASIX-PreTx <.85; green line, EASIX-PreTx >.85, Time on X axis is expressed in months.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jtct.2022.05.001.

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Pharmacokinetics and Efficacy of Generic Melphalan Is Comparable to Innovator Formulation in Patients With Multiple Myeloma Undergoing Autologous Stem Cell Transplantation

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Abstract

Pharmacokinetics and therapeutic efficacy were investigated in patients with multiple myeloma receiving both generic and innovator melphalan (MEL) formulation for conditioning pre autologous stem cell transplantation. Both the MEL formulations were comparable in terms of pharmacokinetics and efficacy, suggesting generic MEL as a low-cost alternative to innovator MEL for autologous stem cell transplantation conditioning in multiple myeloma.

Background—High-dose melphalan (MEL) is the standard conditioning regimen used for autologous stem cell transplantation (ASCT) in patients with multiple myeloma (MM). Generic MEL is routinely used in various transplant centers across the world including ours due to its reduced cost and ease of availability. We compared the pharmacokinetics (PK) and the clinical efficacy of generic MEL with that of the innovator formulation in MM patients undergoing ASCT.

Patients and Methods—Sixty-three patients diagnosed with MM receiving high-dose MEL were included in this study. MEL levels in plasma were measured using a liquid chromatography tandem mass spectrometry (HPLC/MS-MS) protocol and non-linear mixed effects modeling was used to evaluate the PK of the data.

Results—The interindividual variability (IIV) in MEL area under the concentration versus time curve (AUC) and clearance (CL) were 4.39, 5.88-fold for generic, and 4.34, 6.85-fold for the innovator formulation, respectively. The median MEL AUC and CL were comparable between the 2 formulations. The population PK analysis showed age and creatinine CL as the only significant covariates explaining IIV in MEL AUC/CL. Analysis of MEL PK parameters with clinical outcome showed no significant differences in terms of onset and severity of mucositis, day to neutrophil and platelet engraftment, as well as response status on day 100 post ASCT between

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patients receiving generic or innovator formulations of MEL. In addition, neither MEL AUC nor CL was found to be associated with day +100 response.

Conclusion—Our study suggests that the PK and efficacy of the generic MEL is comparable to the innovator formulation.

Keywords

Autologous HSCT; Generic melphalan; Multiple myeloma; Population pharmacokinetics

Introduction

High-dose melphalan (MEL) is the most common conditioning regimen being used prior to autologous stem cell transplantation (ASCT) in patients with multiple myeloma (MM) since the 1980s.[1–6] Common toxicities after MEL administration include myelosuppression, nausea, vomiting, diarrhea, alopecia, and gastrointestinal mucositis. Oral and esophageal mucositis occurs frequently, affecting the nutritional status, hydration, and quality of life in patients during ASCT, thereby increasing the hospitalization duration and cost of care.[2] Several studies have evaluated pharmacokinetics (PK) of MEL and have related high MEL exposure with either increased toxicity,[7–11] improved therapeutic efficacy,[12,13] or no obvious perceptible effect.[14–16] However, PK-guided dosing for MEL is limited except for a few reported studies using the test dose PK approach.[17–22]

In many centers across the world, generic and innovator formulations of MEL are in clinical use. Generic MEL (Megval, Emcure Pharmaceuticals, Pune, India) costs less than 25% of innovator MEL (Alkeran, Aspen Pharmacare, New South Wales, Australia). In a country like India, majority of the patients pay from their own pocket for health care.[23] In this scenario, the availability of a low-cost generic formulation would enable the patient and the physician to maintain the cost of the transplantation within affordable limits. However, there is a paucity of data on the therapeutic equivalence of many of the generic drugs. To this date, there have been no reports on the PK and efficacy of the generic MEL in patients with MM undergoing ASCT. In this study, we evaluated the PK and compared therapeutic efficacy of both formulations in terms of ASCT outcomes and PK.

Patients and Methods

Reagents and Chemicals

MEL (Cat no: 148-82-3), internal standard (IS) N-phenyldiethanolamine (NPEA; Cat no: P22400), mass spectrometry grade acetonitrile (Cat no: 900667), formic acid (Cat no: F0507), and methanol (Cat no: 900688) were purchased from Sigma Aldrich (St Louis, MO).

Patients

All patients diagnosed with MM and undergoing ASCT using a high-dose MEL conditioning regimen between March 2016 and August 2018 in the Department of Hematology, Christian Medical College, Vellore, India, were included in this study. Written

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informed consent was obtained from the patients. This study was approved by the institutional review board.

Stem Cell Mobilization and Collection

Stem cell mobilization was done with granulocyte-colony stimulating factor (G-CSF) given for 4 consecutive days at 10 μg/kg in 2 divided doses followed by stem cell harvest using a COBE Spectra apheresis system on day 5. A stem cell dose of 4×10^6 $CD34^+$ cells was targeted for ASCT. In case the stem cell dose was inadequate after the first day collection, patients were taken up for a second stem cell collection on the next day after administering G-CSF alone or G-CSF with plerixafor (0.24 mg/kg). Stem cells after collection were stored at 4°C in the blood bank refrigerator before infusion and were infused without cryopreservation.

Conditioning Regimen

MEL was administered on day −1 as a single dose of 200 mg/m^2. The dose was reduced to 140 mg/m^2 in cases for which the creatinine clearance (CL) was <60 mL/min or if the age was more than 65 years. MEL was administered as an intravenous bolus injection through a central venous catheter over 30 minutes. Cryotherapy with ice chips was initiated along with MEL to reduce the severity of mucositis. The choice of the MEL used (innovator vs. generic) was non-randomized and was purely on the basis of the discretion of the treating physician/resources of the patient. The generic MEL used was Megval (Emcure Pharmaceuticals, Pune, India).

Sample Collection for PK Analysis

Peripheral blood (5 mL) was collected in sodium heparin tubes before the start (0 hours), end of infusion, and 1, 2, 3, 6, and 24 hours after the end of MEL infusion. Plasma was separated immediately and stored at −80° C until further analysis.

Measurement of Plasma MEL in High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC/MS-MS)

Measurement of plasma MEL levels was carried out as per the method reported previously with some modifications.[24] Briefly, MEL levels in plasma samples were measured using a validated LC/MS-MS using a Shimadzu-Nexera X2 ultra HPLC consisting of binary gradient pumps (LC-30AD), auto sampler (SIL-30AC), mobile phase degasser (DGU20ASR), and a column oven (CTO-20AC) coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The data were analyzed using LC Solutions software (Shimadzu, Kyoto, Japan). The parameters were adjusted to yield maximum multiple reaction monitoring signals. The Q1/Q3 for MEL was set at 304.80 > 288.10 m/z and 182.70 > 119.80 m/z for IS NPEA in the positive electrospray ionization mode, respectively. Chromatographic separation of the analytes was done using Luna C_{18} (4.6 × 150 mm, 5 μm, Phenomenex, USA) protected with a C_{18} guard column from the same source. The liquid chromatography conditions were as follows: solvent A: water containing 0.1% formic acid and solvent B: 0.1%; formic acid in acetonitrile was used as mobile phase with gradient elution of solvent B at 20% (0-4.0 minutes); 80% (4.0-6.0 minutes); 20%

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(7.0-10.0 minutes) at a flow rate of 0.8 mL/min. The total run time was 10 minutes. Retention time for MEL was 2.2 minutes and the IS was 2.7 minutes. The concentration of MEL was expressed as ng/mL.

Population Pharmacokinetics (PopPK) Modeling

Non-linear mixed effects modeling analysis was performed with Monolix (version 5.0.1; Lixoft, France) using the stochastic approximation expectation-maximization (SAEM) method. A 2-compartment PK model was used to describe the data. The PK parameters estimated included CL (in L/h/m^2) and volume (V; in L/m^2) along with the intercompartmental CL and peripheral compartment (Q (L/h/m^2) and V_2 in L/m^2). In addition, the individual post hoc parameter values were used to estimate the area under the concentration versus time curve (AUC). The interindividual variability of the parameters was assumed to be log-normally distributed. A proportional residual error model was used with assumed normal distribution of the residuals.

The relationships between the PK parameters and covariates (age, sex, MEL dose, hemoglobin, albumin, and creatinine CL) were described using the following model: $\theta = \theta_{Base} \times \exp(\beta \times \text{covariate})$. A covariate was considered significant in the univariate analysis, if the addition of the covariate to the model reduced the objective function value at least 3.84 units ($P < .05$, on the basis of the χ^2 test for the difference in the −2 log likelihood between 2 hierarchical models that differ by 1 degree of freedom).

Stem Cell Transplantation and Post-Transplantation Care

The patients underwent ASCT as an in-patient either in HEPA filtered or non—HEPA filtered rooms. The collected stem cells were infused fresh without any modifications 12 hours after the MEL administration in patients with normal renal function. For patients with creatinine CL <60 mL/min, the stem cells were infused 24 hours after the administration of MEL. Post stem cell infusion, the patients were managed for the neutropenic period with antibiotics and blood products. Oral mucositis was graded as per the World Health Organization (WHO) grading system. During the period of mucositis, patients were supported with analgesics and total parenteral nutrition as and when required according to the severity reported by them. All patients were given G-CSF from day +7 post-transplant to enhance neutrophil engraftment.

ASCT Outcome

The influence of MEL PK parameters (CL or AUC) on the various outcome measures were analyzed. Patient demographic characteristics as well as clinical response such as duration to neutrophil and platelet engraftment, onset and grade of mucositis, duration of hospitalization, and response on day + 100 post ASCT were analyzed. Neutrophil engraftment was defined as the first day of a neutrophil count >0.5 × 10^9/L or greater over 3 consecutive days. Platelet engraftment was defined as the first day of platelet count >20 × 10^9/L or greater without needing transfusion support for at least 1 week in accordance with the Center for International Blood and Marrow Transplant Research (CIBMTR). Toxicity was graded as per National Cancer Institute Common Terminology Criteria for Adverse

Events (CTCAE) V.5.0. In our center, post-transplant minimal residual disease monitoring is not done.

Statistical Analysis

All statistical analysis was carried out using IBM SPSS statistics 21.0 (Armonk, NY) and GraphPad PRISM5 software (San Diego, CA). Relative risk of variables on ASCT outcome was performed using logistic regression.

Results

Patients

A total of 63 patients with MM underwent ASCT during the study period. Thirty-three received generic MEL, and 30 patients received innovator MEL. The 2 treatment groups were similar with respect to age, sex, pre-ASCT response, and creatinine CL. Baseline patient demographic characteristics are summarized in Table 1.

MEL Assay Validation

MEL assay was validated for its specificity, linearity, precision, accuracy, and recovery before it was used for measurement in patients' plasma. There was no peak detected in unspiked blank plasma at the retention times of MEL (2.2 minutes) and IS (2.7 minutes). The method was linear for a concentration range from 1 to 2000 ng/mL with mean R^2 = 0.99 ± 0.001. MEL was detected in most patient's plasma even at 7 hours after infusion but no traces of MEL were found after 24 hours. Linearity, accuracy, and interday precision are as shown in Supplemental Table 1 in the online version)

PopPK of MEL

The PopPK model parameters comprising body surface area normalized dose are shown in Table 2. The median MEL AUC and CL were comparable between the 2 formulations as well as with previous reports on MEL PK (Table 3). In a univariate analysis, age and creatinine CL were significant covariates explaining variability in MEL CL. Specifically, MEL CL decreased (P = .01) with increasing age and increased (P = .02) with increasing creatinine CL (Figure 1). Since age and creatinine CL are correlated (r = −0.48; P = .0002) the model including both covariates were not significantly different from either one alone.

Comparison of ASCT Outcomes Between 2 Formulations

There were no significant differences in terms of onset and severity of mucositis and duration of hospital stay between patients receiving generic or innovator formulations of MEL (Table 4). The time to platelet and neutrophil engraftment also were comparable between the 2 formulations. The post-transplant responses on day + 100 was also comparable in the 2 arms. There was no difference in the progression-free survival (74.3% ± 10.2% versus 82.6% ± 12.0%; P = .917) and overall survival (89.8% ± 5.6% vs. 82.6% ± 12.0%; P = .544) between the 2 groups of patients who received generic or innovator formulations of MEL (Figure 2). Also, there was no statistically significant difference in the status pre-transplant versus post-transplant between the generic and innovator MEL groups

(pretransplant to post-transplant status remained the same or improved: 30/33 in generic and 28/30 in innovator groups; 3/33 and 0/30 progressed; *P* = not significant).

Influence of MEL PK on ASCT Outcome

None of the MEL PK parameters (AUC and CL) influenced ASCT outcomes such as mucositis onset and severity, platelet and neutrophil engraftment, and duration of hospitalization.

Discussion

Generic MEL is widely used across the world as conditioning regimen for patients with MM undergoing ASCT. Despite their wide clinical utility, to our knowledge there are no reports comparing PK and therapeutic efficacy of generic and innovator MEL. Previous studies have shown wide variability in MEL PK with higher MEL exposure associated with increased toxicity with improved or no significant effect on efficacy.[8–17] In the present study, we compared PK and therapeutic efficacy between generic and innovator MEL.

We developed a PopPK model of MEL in patients who received generic or innovator MEL. Age and creatinine CL were identified as the most significant covariates accounting for a large proportion of the interindividual variability in MEL CL. MEL elimination is primarily through renal excretion and hence creatinine CL is a well-known predictor of MEL CL. We also observed that MEL CL increases with increasing creatinine CL, which is consistent with previous findings.[7,11,25,26] In addition, our model showed MEL CL decreases with increasing age, probably because of the decreased renal function with age.[27] However, age was not a significant covariate in previous PopPK studies in MEL PK.[7,11,25,26]

Comparison of PK profiles of both generic and innovator formulations of MEL showed that the PK estimates (AUC and CL) were comparable to each other and previously reported studies on MEL PK.[7,11,25,28,29]

ASCT outcomes such as onset and severity of mucositis, day to neutrophil and platelet engraftment, duration of hospitalization between both formulations were also comparable. None of the MEL PK parameters (irrespective of formulation) showed any association with ASCT outcomes including overall survival, which was consistent with a previous study.[11] However, this observation was contrary to a previous finding, which showed that high MEL exposure improves overall survival.[13] One of the limitations of our study is its non-randomized nature. This study is also not powered to address the influence of MEL PK on ASCT outcomes/toxicity.

Conclusion

This study demonstrates that the therapeutic efficacy and PK of generic MEL is comparable to innovator MEL. Generic MEL is therefore a good alternative to its innovator formulation to cut the cost of transplantation in a country like ours.

Clinical Practice Points

- In many centers across the world, both generic and innovator formulations of high-dose MEL are in use as conditioning regimen in ASCT.
- Previous studies on MEL PK were demonstrated in innovator formulations, and to date, there are no reports on the PK and efficacy of the generic MEL in patients with MM undergoing ASCT.
- The present study evaluated the PK and compared the therapeutic efficacy of both formulations in terms of ASCT outcomes and PK, demonstrating that the therapeutic efficacy and PK of generic MEL is comparable to innovator MEL.
- Generic MEL is therefore a good alternative to its innovator formulation to cut the cost of transplantation, especially in developing countries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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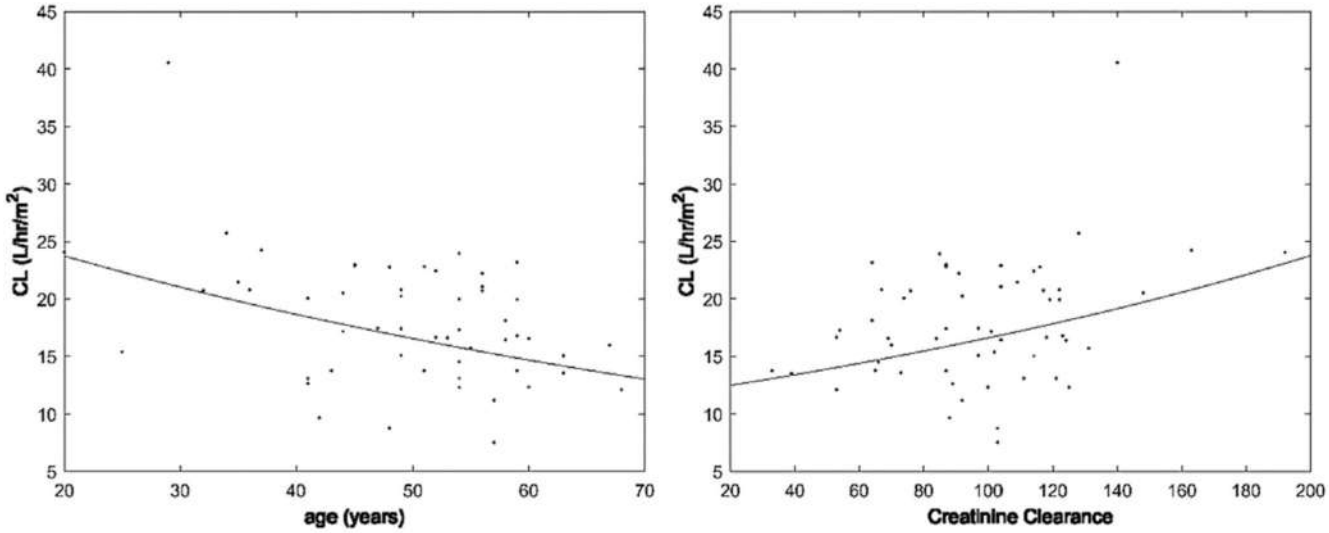

Figure 1.
Linear Dependency Between Melphalan (MEL) Clearance (CL) and Age (Left) and Between MEL CL and Creatinine CL (Right)

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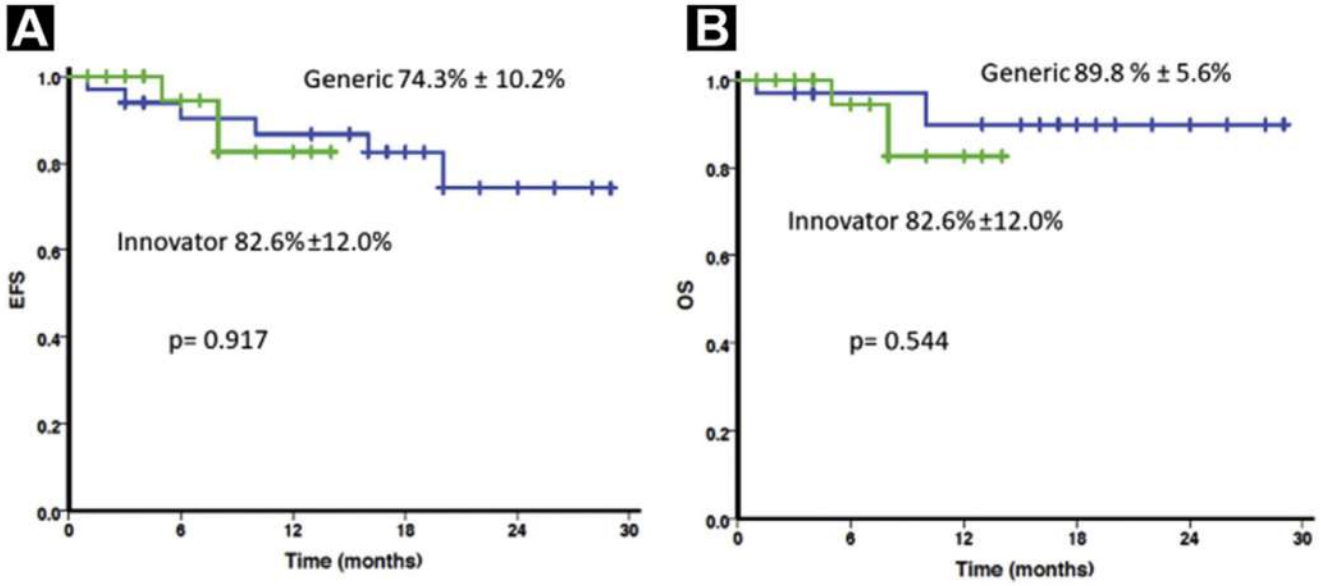

Figure 2.

Kaplan—Meier Survival Curves Showing Influence of MEL Formulation on Overall Survival (OS) and Progression-Free Survival (EFS). No Significant Differences in EFS (A) and OS (B) Were Observed Between Generic (n = 33) and Innovator (n = 30) MEL Formulations

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Table 1

Patient Demographic Characteristics

Characteristic	Generic MEL	Innovator MEL	*P*
Patients, n	33	30	NS
Age, y	52 (26-63)	54 (20-68)	NS
Male:Female Sex	22:11 (2:1)	20:10 (2:1)	NS
Pretransplantation Response			NS
CR	19 (58%)	13 (43%)	
VGPR	10 (30%)	9 (30%)	
PR	4 (12%)	8 (27%)	
MEL Dose			NS
200 mg/m^2	33	22	
140 mg/m^2	-	8	
CD34, × 10^6/kg	5.39 (3.22-13.36)	5.2 (3.05-13.58)	NS

Data are presented as n (%) or median (range) except where otherwise noted. Abbreviations: CR = complete response; NS = not significant; PR = partial response; VGPR = very good partial response.

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Table 2
Population Pharmacokinetics of Melphalan

Parameter	Base	RSE, %	Age	RSE, %	*P*	Creatinine CL	RSE, %	*P*
CL, L/h/m^2	16.4	4.7	19.8	8.7		11.6	15.5	
β [a]			–0.012	38.9	0.010	0.0036	42.9	.020
V, L/m^2	14.5	6.5	14.4	7.3		14.6	6.5	
Q, L/h/m^2	4.8	23.3	5.2	25.9		4.8	29.1	
V_2, L/m^2	8.9	12.9	8.9	14.7		8.7	15.4	
σ Additive, mg/mL	0.0005	Fixed	0.0005	Fixed		0.0005	Fixed	
σ Prop, CV%	0.32	6.9	0.31	6.6		0.31	6.4	
—2 Log-Likelihood [b]	469.7		464.3		0.020		465.0	.030
IIV	**CV%**	**RSE, %**	**CV%**	**RSE, %**	*P*	**CV%**	**RSE, %**	*P*
CL	0.31	11.3	0.32	12.1		0.31	11.6	
V	0.32	16.9	0.37	15.6		0.34	15.7	
Q	0.85	21.8	0.97	22.0		0.93	27.4	
V_2	0.73	14.9	0.71	15.5		0.77	15.6	

Abbreviations: CL = clearance; CV% = coefficient of variation; IIV = interindividual variability; Q = intercompartmental clearance; RSE = relative standard error; V = volume of distribution into the central compartment; V_2 = volume of distribution into the peripheral compartment.

[a] Covariate model: θ × exp (β × covariate).

[b] *P* value represents the significance of the change in the —2 log likelihood (on the basis of the χ^2 test) relative to base model.

Table 3
Comparison of MEL PK With Existing Literature

PK Parameter	Generic MEL (n = 32) [a]	Innovator MEL (n= 23) [a]		Cho et al[11] (n = 146)[b]	Nath et al[7] (n = 100)[b]
Age (Range), Years	52 (26-63)	54 (20-68)		59 (35-72)	57 (36-73)
Median AUC (Range), mg/h/L	10.7 (6.7-23.7)	MEL 140	MEL 200	14.4 (5.6-27.3)	12.8 (4.9-24.6)
		9.9 (8.3-11.1)	11.6 (5.6-22.4)		
Mean Clearance, L/h/m^2	27.12	27.07		29.0	27.8

Abbreviations:AUC = area under the curve; MEL = melphalan; PK = pharmacokinetics.

[a] Present study.

[b] Reported studies that evaluated innovator MEL.

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Table 4
Comparison of ASCT Outcomes in Patients Who Received Generic and Innovator MEL

ASCT Outcomes Days (Range)	Generic MEL (n = 33)	Innovator MEL (n = 30)	*P*
Day of Onset of Mucositis (Range)	3(1-7)	3(1-7)	NS
Mucositis Grade			NS
3	1	–	
2	18	16	
1	14	14	
Duration to Neutrophil Engraftment	12 (10-16)	12 (10-21)	NS
Duration to Platelet Engraftment	16 (13-33)	17 (14-33)	NS
Duration of Hospitalization	22 (17-55)	21 (15-42)	NS
Day 100 Post ASCT Response			NS
CR	20	16	
VGPR	9	4	
PR	1	4	
Others [a]	3	6	

Abbreviations: ASCT = autologous stem cell transplantation; CR = complete response; MEL = melphalan; NS = not significant; PR = partial response; VGPR = very good partial response.

[a]Others includes in generic MEL (1 expired, 1 progression, and 1 response not reached) and in innovator MEL (4, response not reached and 2, lost to follow-up).

Pharmacogenomics and Personalized Medicine

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ORIGINAL RESEARCH

NUDT15 c.415C>T Polymorphism Predicts 6-MP Induced Early Myelotoxicity in Patients with Acute Lymphoblastic Leukemia Undergoing Maintenance Therapy

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Purpose: Severe myelosuppression in patients with acute lymphoblastic leukemia (ALL) undergoing 6-MP-based maintenance therapy is attributed to *TPMT* gene polymorphisms, which is rare in Asian populations. This study aims to evaluate the role of selected polymorphisms in *NUDT15, ITPA*, and *MRP4* genes in addition to *TPMT* in predicting 6-MP intolerance during ALL maintenance therapy.

Patients and Methods: We screened for the presence of *NUDT15*3* (c.415 C>T, rs116855232); *MRP4* c.2269 C>T (rs3765534), *ITPA* c.94 C>A (rs1127354) polymorphisms in addition to *TPMT* *2 (rs1800462), *3A (*3B and *3C; rs1800460 and rs1142345) in ALL patients with documented severe neutropenia (cohort-1; n=42). These polymorphisms were then screened in a prospective cohort of ALL patients (cohort-2; n=133) and compared with 6-MP dose reduction, early/late myelotoxicity.

Results: Nineteen (45%) patients in cohort-1 and 18 (14%) in cohort-2 had *NUDT15* c.415 C>T variant while 4 (3%) patients in cohort-2 had *TPMT*3C* variant. Five (12%) in cohort-1 and 30 (24%) in cohort-2 had *ITPA* c.94 C>A variant while 9 (22%) and 15 (12%) had *MRP4* c.2269 C>T variant in cohorts-1 and 2, respectively. All in cohort-1 and 36 (27%) in cohort-2 had severe myelotoxicity. Twenty-eight patients (66.6%) in cohort-1 and 40 (30%) patients in cohort-2 had significant 6-MP dose reduction. *NUDT15* c.415 C>T variant explained severe myelotoxicity in 63% and 33% in cohort 1 and 2. *TPMT**3C and *ITPA* c.94 C>A variants also explained myelotoxicity in cohort-2 (Median ANC: 376 vs 1014 mm^3; p=0.04 and 776 vs 1023 mm^3; p=0.04 respectively). *NUDT15* c.415 C>T polymorphism explained significant myelotoxicity (507 vs 1298 mm^3; p<0.0001) in the multivariate analysis as well (β=−0.314, p<0.0001).

Conclusion: *NUDT15* c.415 C>T (15*3), TPMT*3C, as well as ITPA c.94 C>A and MRP4 c.2269 C>T polymorphisms explain hematotoxicities. Preemptive genotype-based (*NUDT15*3, TPMT, ITPA* c.94 C>A) 6-MP dosing could improve the outcome after maintenance therapy.

Keywords: leukemia, mercaptopurine, myelotoxicity, pharmacogenomics

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer and also occurs in adults.[1] The last phase of the treatment of ALL involves maintenance therapy with daily oral 6-mercaptopurine (6-MP) and weekly methotrexate (MTX) for nearly 2 years in most treatment protocols. However, the significant dose-limiting toxicity is life-threatening myelosuppression[2] owing to the narrow

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therapeutic indices of these drugs and a wide inter-individual variation in drug response. These toxicities often lead to extended hospitalization resulting in increased cost of treatment. Genetic polymorphisms in drug-metabolizing enzymes and transporters contribute significantly to this variability in response to drugs. It is now well acknowledged that polymorphisms in Thiopurine Methyltransferase (*TPMT*)[3] and Nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*)[4–6] genes explain 6-MP mediated cytopenia.

We have reported previously that *TPMT* variants are rare in our population and do not fully explain the severe myelosuppression occurring in patients with ALL undergoing maintenance therapy.[7] Further screening for *NUDT15* c.415 C>T polymorphism in a cohort of patients with severe myelosuppression (but lacking *TPMT* variants) showed that only ~53% of these patients carried *NUDT15* c.415 C>T[8] suggesting that additional genetic factors could play a role. Polymorphisms in Multidrug resistance protein-4 (MRP4 c.2269 C>T)[9,10] and Inosine triphosphate pyrophosphatase (ITPA c.94 C>A)[11–13] have also been shown to explain toxicity to 6-MP in specific ethnic populations. We have previously reported that *TPMT* polymorphisms are rare in our populations and do not entirely explain the variation in 6-MP toxicity.[7] We have recently reported that *NUDT15 c.415C>T* polymorphism is a significant determinant of myelosuppression related to the intake of thiopurines in patients with Immune thrombocytopenic purpura and autoimmune hemolytic anemia.[14] A recent report from the Clinical Pharmacogenetics Implementation Consortium (CPIC) has recommended preemptive genotyping of *NUDT15* apart from *TPMT* variant testing before 6-MP dosing.[6]

Although previous studies in the Indian population have highlighted the role of *NUDT15 c.415 C>T* polymorphism associated with 6-MP toxicity,[15–17] there are no comprehensive reports on all the identified genetic polymorphisms related to 6-MP intolerance and early hematological toxicities in patients with ALL. This study aims to evaluate the role of selected polymorphisms in *ITPA*, and *MRP4* genes in addition to CPIC recommended *NUDT15* and *TPMT* testing in predicting 6-MP intolerance/6-MP dose reduction and toxicities during ALL maintenance therapy.

Patients and Methods

Patients

Patients with ALL who experienced severe clinical thiopurine-related myelotoxicity requiring dose-reduction referred for *TPMT* genetic testing from 2009 to 2017 were included (cohort-1). In addition, all consecutive patients diagnosed with ALL undergoing maintenance therapy between September 2018 and March 2020 in the Department of Hematology, Christian Medical College, Vellore, India, were prospectively enrolled (Cohort-2). The purpose of retrospective cohort 1 is to identify genetic polymorphisms (in addition to *TPMT*) that could explain myelotoxicity, while prospective cohort-2 is to study the influence of genetic polymorphisms on 6-MP intolerance, toxicities, and survival. Patients with less than six-month follow-up or lost follow-up, who underwent consolidation therapy with 6-MP, and those who refused to consent, were excluded from the analysis. Written informed consent was obtained from the patients/parents. This study was approved by the Institutional Review Board [IRB (EC)-ER-1-23-07-2014]. The initial doses of 6-MP and MTX for maintenance therapy were 50 mg/m^2 daily and 20 mg/m^2 weekly, respectively. The treating physicians adjusted the 6-MP/MTX doses to maintain a white blood cell (WBC) count of 3.0 × 10^9/L and avoid infections and hepatotoxicity.

Genotyping

Before starting maintenance therapy, peripheral blood was collected in EDTA tubes, and DNA was extracted using the Qiagen Gentra kit. The samples were screened for selected polymorphisms: *NUDT15*3* (c.415 C > T, rs116855232, p.Arg139Cys), *ITPA* (c.94 C > A, rs1127354, p.Pro32Thr) and *MRP4* (c.2269 G>A, rs3765534, p.Glu757Lys) by bidirectional sanger sequencing[14] and *TPMT**3A [*3B (460 G>A, rs1800460, p.Ala154Thr) and *3C (719 A > G, rs1142345, p.Tyr240Cys)] by Restriction Fragment Length Polymorphism (RFLP) and [*2(238G > C, rs1800462, p.Ala80Pro)] by PCR using allele-specific oligonucleotides (ASO) as reported previously.[7] Additionally, polymorphisms in *NUDT15* exon1 were screened by Sanger sequencing as reported previously.[18]

Clinical Outcomes

Clinical data such as dose reduction, WBC/ANC counts, and ALT/AST levels were monitored and documented longitudinally throughout the maintenance therapy. %6-MP dose intensity was defined as the ratio between clinician prescribed 6-MP dose to protocol dose (%) and was captured monthly for the first 6-months since the start of 6-MP. We calculated the average 6-MP dose intensity (up to 6 months) for association analysis in the present study. In addition, information on the total direct hospital costs incurred during the first 6 months of

maintenance therapy was obtained for all the patients. Therapy interruption was defined as the cessation of medicine administration resulting from cytopenia, infections, or hepatotoxicity. Hepatotoxicity (Grade 3 and above) was defined based on the Common Terminology Criteria for Adverse Events version 5.0) (CTCAE5.0)[19] (ALT/AST levels >5.0–20.0 x ULN if the baseline was normal; >5.0–20.0 x baseline if the baseline was abnormal at any time point during maintenance therapy). Severe myelotoxicity/neutropenia was defined as ANC (Absolute Neutrophil Count) below 500/mm^3. Early and late myelotoxicity were documented during 1–3 and 4–6 months, respectively, after maintenance therapy. Overall survival (OS) was calculated from the start of maintenance therapy to the date of death or the last follow-up as applicable. Event Free Survival (EFS) was defined as the percentage of patients who were alive without relapse or death at last follow-up through maintenance therapy. Relapse Free Survival (RFS) was defined as the percentage of patients who had no relapse event at last follow-up from the start of maintenance therapy. Relapse and survival were documented for the prospective cohort only.

Statistics

All statistical analyses were performed using SPSS (IBM SPSS statistics version 21.0, Armonk, NY) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA); p-value <0.05 was used for significance testing. Comparison of clinical response indices such as myelotoxicity (ANC), leukotoxicity (WBC), hepatotoxicity (ALT/AST levels), and %6-MP dose intensities with genotype was done using one-way ANOVA or Mann–Whitney *U*-test. Associations between genotypes and clinical responses were evaluated using a linear regression model, and multivariate analysis was performed with significant covariates from univariate linear regression model. Haplotype analyses for all genes were performed using SNPStats programs (Institut Català d'Oncologia, Barcelona, Spain). We used Firth logistic regression to test the association between polymorphism and outcomes (R Statistical software version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria). Log-rank Cox regression was used for the survival analysis, and the Kaplan– Meier curves were generated for OS, EFS, and RFS.

Results

Patient Demographics

The baseline characteristics of patients included in cohort-1 (n=42) are listed in Table 1. During the study period, 241 patients with ALL underwent maintenance therapy in our center. Of these, patients who gave consent to participate in the study and those with regular follow-up were prospectively enrolled (cohort-2; n=133). The study design is illustrated in Figure 1. There was no significant difference in demographics between the patients enrolled in the study and those not enrolled (Table S1). Most of the patients had B-ALL (82.5%) and belonged to intermediate cytogenetic risk (58%) based on stratification reported previously.[20,21]

Genotype

None of the patients carried *TPMT* polymorphisms in cohort-1, while four patients (3%) were heterozygous for the *TPMT**3C variant in cohort-2. Nineteen patients in cohort-1 had *NUDT15 c.415C>T* variant (14 heterozygous and five homozygous), while 18 in cohort-2 were heterozygous for this variant. None of the patients included in the study had polymorphisms in exon 1 of the *NUDT15* gene. The allelic and genotypic frequencies are tabulated in Table S2.

6-MP Dose

In cohort-1, eight patients (19%) required drastic 6-MP/MTX dose reduction (less than 50%), 20 (47.6%) received 50 −80% of the total dose, and 14 (33.4%) received more than 80% of the planned dose of 6-MP.

The majority of the patients in cohort-2 (n=93; 70%) received more than 80% of the planned dose while 36 (27%) received 50–80% of the full dose, and four (3%) patients received less than 50% of the total planned dose of 6-MP.

Incidence of Myelotoxicity and Hepatotoxicity

Although all the 42 patients in cohort-1 had severe myelotoxicity, 16 patients (38%) had severe neutropenic episodes [median ANC<500 (range 32–504/mm^3)], while remaining 26 (62%) had a median ANC of 1100 (range: 528–1760/mm^3) during the study period. Three patients (7%) had a high ALT level (Grade 3), and one patient (2%) had a high AST level (Grade 3) based on NCI CTCAE criteria.

Thirty-six patients (27%) in cohort-2 had severe neutropenic episodes [median ANC<500 (range: 36–507/mm^3)]. Twelve patients (9%) had high ALT levels (Grade 3), and two patients (2%) had high AST levels (Grade 3).

Further outcome analysis was carried out only in cohort-2, where 21 patients (16%) relapsed during or after maintenance therapy, and the 3-year RFS in this cohort was 84.2% at a median follow-up of 18 (6–38) months. One hundred and twenty-eight patients were

Table 1 Baseline Characteristics of Patients

Patient Parameters	Cohort-1 (n=42) N (%)	Cohort-2 (n=133) N (%)
Median Age at Diagnosis (range)	16.5 (2–56)	17 (1–63)
Sex		
Male	35 (83.3)	88 (66)
Female	07 (16.7)	45 (34)
BSA (m^2)	1.5 (0.5–2.2)	1.5 (0.4–2.1)
Immunophenotype		
B cell	31 (73.8)	111 (83.4)
T cell	09 (21.4)	20 (15)
MP-ALL	–	1 (1)
NA	2 (4.7)	1 (1)
Cytogenetics Risk		
Favourable	6 (14.2)	25 (18.7)
Intermediate	25 (59.5)	81 (61)
Poor	7 (16.7)	7 (5.3)
NA	4 (9.6)	20 (15)
ALL Risk		
Standard Risk	17 (40.4)	20 (15)
Intermediate Risk	20 (47.6)	70 (52.6)
High Risk	5 (12)	5 (3.8)
NA	–	38 (28.6)
Polymorphisms		
TPMT variants		
***2A**		
Homozygous reference	42 (100)	130 (100)
Not available	0	3 (-)
***3A (*3B&C)**		
Homozygous reference	39 (100)	123 (96.8)
Heterozygous variant	–	4 (3.2)
Not available	3 (-)	6 (-)
ITPA c.94 C > A		
Homozygous reference	37 (88)	97 (76.4)
Heterozygous variant	5 (12)	28 (22)
Homozygous variant	–	2 (1.6)
Not available	–	6 (-)
MRP4 c.2269G>A		
Homozygous reference	32 (76.2)	107 (80)
Heterozygous variant	9 (21.4)	13 (10)
Homozygous variant	1 (2.4)	2 (2)
Not available	–	11 (8)
NUDT15 c.415C>T		
Homozygous reference	23 (54.7)	115 (86)
Heterozygous variant	14 (33.3)	18 (14)
Homozygous variant	5 (12)	–

alive at the time of the last follow-up. The 3-year OS and EFS were 96.2 and 83.5%, respectively, at a median follow-up of 18 (6–38) months.

NUDT15, TPMT, ITPA, MRP4 Polymorphisms, and 6-MP Dose Reduction/Therapy Intervention

Cohort-1

Although all the patients in cohort-1 had neutropenic episodes and required dose reduction, the *NUDT15 c.415C>T* was identified only in 19 patients and *ITPA* c.94C > A in 3 and *MRP4* c.2269G>A in 2 patients, respectively. While five patients homozygous for *NUDT15* c.415C>T polymorphism were highly sensitive to 6-MP and tolerated only 25% of the median dose intensity (range 14–34% of the protocol dose), those with heterozygous (n = 14) or wild-type genotype (n = 23), tolerated an average dose intensity of 58% (31–94) and 77% (46–96), p= 0.040 and 0.0005, respectively (Figure 2A).

With respect to therapy intervention, patients in cohort-1 who were homozygous for *NUDT15* c.415C>T polymorphism had the maximum duration of cessation of 6-MP therapy [median: 80 (25–94) days] compared to patients who were heterozygous or wild type [21 (7–54) and 28 (7–70) days, p=0.080 and 0.009, respectively] (Figure 2B).

Cohort-2

None of the patients in cohort-2 were homozygous for *NUDT15* c.415C>T polymorphism. Patients heterozygous (n=18 (14%)) for *NUDT15* c.415C>T polymorphism were sensitive to 6-MP compared to those with wild type (n = 115) genotype [%6-MP dose intensity: 82% (45–96) vs 90% (27–100), p=0.034] (Figure 2C).

None of the other polymorphisms screened (*TPMT**3C, *ITPA* c.94 C > A, and *MRP4* c.2269 G>A) were significantly associated with 6-MP dose intensity. With respect to therapy intervention, patients who were heterozygous for *NUDT15* c.415C>T polymorphism had the maximum duration of cessation of 6-MP therapy compared to those who were wild type [23 (0–67) vs 14 (0–67) days, respectively, p=0.033] in cohort-2 (Figure 2D).

In the multivariate analysis, there was no significant association between NUDT15 c.415C>T polymorphism and 6-MP dose reduction (Table 2).

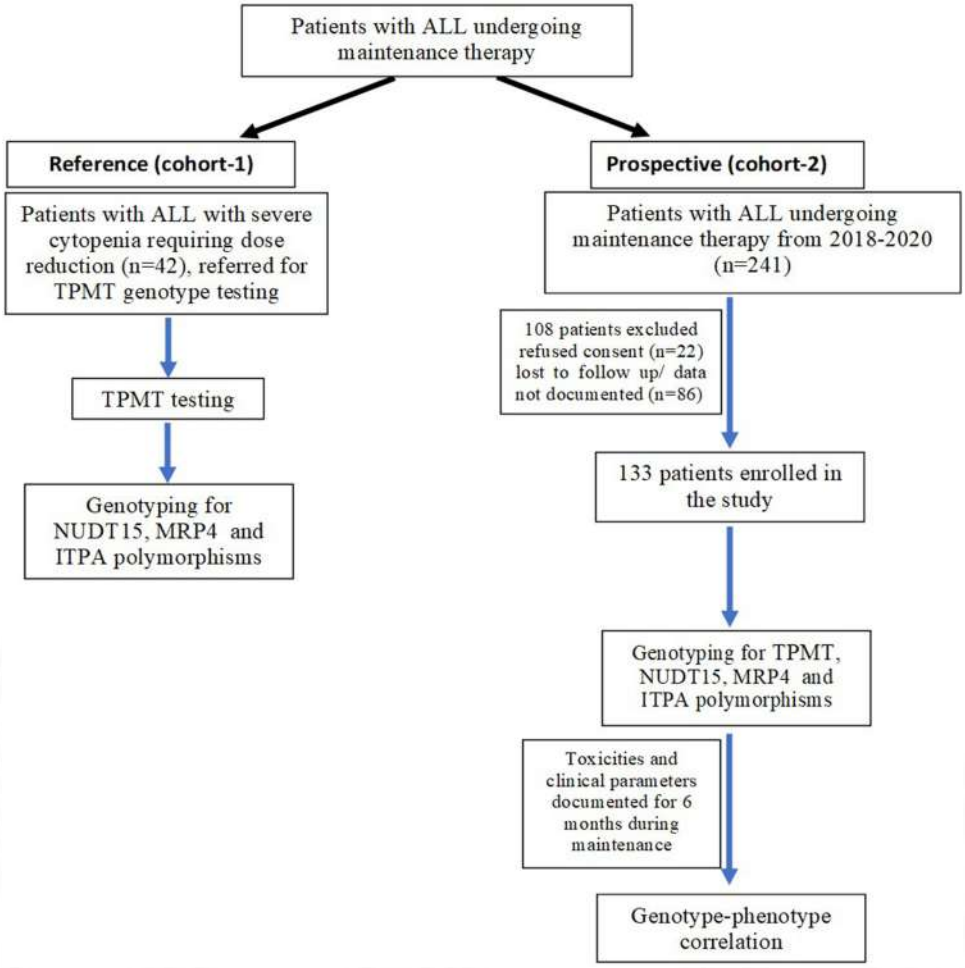

Figure 1 Study design.

NUDT15, ITPA, and TPMT Polymorphisms Explain Myelotoxicity but Not Hepatotoxicity

We then evaluated the association between these polymorphisms and myelotoxicity, hepatotoxicity, relapse, and survival. In cohort-1, patients who were homozygous/heterozygous for *NUDT15* c.415C>T polymorphism had significantly lower ANC [Median ANC-504 (32–1443) mm^3] compared to patients with wild-type genotype [988 (280–1760) mm^3, p=0.006].

In cohort-2, patients who were heterozygous for *NUDT15* c.415C>T polymorphism had significantly higher early (Median ANC: 507 vs 1298 mm^3; p<0.0001) and late myelotoxicity (Median ANC: 982 vs 1517 mm^3; p=0.015) compared to patients carrying wild-type genotype (Figure 3). Additionally, *ITPA* c.94C > A and *TPMT**3C polymorphisms also contributed to myelotoxicity; patients who had risk allele for *TPMT**3C and *ITPA* c.94C > A polymorphism had significantly lower ANC (Median ANC: 376 vs 1014 mm^3; p=0.04 and 776 vs 1023 mm^3; p=0.04 respectively) during maintenance therapy (Figure S1). Upon multivariate analysis (cohort-2), although *NUDT15* c.415C>T, and *TPMT**3C (β=−0.209, p=0.02) were significantly associated with myelotoxicity, *NUDT15* c.415C>T was the most significant variable associated with both early (β=−0.314, p<0.0001) and late myelotoxicity (β=−0.197, p=0.018) (Table 2). None of the polymorphisms tested, including *NUDT15*, was associated with hepatotoxicity, relapse (RFS), and survival (OS & EFS) (Figure S2).

Interestingly, a significant proportion of patients (10 of the 36 patients with severe myelotoxicity in cohort 2 (28%)) carried no variants for any of the screened polymorphisms but still had severe neutropenia [Median ANC-214 (88–420 mm^3)]. Also, 53% of patients (n=21) who received ≤80% of the planned dose of 6-MP [Median % 6-MP intensity-67 (45–79)] did not bear any genetic polymorphisms.

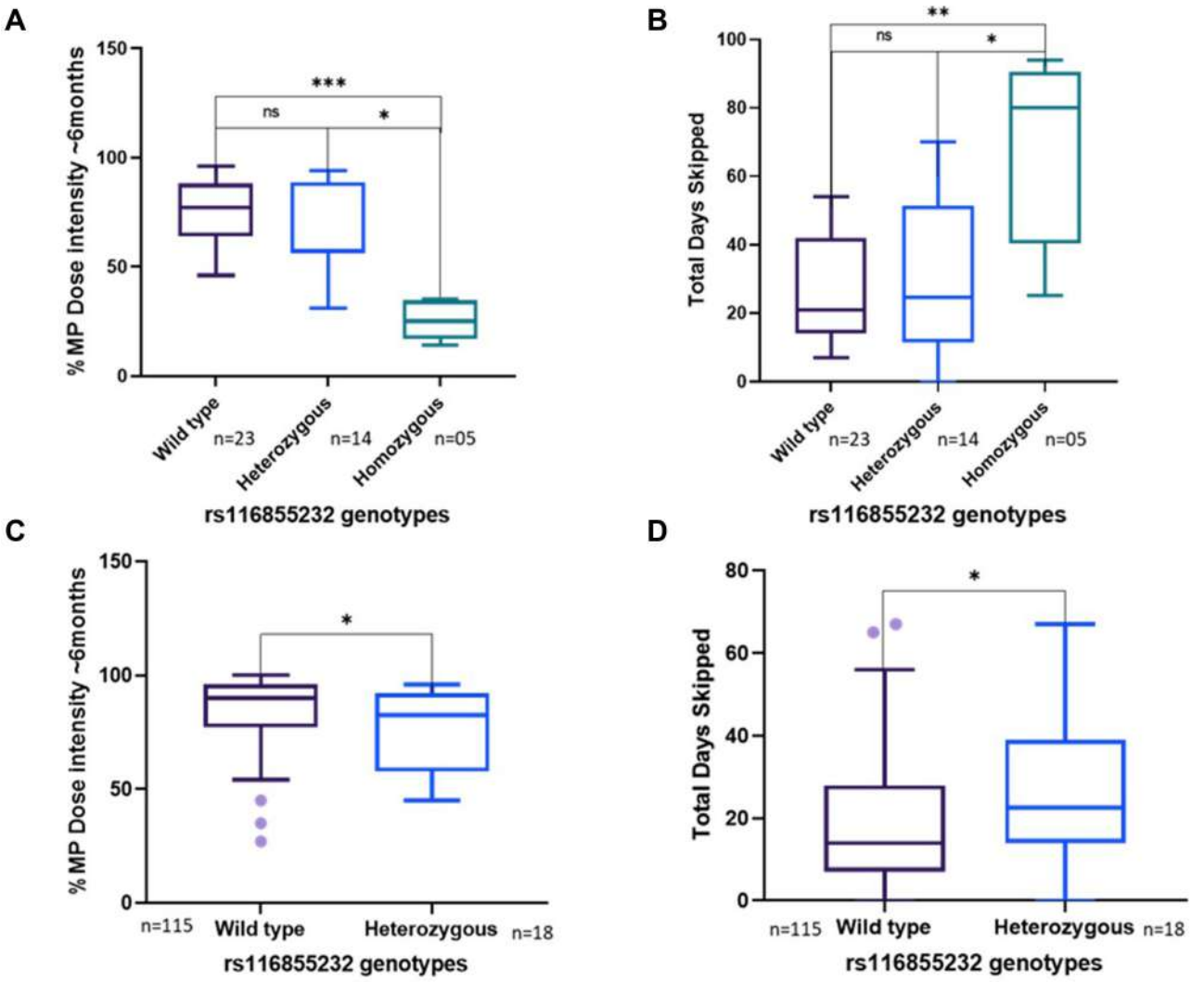

Figure 2 Associations between *NUDT15* c.415C>T polymorphism (rs116855232) and % 6-MP dose intensity (**A**) and therapy interruption (**B**) in cohort-1 and cohort-2 (**C**) and (**D**). *Asterisks indicate the level of the significance (p-value); *Means p< 0.05, **Means p< 0.01, ***Means p< 0.001 and ns-not significant.

Combined Effect of Genotypes on 6-MP Intolerance and Toxicities

To evaluate the combined effects of *TPMT, NUDT15, ITPA*, and *MRP4* variants on 6-MP intolerance and toxicities, the patients in cohort-2 with different genotype combinations were grouped-

Group 1- patients wild type for NUDT15, TPMT, ITPA, and MRP4 (n=66);

Group 2- patients with *MRP4* c.2269G>A alone (n=11);

Group 3- patients with *ITPA* c.94 C > A alone (n=21) and

Group 4- patients with *NUDT15* c.415C>T alone (n=12).

Patients carrying the risk allele for *NUDT15* c.415C>T polymorphism alone (Group 4) had increased myelotoxicity (OR-7.33; 95% CI-0.72–3.33; p=0.002) compared to Group 1. Similarly, patients harboring the risk allele for *ITPA* c.94 C > A polymorphism alone (Group 3) also had increased myelotoxicity (OR-3.49; 95% CI-0.12–2.31; p=0.030) compared to patients in Group 1.

Cost Analysis

We then compared the total direct hospital costs incurred during the first 6 months of maintenance therapy between patients with wild-type genotype and those with heterozygous or homozygous for *NUDT15* c.415C>T polymorphism among all patients (Cohort-1 + Cohort-2). The median total direct hospital cost incurred during the first 6 months of maintenance therapy was higher for patients with heterozygous or homozygous compared to patients with wild-type genotype for *NUDT15* c.415C>T polymorphism [20245 (IQR: 12192 to 28746 INR) vs 14356 (IQR: 9939 to 19894 INR), p=0.07]

Table 2 Association Analysis of Genetic Variants and Clinical Response in Prospective Patients with ALL Undergoing Maintenance Therapy

Clinical Response/ Toxicities	Association Analysis*			
Covariates	**Univariate Linear Regression**		**Multivariate Regression**	
	β	**p value**	**β**	**p value**
% MP Dose intensity				
Age	−0.195	0.063	−0.044	0.760
ALL risk group	−0.228	0.027	−0.169	0.134
BSA	−0.191	0.028	−0.166	0.265
MRP4 c.2269G>A	−0.178	0.050	−0.029	0.789
NUDT15 c.415C>T	−0.173	0.047	0.138	0.203
ITPA c.94 C > A	0.155	0.081	0.137	0.200
Myelotoxicity				
Early				
Age	0.162	0.063	−0.062	0.590
BSA	**0.245**	**0.005**	**0.263**	**0.024**
NUDT15 c.415C>T	**−0.328**	**<0.001**	**−0.314**	**<0.001**
Late				
*TPMT**3A	**−0.181**	**0.042**	**−0.209**	**0.025**
NUDT15 c.415C>T	**−0.191**	**0.028**	**−0.197**	**0.018**

Notes: *Amongst covariates tested in both univariate and multivariate regression, only significant variables are listed above. Bold values denote statistical significance at the p ≤ 0.05 level.

Discussion

The present CPIC guidelines[6] recommend *NUDT15* and *TPMT* testing in patients before thiopurine therapy to prevent dramatic myelotoxicity. The frequency of these genetic polymorphisms is noticeably lower in the Indian population than in other populations (Table S3). Therefore, we aimed to explore the role of additional genetic variants over and above CPIC recommended genetic variants. This is the first single centre study to explore the association between *NUDT15* (c.415C>T), *TPMT* (G238C, G460A & A719G), *ITPA* (c.94C>A), and *MRP4* (2269 G>A) polymorphisms vs early and late myelotoxicity to 6-MP in Indian patients with ALL on final maintenance therapy.

Similar to previous reports from other Asian studies[22] and ours,[7] except for the rare occurrence of the *TPMT**3C variant, other *TPMT* polymorphisms were not identified in this cohort. The frequency of the *NUDT15* c.415T allele in the present study is similar to previous studies in India,[15–17] but higher than that of the West Asian population,[23] and lower than East Asian population[24–26] (Table S3).

Patients belonging to Asian ancestry with *NUDT15* c.415C>T polymorphism have been shown to be poor metabolizers of 6-MP and may show intolerance to the drug.[27] Patients homozygous for *NUDT15* c.415C>T polymorphism in the present study tolerated ~25% of the total planned 6-MP dose, which is higher than the previously reported studies[4,5] and the current recommended CPIC guidelines on 6-MP dosing.[4–6] This discrepancy in 6-MP dose intensity (25% in the present study vs 8%[4,5] as per previous reports) can be attributed to different treatment practices. Apart from the *NUDT15*3*, we failed to identify additional polymorphisms in *NUDT15* exon 1 that has been reported to play role in 6-MP intolerance.[28,29] It is possible that additional confounding genetic/non-genetic factors could also contribute to increased tolerance,

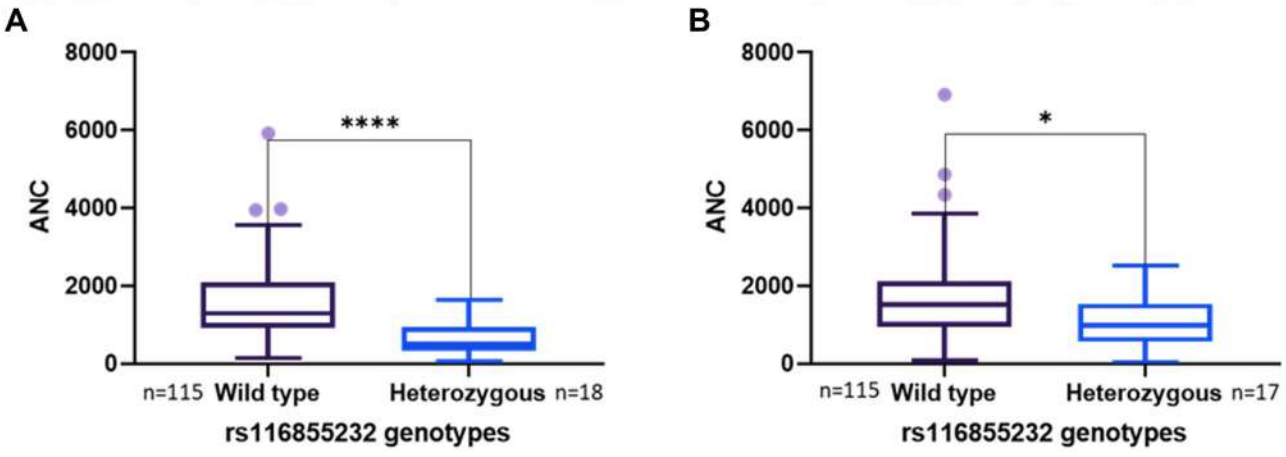

Figure 3 Associations between *NUDT15* c.415C>T polymorphism (rs116855232) and (**A**) Early, (**B**) Late Myelotoxicity. *Asterisks indicate the level of the significance (p-value); *Means p< 0.05 and ****Means p<0.0001.
Abbreviation: ANC, absolute neutrophil count.

especially in patients homozygous for *NUDT15* c.415C>T polymorphism in the Indian population compared to other ethnicities, which needs to be further evaluated.

Myelotoxicity is a common adverse drug reaction during 6-MP maintenance therapy that may result in frequent febrile neutropenic episodes affecting treatment outcomes, risk of relapse, and quality of life.[30] Similar to previous studies,[15,25–27,31,32] patients who were homozygous for *NUDT15* c.415C>T polymorphism experienced a significantly higher incidence of marrow toxicities and, as a result, experienced considerably more therapy interruptions/6-MP dose reduction in comparison to patients who were wild-type or heterozygous for this polymorphism (cohort-1). Although there were no patients with homozygous genotype for *NUDT15* c.415C>T polymorphism in the prospective cohort, patients with heterozygous genotype experienced moderate/severe neutropenia leading to considerable dose reduction (Table 3). Although the *TPMT* polymorphisms were rare in our study cohort, patients who were heterozygous for *3C polymorphism experienced significantly increased neutropenic episodes similar to previous studies,[33–35] but no significant 6-MP dose reduction.

ITPA is another crucial enzyme in thiopurine detoxification whose enzyme activity is genetically determined.[11] A missense variant (c.94 C > A) was observed to be a significant determinant of mercaptopurine metabolism and severe febrile neutropenia.[35–37] Consistent with previous reports,[9,10,22] we observed that patients with variant genotype for ITPA c.94 C > A polymorphism experienced neutropenic episodes due to low ANC. Unlike previous studies[13,38] that showed *ITPA* c.94 C > A polymorphism to be associated with lower EFS, we did not find this association in the present study.

A missense polymorphism (c.2269G>A) identified in the *MRP4*/ATP binding cassette subfamily C member-4 (*ABCC4*) has been shown to dramatically enhance 6-MP sensitivity, especially in the Asian population.[39] Tanaka et al[10] have reported a high frequency of 6-MP dose reduction in Japanese children with ALL bearing homozygous variant allele for this polymorphism. These authors subsequently reported a higher incidence of leukopenia in children with risk allele for *MRP4* c.2269G>A polymorphism but not with %6-MP dose intensity.[24] Similar to these reports,[10,24] we observed that patients carrying risk allele for *MRP4* c.2269G>A polymorphism experienced leukopenia (data not shown), albeit no 6-MP dose reduction. Our data suggest the limited clinical utility of *MRP4* pharmacogenetics as compared to *NUDT15* /*TPMT* in ALL.

In addition, we also identified a significant proportion of patients with severe neutropenia yet did not bear any genetic variants screened in this study, suggesting that additional genetic polymorphisms related/unrelated to the thiopurine metabolic pathway could explain 6-MP intolerance/toxicities. It is now well acknowledged that severe neutropenia can also be attributable to methotrexate intolerance[15,40] during maintenance therapy, which was not evaluated in the present study. An exploratory approach to identify additional genetic variants in patients who required dose reduction due to toxicities with no polymorphisms in *NUDT15, ITPA, TPMT*, or *MRP4* genes is ongoing in our laboratory.

Table 3 Influence of *NUDT15* c.415C>T Polymorphism in Early 6-MP Induced Toxicities (Cohort-2)

Clinical Response	Patients with *NUDT15* c.415C>T Polymorphism		
	Wild Type, N=/115 (%)	Heterozygous, N=/18 (%)	Odds Ratio (95% CI), p-value*
MP dose reduced (<100%)	99 (86)	18 (100)	1.6 (−0.56–1.46), 0.361
% MP Dose intensity			
>80%	66 (57)	11 (61)	
50–80%	30 (26)	6 (33)	
<50%	3 (3)	1 (6)	
Neutropenia (ANC <1500 mm^3)	84 (73)	18 (100)	**13.79 (0.57–7.48), 0.005**
Severe neutropenia (ANC <500 mm^3)	24 (21)	12 (67)	**7.18 (0.95–3.07), 0.0001**
Dose interruption	98 (85)	17 (94)	2.07 (−0.75–2.97), 0.373

Notes: *ORs and p-values were calculated using penalized likelihood test-Firth logistic regression method using R. Bold values denote statistical significance at the p ≤ 0.05 level.

Neutropenia and other thiopurine-induced toxicities culminate in increased cost of maintenance therapy. We observed that patients with heterozygous or homozygous NUDT15 c.415C>T polymorphism had higher maintenance therapy-related costs. Therefore, pre-emptive genotype-based (*NUDT15* c.415C>T, TPMT) dosing could benefit Indian patients by decreasing the cost of maintenance therapy, minimizing hospital visits and therapeutic interventions.

The major limitation of the study is its retrospective nature, wherein none of the patients received tailored dosing based on genotype, including *NUDT15* c.415C>T polymorphism. Other limitations include small sample size, wide window of the study period, and lack of validation cohorts. MTX is administered along with 6-MP, which also possesses similar myelotoxicity and hepatotoxicity.[41] However, the present study did not address the role of MTX pharmacogenetics, which again is a major limitation.

Conclusion

Our results suggest that *NUDT15*3, TPMT*3C*, as well as *ITPA* c.94 C > A and *MRP4* c.2269 C>T polymorphisms explain hematological toxicity to 6-MP in patients with ALL undergoing maintenance therapy. Genotyping for *NUDT15, TPMT,* and *ITPA* is routinely performed in ALL patients undergoing maintenance therapy as well as in patients on thioguanine therapy for other conditions including AHA, ITP, inflammatory bowel disease (IBD), and autoimmune disorders at our centre. Preemptive genotype based (*NUDT15*3, TPMT, ITPA* c.94 C>A) 6-MP dosing could improve the outcome after maintenance therapy. Further whole-genome sequencing in patients with severe myelotoxicity but not carrying *TPMT* or *NUDT15* variants is ongoing to identify potential genetic risk for intolerance to maintenance therapy in ALL.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

Approval was obtained from the Institutional Review Board [IRB (EC)-ER-1-23-07-2014] of Christian Medical College, Vellore, India. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Informed Consent

Informed consent was obtained from all individual participants/legal guardians included in the study.

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Author Contributions

All authors contributed to data analysis, drafting, and revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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Biomarkers for early complications post hematopoietic cell transplantation: Insights and challenges

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Hematopoietic cell transplantation is an established curative treatment option for various hematological malignant, and non-malignant diseases. However, the success of HCT is still limited by life-threatening early complications post-HCT, such as Graft Versus Host Disease (GVHD), Sinusoidal Obstruction Syndrome (SOS), and transplant-associated microangiopathy, to name a few. A decade of research in the discovery and validation of novel blood-based biomarkers aims to manage these early complications by using them for diagnosis or prognosis. Advances in this field have also led to predictive biomarkers to identify patients' likelihood of response to therapy. Although biomarkers have been extensively evaluated for different complications, these are yet to be used in routine clinical practice. This review provides a detailed summary of various biomarkers for individual early complications post-HCT, their discovery, validation, ongoing clinical trials, and their limitations. Furthermore, this review also provides insights into the biology of biomarkers and the challenge of obtaining a universal cut-off value for biomarkers.

KEYWORDS
biomarker, GVHD, endothelial, SOS, HSCT

Introduction

Hematopoietic cell transplantation (HCT) from matched related or unrelated donors to recipients with various hematological disease conditions has become a widely accepted curative treatment of choice. Especially with malignant hematological diseases, the graft versus tumor/leukemia effect (GVT) is a beneficial phenomenon expected to improve the outcome of the procedure. However, a similar effect where graft acting against the recipient's cells, such as graft

versus host disease (GVHD), leads to an undesirable outcome. Graft versus host disease (GVHD) still remains a predominant cause of morbidity and mortality in patients following HCT. Clinically GVHD may present as acute (aGVHD) or chronic (cGVHD) based on the symptoms and time of their presentation. The classical pathway of occurrence of GVHD includes damage of the target organs such as skin, eye, gastrointestinal (GI) tract, liver, or lung, followed by the release of a storm of cytokines, which increases the chance of the donor's immunocompetent cells to recognize the host's alloantigens (1). More than half of HCT patients develop GVHD. Although GVHD is treated by several immunosuppressive agents, responsiveness to these agents, GVHD related morbidity and mortality are still concerns that affect HCT outcomes greatly. In addition to GVHD, other serious complications include hepatic or pulmonary sinusoidal obstruction syndrome (SOS), opportunistic infections (bacterial, viral & fungal), and multiorgan damage. Attempts to improve HCT outcomes include predicting patients who are at high risk of developing post HCT complications, predicting their responsiveness to treatment and early diagnosis of these complications. Composite biomarkers of prognostic values have been recently used in confirming the diagnosis of some of these complications (2, 3).

Excluding the known likely causal factors for some of the adverse effects (such as the donor status, age, comorbidity, sex mismatch between donor and recipient, conditioning, and post-HCT immunosuppressive drug levels), various centers performing allogeneic HCT are concentrating on finding efficient, reliable and robust markers from biological fluids for informative, early detection or differential diagnosis of these complications to optimize the treatment as well as improving the outcome (4–6). Many have successfully reported a variety of blood plasma, serum, and fecal biomarkers, while only a handful of these is repeatedly tested and validated and likely to be used as a biomarker routinely (7). The biomarkers from these sources may be soluble factors, cellular markers, or genetic markers. While many candidate biomarkers from plasma were evaluated and verified in independent cohorts, multi-center clinical trials are still needed to validate their clinical applicability. Similarly cell-free DNA have also been recently evaluated for identifying an array of post-HCT complications including aGVHD, relapse, infection, engraftment failure and chimerism status with an objective of employing a single test/technique for elucidating a comprehensive panel of post-HCT complications (8).

However, one of the major limitations of these biomarker studies is the varying cut-off value as a reference to predict or diagnose these complications between centers. Moreover, and not all biomarkers are referenced across the normal cut-off values between healthy individuals and patients undergoing transplantation. Often these biomarkers are tested between HCT patients with and without complications. This review provides insights into the biological significance of biomarkers, their discovery and validation for HCT complications, challenges in quantification or techniques, and lack of universal target cut-offs.

The biological significance of biomarkers

Plasma biomarkers have been extensively evaluated for complications post HCT, since classic clinical risk scores such as HCT related co-morbidity index often fail to predict, diagnose or prognose such complications. The ultimate aim of plasma biomarker evaluation is its clinical translatability in predicting HCT complications, their severity and their response to treatment. On the other hand understanding the biology of these biomarkers would also pave way for developing more rational and effective treatment strategies for HCT complications. However, literature on biology of the biomarkers for HCT complications appear scanty. While increasing evidences suggest endothelial injury as a common cause for most HCT complications, a complete understanding is still lacking. Here we review the biology of a few biomarkers which are extensively evaluated for multiple overlapping complications.

ST2

The suppression of tumorigenicity 2 (ST2) is a receptor belonging to the interleukin (IL)-1 family and binds specifically to IL-33. ST2 is present in two isoforms: a transmembrane form and a soluble form. The membrane-bound ST2 receptor is expressed on various hematopoietic cells such as T helper 2 (Th2) cells, natural killer (NK) cells, mast cells, antigen-presenting cells, and regulatory T cells (Tregs) (9, 10). The IL33/ST2 complex signaling in these cell types has been observed to have proinflammatory and anti-inflammatory responses depending on the disease type (11–13). During acute GVHD, a surge in IL33 has been observed both in the clinical scenario and in mice models of alloHCT. The mucosal barrier tissues, such as the skin, gastrointestinal tract, and liver, have been significant sources of IL33. During the alloHCT conditioning regimen, damage to these tissues increases IL33 production/release that drives donor Th1 cells expansion leading to inflammatory phenotype and further tissue injury. Recently, it was demonstrated that IL-33 acts directly on donor T cells and increases Tbet expression leading to enhanced Th1 cell polarization and expansion. However, despite these observations of elevated IL-33, this could not be used as a specific biomarker for aGVHD due to its pleiotropic effects.

The soluble ST2 (sST2) receptors are expressed in endothelial cells, epithelial cells, fibroblasts, and T cells (14). The soluble ST2 act as decoy receptors, sequestering free IL-33, thereby preventing IL-33-mediated proinflammatory actions (15, 16). Thus, sST2 was generally considered to negatively regulate IL-33 function (Figure 1). However, this contradicts the association of elevated sST2 with GVHD severity in patients. A possible explanation given by earlier studies was that the release of sST2 in the serum occurs very late in the inflammatory response resulting in the inability of sST2 to sequester circulating IL33 (17).

Zhang J et al. demonstrated in a minor mismatch GVHD model and xenograft GVHD model that sST2 was secreted by intestinal stromal cells, endothelial cells, and alloreactive T cells. More importantly, as GVHD progresses, it was shown that pathogenic T cells (Th17 and Tc17) secrete more sST2 and express less mST2, thereby correlating elevated plasma sST2 levels during alloreactivity. Transient blockade of sST2 during GVHD increased Th2 transcription factor GATA3 and cytokine IL-4, improving Th2 phenotype, which protects against severe GVHD (18).

An overall picture of the ST2/IL33 axis in a severe GVHD context remains elusive. Whether sST2 is involved in the pathophysiology of

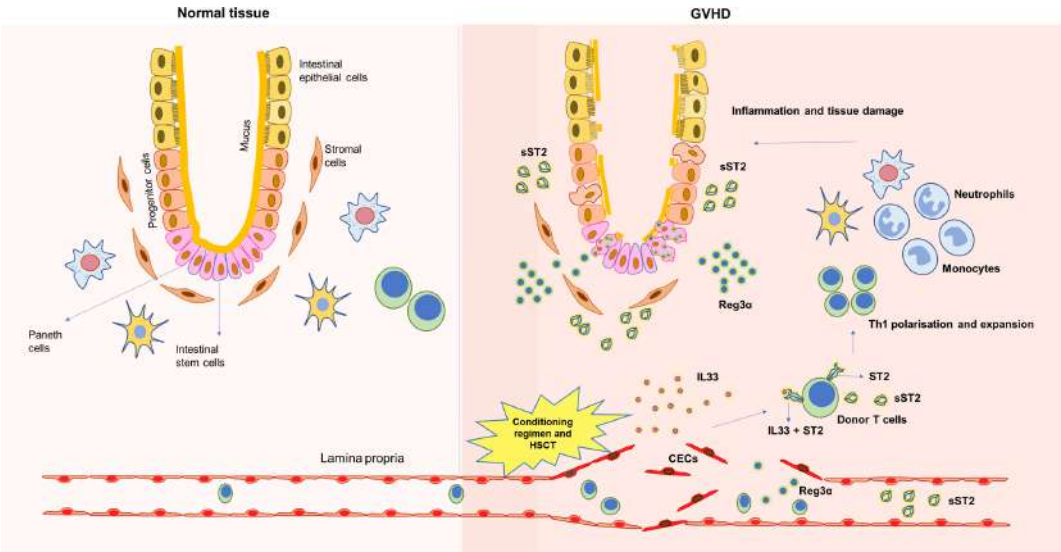

FIGURE 1
Underlying tissue damage during GVHD and release of soluble biomarkers. HSCT transplant procedures, including conditioning regimen, damage underlying endothelium, inflames the tissue and releases soluble factors that could be used as biomarkers during GVHD.

GVHD or it is just a circulating biomarker indicating GVHD severity remains to be clarified.

Reg3α

Regenerating islet-derived -3 α (Reg3α) alpha is one of the antimicrobial peptides secreted by Paneth cells of the gastrointestinal tract and is a C-type lectin having bactericidal actions on most gram-positive bacteria (19). The crypts' innate lymphoid cells 3 (ILC3) secrete IL22, which induces Paneth cells to secrete Reg3α (Figure 1) (20). During HSCT, the crypt cells, including the Paneth cells, are damaged; hence, their numbers are inversely associated with GVHD severity (21). GVHD-induced damage to the gastrointestinal crypt and intestinal mucosa decreases IL22 production and releases antimicrobial peptides stored in these cryptic cells into the bloodstream. Thus, the increased plasma level of Reg3α was strongly associated with GI-GVHD enabling their use as a biomarker (22). It was also observed *in-vivo* in mice models of GVHD that the progression of GVHD suppresses Reg3γ (mouse homolog of human Reg3α) in the GI tract, further worsening GVHD. However, administration of IL22 has been shown to protect the crypt from damage, thereby preventing Reg3γ from being released into circulation. Mechanistically it was demonstrated that Reg3γ functions as an anti-apoptotic protein for intestinal stem cells (ISCs) and Paneth cells (23). Thus, Reg3α has the dual role of being an antimicrobial peptide as well as a survival signal preventing apoptosis of ISCs and Paneth cells.

While Il22 from host cells was recognized to promote intestinal stem cell survival and suppress GI-GVHD (24), a few studies have also shown that IL22 from donor cells augments GI-GVHD (25, 26). In a mouse model of steroid-refractory GVHD, by Song Q. et al. demonstrated that IL22 was produced by donor Th/Tc22 cells, leading to excess production of Reg3γ. However, such excess Reg3γ was shown to result in dysbiosis and worsening of GVHD. Thus, REG3γ could be a therapeutic target for treating steroid-refractory GVHD (27). Hence, whether Reg3alpha is a therapeutic target or a biomarker remains an enigma.

TIM3

T cell immunoglobulin and mucin domain 3 (TIM3) is a transmembrane receptor protein expressed on interferon γ producing T cells, Tregs, myeloid cells, natural killer cells, and mast cells (28). The primary function of TIM3 is to inhibit Th1 responses and cytokine expressions. Hence, its dysregulation correlates with most autoimmune diseases, such as multiple sclerosis (29) and type I diabetes (30). Increased expression of TIM3 has been observed in solid tumors such as lung cancer, gastric cancer, colon cancer, etc., and their high expression levels were associated with low overall survival (31).

Elevated levels of TIM3 in the plasma of patients with GVHD (32) and osteosarcoma (33) have been observed, facilitating their use as potential biomarkers. However, the mechanism of soluble TIM3 release remains an enigma. It could be a splice variant, a metalloproteinase-dependent cleaved product, or a soluble fragment from apoptotic cells. While soluble TIM3 was found to express as a splice variant in mice splenocytes (34), their existence in humans is still debatable.

The mechanistic understanding of TIM3's action in aGVHD remains incomplete and is not explored much. Oikawa et al. demonstrated in a murine model of GVHD that TIM3 plays a crucial role in the activation of CD8+ T cells, which are the primary effectors in target organ destruction in aGVHD. Two weeks post-transplantation, the CD8+ T cells in the spleen and liver of GVHD mice showed enhanced TIM3 and interferon γ (IFNγ) expression. Moreover, the CD8+T cell infiltration was dominant in

the liver of GVHD mice (35). However, the exact mechanism of TIM3 induction in these cells and their shedding into peripheral circulation remains unclear.

Elafin

Elafin is a biomarker associated with the diagnosis and prognosis of skin GVHD. It is an epithelial protein secreted by keratinocytes in response to inflammatory cytokines. Hence elafin's expression is higher in the inflamed epidermis and absent/low in the normal epidermis. It is a peptidase inhibitor-3 or skin-derived anti-leukoprotease (SKALP) with antimicrobial activity and priming innate immune responses (36). It was observed that when induced by GVHD mediating inflammatory cytokines, human keratinocytes express elafin significantly (37, 38). However, the mechanism by which elafin from keratinocytes is released into circulation during GVHD remains unclear.

Biomarkers for acute GVHD

Biomarkers for acute GVHD have been extensively evaluated over the past decade in multiple HCT centers worldwide. These range from plasma, cellular, genetic and in a few cases, a combination. Biomarkers for aGVHD have been measured pre-HCT to personalize GVHD prophylaxis, post-HCT before or at the onset of GVHD, to confirm the diagnosis of GVHD or after treatment to predict treatment response.

Biomarkers measured pre-transplant for modulating GVHD prophylaxis

CD86 is the ligand for costimulatory (CD28) and coinhibitory (CTLA-4) molecules. Karaban et al. reported that the recipients' CTLA-4 CT60GA[GG] genotype, myeloablative conditioning regimen, and use of an unrelated donor were independent predictors of acute GVHD (39). Also, the same group has shown that donor and recipient CTLA-4 mRNA and recipient membrane protein expression measured before transplantation are prognostic for acute GVHD (40). Later they also reported a lack of association of CD86 gene polymorphisms with GVHD. However, they noted a gene-gene interaction wherein patients with a specific CD86 genotype and a CTLA-4 genotype was associated with an increased risk of aGVHD. With a combination of specific donor CD86 genotype and recipient CTLA-4 genotype there was an elevated GVHD risk (41).

In a study exploring the role of donor genetic variations in glucocorticoid pathway on steroid responsiveness of GVHD, although donor SNPs in *ZAP70* and *DUSP1* genes were associated with response, these were not statistically significant on adjustment for multiple testing (42). Cytokine biomarkers – TLR4 and TNFR1 are significantly increased in steroid-refractory acute GVHD compared to those with steroid-responsive GVHD (43).

DNAX accessory molecule-1 (DNAM-1, or CD226) is a leukocyte adhesion molecule constitutively expressed on most CD4+ T cells, CD8+ T cells, natural killer (NK) cells, and monocytes. A retrospective study from Japan showed that higher soluble DNAM-1 measured between day -7 to day 0 of an allotransplant was predictive of a higher risk of acute GVHD. Using a cut-off of 30pM for soluble DNAM-1, the sensitivity for predicting acute GVHD was 43.8%, while the specificity was 82.6% (44). Serum IL-6 levels measured pre-conditioning and one week after transplant were predictive of acute GVHD and transplant-related mortality (45), although, the pleiotropic nature of IL-6 may be a concern. Specific donor graft characteristics like an elevated proportion of T cells with low CD127 and high PD-1 expression have been associated with subsequent acute GVHD (46).

Biomarkers measured post-transplant before or at GVHD onset

Specific patterns of immune reconstitution following transplantation, such as increased CD8+ T cells (both naïve and memory) in the early post-transplant period (on day 15), have been associated with development of subsequent acute GVHD (47).

Elevated plasma REG3α measured at the onset of GVHD predicted non-response to treatment at 4 weeks and also 1 year non-relapse mortality (22). Elevated plasma elafin at the onset of skin GVHD is associated with higher maximum grade of GVHD and also non-relapse mortality (48). Also, elevated ST2 predicts steroid resistance in acute GVHD and non-relapse mortality (4). In non-myeloablative transplants, elevated plasma ST2, REG3α, and elafin measured early post-transplant were predictive of acute GVHD (49). Similarly, in T-replete haplotransplants, elevated plasma ST2 and REG3α measured early post-transplant were predictive of acute GVHD and non-relapse mortality (50). In patients undergoing matched donor T deplete transplantation using anti-thymocyte globulin or alemtuzumab, a biomarker panel including HGF, elafin, sIL-2Rα, sTNFR1, and REG3α was predictive of GVHD and its severity (51).

Lower serum sIL-27Rα at the time of neutrophil engraftment is predictive of acute GVHD and has been shown to correlate with other serum GVHD biomarkers (52). Elevated plasma levels of sIL2-Rα and TIM-3 in the early post-transplant period predicted increased transplant-related mortality and acute GVHD (53).

Similarly, expression patterns of genes and a few microRNAs have also been evaluated as biomarkers post HCT. Transcripts levels of *FOXP3*, *ICOS*, *CD52*, and *CASP1* genes involved in alloreactive immune responses and immune cell interactions were predictive of acute GVHD using a personalized modeling-based gene selection (PMGS) method (54). A risk score developed using metabolite and transcriptome analysis incorporating 5 metabolite markers from glycerophospholipid metabolism was predictive of acute GVHD (55). miR-155 and miR-146a measured in target tissues at the time of GVHD onset and measured in extracellular vesicles in serum and urine in the early post-transplant period before GVHD onset have been predictive of acute GVHD (56).

Biomarkers measured at symptom onset for supporting the diagnosis of GVHD (elafin, calprotectin)

Fecal, and not serum calprotectin is a biomarker for acute gut GVHD and can potentially help diagnose gut GVHD (57). Also, low

tissue amphiregulin expression on immunohistochemistry has been reported in 74% of patients with acute gut GVHD and might aid in diagnosis without classic apoptotic changes (58). Similarly, tissue, and not plasma, elafin on immunohistochemistry can aid in diagnosing acute GVHD involving the skin (59, 60).

Analysis of exosomal miRNA expression using quantitative RT-PCR on plasma samples showed that miR-128 was elevated in late-onset GVHD and is a promising diagnostic marker of late-onset GVHD (61). However, the turnaround times for these biomarkers may limit their practical utility.

Biomarkers measured at the onset of GVHD and after treatment of GVHD for potentially predicting response

The Mount Sinai Acute GVHD International Consortium (MAGIC) algorithm probability score (MAP score) based on plasma ST2 and REG3α is a response biomarker for acute GVHD. After four weeks of therapy, it was shown to predict non-relapse mortality better than the change in clinical symptoms. The MAP score was predictive of non-relapse mortality within every clinical grade of acute GVHD (62). The MAP score has been shown to be helpful when measured at day 28 along with the disease risk index could also identify patients at high relapse risk and low non-relapse mortality risk who can potentially benefit from strategies to enhance the graft versus leukemia effect for relapse prevention (63). Rising REG3α following treatment for GVHD using a novel combination of upfront steroids+ruxolitinib was shown to be a predictor of refractory GVHD (64). However, there is no prospective clinical study on biomarker-based intervention for adding second-line therapy for acute GVHD. A list of various biomarkers measured at different stages during HCT procedure and/or at GVHD onset, with potential clinical values that could help in prediction, diagnosis or prognosis for acute GVHD is summarized in Table 1.

Biomarker guided pre-emptive therapy for GVHD

Initial discovery and validation of ST2 as a biomarker for aGVHD also led to studies investigating inhibitors for ST2 in animal models (65). While this is still in progress, biomarker evaluation has progressed towards guided therapy/intervention for aGVHD with already existing anti-GVHD strategies. Gergoudis et al. have recruited patients at high risk for developing steroid-refractory GVHD (SR-GVHD) based on the MAGIC algorithm probability (MAP) scores on days 7 and 14 post-HSCT. These patients were then treated with alpha-1 anti-trypsin (AAT), a serine protease inhibitor with proven activity against GVHD. Although AAT treatment was well tolerated, the incidence of SR-GVHD was not lowered (66). Nevertheless, the power of biomarker-based SR-GVHD prediction could not be undermined. Instead, such studies pave the way for investigating more treatment options. A recent study involving a prospective phase 2 trial stratified patients based on sIL-2Rα and IL-15 levels. High-risk patients (sIL-2Rα 4500 ng/L or IL-15 31 ng/L) were treated with rabbit anti-thymocyte globulin (ATG) 3 mg/kg on day 8 post-transplant and were compared with controls who had the biomarkers measured but did not participate in this interventional trial. A reduction in GVHD was observed in these patients compared to high-risk controls who did not receive ATG (Hazards ratio of 0.48), signifying the feasibility and effectiveness of such an approach (67).

Biomarkers for sinusoidal obstruction syndrome

Sinusoidal Obstruction Syndrome (SOS), previously, known as veno-occlusive disease, is a severe complication post HSCT affecting liver sinusoidal endothelial cells. About 13 to 20% of allogeneic HSCT recipients develop SOS and the severe form of SOS is associated with multiorgan failure and mortality (68).

Typically, SOS has been observed between one to three weeks post-HSCT. Often clinically indistinguishable from other causes of weight gain, ascites, abdominal pain, and jaundice.

Factors such as conditioning regimen drugs or radiation, releasing cytokines from injured tissues, and the endogenous microbial substances that cross the compromised mucosal barriers lead to the activation of sinusoidal endothelial cells. Sustained activation can progress to endothelium damage (69). The sinusoidal endothelial cells swell and round, forming gaps in the sinusoidal barrier. These alterations facilitate the egress of leucocytes, RBCs, and cellular debris into the perisinusoidal space beneath the endothelial cells and disrupt the endothelial lining leading to sinusoidal embolisms and obstruction of the sinusoidal flow, liver dysfunction, ascites ultimately leading to multiorgan failure (70, 71).

Some reliable markers of endothelial activation and damage are soluble cellular adhesion molecules (sVCAM1, sICAM1, and sP-selectin), coagulation factors (Von Willebrand factor (VWF), thrombomodulin (TM) and plasminogen activator type-1 (PAI-1)) (Table 2).

The microenvironment of the endothelium is significantly altered in patients who undergo allo-HCT. Allo-HCT patients who develop SOS have a significant increase in both VWF and TM levels (69, 84). Furthermore, in patients receiving both tacrolimus and sirolimus as GVHD prophylaxis, levels of VWF and TM (together with ICAM-1 and E-selectin level) serve as SOS predictive biomarkers one-week post HCT (69). Two weeks post- HCT, plasma levels of REG3α, sVCAM1, sICAM1, and TIM3 are shown to be consistently elevated in patients who developed SOS (80). P-selectin levels are shown to be selectively higher in patients who develop severe SOS and elevated circulating levels of PAI-1 allow differential diagnosis between SOS and GVHD, as patients with SOS show elevated PAI-1 but not those with GVHD (81, 85, 86).

In a recent study, a composite diagnostic panel of three biomarkers: L-Ficolin, hyaluronic acid (HA), and VCAM-1, was reported to detect patients at high risk of SOS as early as the first day after HCT, even before clinical manifestation of SOS (82). Additionally, it was proposed that the biomarker panel ST2, ANG2, L-Ficolin, HA, and VCAM-1 could be helpful in the diagnosis of SOS (82). Inflammatory cytokines such as IL2, IL6, IL33, IFNγ, and TNFα are mediators of EC activation and damage. Both TNFα and soluble IL2 receptor α (sIL2Rα) are shown to be elevated during GVHD and SOS (90, 93, 94).

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TABLE 1 List of various types of biomarkers with clinical values that could help in the prediction, diagnosis, or prognosis for Acute GVHD.

S.No	Biomarker	Type	Biological Specimen(s)	Detection Method(s)	Biomarker level(s)	Clinical Value	Ref(s)
1	CD86/CTLA4 polymorphisms	Immunological	DNA	Genotyping	Polymorphism	Predictive	(12)
2	DNAM-1/CD226	Immunological	Serum	ELISA	Increased levels	Predictive	(9)
3	IL-6	Immunological	Serum	ELISA	Increased levels	Predictive	(13)
4	CD127/PD-1	Immunological	Peripheral Blood	Flow cytometry	High frequency of PD-1$^+$ T cells and low frequency of CD127$^+$ T cells in donor graft associated with grades II-III aGVHD	Predictive	(14)
5	*FOXP3* and *ICOS*	Immunological	Peripheral Blood	RT-PCR	Low levels associated with aGVHD Increasing levels correlate with response to anti-GVHD therapy	Diagnostic /Prognostic	(21)
6	PAF, LysoPC, PE, PC, and LysoPE	Metabolic (Biochemical)	Plasma	LC-MS	aGVHD risk score developed	Predictive /Prognostic	(22)
7	sIL2Ra	Immunological	Plasma/Serum	ELISA	Decreased levels	Prognostic	(23)
8	miR-146a and miR-155	MicroRNA	Plasma/Serum	RT-PCR	Increased expression	Diagnostic /Prognostic	(24)
9	Calprotectin	Immunological	Serum	ELISA	Increased levels	Diagnostic /Prognostic	(26)
10	Amphiregulin	Immunological	Serum	ELISA	Decreased levels	Diagnostic /Prognostic	(27)
11	Exosomal miR-128	MicroRNA	Plasma	RT-PCR	Increased expression	Diagnostic Biomarker for Late-Onset aGVHD	(30)
12	Elafin	Epidermal	Tissue	Immunohistochemistry	Increased expression	Diagnostic	(28, 29)
13	Donor *ZAP70* and *DUSPI* SNPs	Biochemical	DNA	Genotyping	Polymorphism	Prognostic	(31)
14	TLR4 and TNFR1	Immunological	Serum	ELISA	Increased levels	Prognostic	(32)
15	ST2, REG3α and Elafin	Immunological	Plasma	ELISA	Increased levels	Predictive/ Prognostic (Non-myeloablative HCT setting)	(17)
16	REG3α and Elafin	Immunological	Plasma	ELISA	Increased levels	Predictive/ Prognostic (Myeloablative HCT setting)	(16)
17	ST2 and REG3α	Immunological	Plasma	ELISA	Increased levels	Predictive/ Prognostic (Haploidentical HCT setting)	(18)
18	HGF, Elafin, sIL-2Rα, sTNFR1, and REG3α	Immunological	Plasma	ELISA	Increased levels	Predictive/ Prognostic (Matched T-cell deplete HCT setting)	(19)

CD, Cluster of Differentiation; CTL14, cytotoxic T-lymphocyte–associated antigen 4; HGF, Hepatocyte growth factor; miRNAs, microRNAs; DNAM1, DNAX accessory molecule-1; IL-6, Interleukin-6; PD-1, Programmed Cell Death Protein 1; FOXP3, forkhead box P3; ICOS, Inducible T-cell COStimulator; PAF, Platelet-activating factor; LysoPC, Lysophosphatidylcholines; LysoPE, Lysophosphatidylethanolamine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; LC-MS, Liquid Chromatography-Mass Spectrometry; ZAP70, Zeta Chain Of T Cell Receptor Associated Protein Kinase 70; DUSP1, Dual specificity protein phosphatase 1; TLR4, Toll-like receptor 4; TNFR1, Tumor necrosis factor receptor 1; ST2, soluble suppressor of tumorigenicity 2; REG3a, regenerating islet-derived protein 3a; sIL2Rα, soluble interleukin-2 receptor alpha-chain; ELISA, Enzyme-linked immunosorbent assay; RT-PCR, Reverse transcription–polymerase chain reaction.

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TABLE 2 List of various types of biomarkers with clinical values that could help in the prediction, diagnosis, or prognosis for SOS.

S.No	Biomarker	Type	Biological Specimen(s)	Detection Method(s)	Biomarker level(s)	ClinicalValue	Ref(s)
1	Ferritin	Biochemical	Serum	Biochemical tests	Increased levels	Predictive	(72)
2	Uric Acid	Biochemical	Serum	Biochemical tests	Increased levels	Predictive	(73)
3	Liver Profiling	Physiological	Liver	MRI	Increased iron overload	Predictive/ Diagnostic	(74)
4	HGF	Immunological	Serum	Immunoassay	Increased levels	Predictive	(75)
5	GSTs GSTA1 GSTM1	Genetic	DNA	Genotyping	Polymorphism	Predictive	(76, 77)
6	MTHFR	Genetic	DNA	Genotyping	Polymorphism	Predictive	(78)
7	HPSE	Genetic	DNA	Genotyping	Polymorphism	Predictive	(79)
8	sICAM-1	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Predictive	(69, 80–82)
9	sVCAM-1	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Diagnostic	(80)
10	sE-Selectin	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Predictive	(69)
11	sP-Selectin	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Predictive	(81, 83)
12	VWF	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic	(69, 84)
13	TM	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic	(69, 84)
14	PAI-1	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Diagnostic	(84–87)
15	VEGF	Endothelial	Plasma/Serum	ELISA	Increased levels	Predictive	(88)
16	ANG2	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Diagnostic	(82)
18	miRNAs	MicroRNA's	Plasma/Serum	RT-PCR/Micro-seq Microarray	miRNA dependent	Prognostic	(89)
19	TNFa	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic	(90)
20	ST2	Immunological	Plasma/Serum	ELISA	Increased levels	Diagnostic	(82)
21	REG3a	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic	(80)
22	TIM3	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic	(80)
23	HA	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic/ Diagnostic	(82)
24	L-Ficolin	Immunological	Plasma/Serum	ELISA	Decreased levels	Diagnostic	(82)
25	sIL2Ra	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic	(90)
26	IGF-1	Immunological	Plasma	Immunoassay (Chemiluminescence)	Decreased levels	Predictive	(91)
27	EASIX	Biochemical	Panel (Serum/Blood)	Biochemical tests	Increased levels	Diagnostic	(92)

SOS, sinusoidal obstruction syndrome; HGF, Hepatocyte growth factor; GST, Glutathione S-transferases; MTHFR, Methylenetetrahydrofolate Reductase; HPSE, Heparanase; sICAM-1, soluble Intercellular CAM protein 1; sVCAM-1, soluble vascular CAM protein; VWF, Von Willebrand factor; TM, thrombomodulin; PAI-1, plasminogen activator type-1; VEGF, vascular endothelial growth factor; ANG2, Angiopoietin2; EV, extracellular vesicles; miRNAs, microRNAs; TNFa, tumor necrosis factor alpha; ST2, soluble suppressor of tumorigenicity 2; REG3a, regenerating islet-derived protein 3a; TIM3, T-cell immunoglobulin and mucin domain-containing protein 3; HA, hyaluronic acid; sIL2Ra, soluble interleukin-2 receptor alpha-chain; EASIX, Endothelial Activation, and stress index panel; IGF1, Insulin-like growth factor 1; ELISA, Enzyme-linked immunosorbent assay; RT-PCR, Reverse transcription–polymerase chain reaction; micro-seq, miRNAs sequencing.

Biomarkers for other early complications post-HCT

Promising results from the studies evaluating biomarkers for GVHD and SOS have also led to the identification of similar plasma biomarkers for other early HSCT complications, such as transplant-associated- thrombotic microangiopathy (TA-TMA) and engraftment syndrome (ES). TA-TMA is characterized by occlusion and disruption of microcirculation as a result of micro-thrombi deposition. It is believed that the disruption of microcirculation results from endothelial dysfunction. Lia G et al. reviewed that the endothelial dysfunction could be due to persistent insult to the endothelium caused throughout the HSCT procedure, starting from the conditioning regimen and subsequently through calcineurin inhibitors (95).

While there are multiple causes for endothelial injury, neutrophil extrusion traps (NETs) also appear to be one component evaluated in the TA-TMA context. A significantly elevated level of NETS, within the first 4 weeks post-HSCT, has been reported to be associated with an increased risk of TA-TMA (96). In contrast, the same study could not find a possible association of thrombomodulin (expressed by endothelial cells and serves as a cofactor for thrombin) with the occurrence of TA-TMA, indicating challenges in understanding the pathophysiology of TA-TMA. Interestingly, elevated ST2 levels on day 14 post-HSCT was also reported to be associated with TA-TMA. The clinical overlap between GVHD and TA-TMA occurrence and endothelial injury as a common factor for both conditions, indicates that ST2 could also be a possible biomarker for TA-TMA (97). A recent study by Okamura H et al. has shown that elevated levels of complement factor Ba on day 7 post-HSCT significantly predicted TA-TMA (98).

Similarly, the symptoms of engraftment syndrome (ES) post HCT appears overlapping with that of either GVHD or with infections. Biomarkers that could help in the early differential diagnosis of ES from other conditions with overlapping symptoms could improve HCT outcomes. Procalcitonin (PCT), a hormokine has been reported to be elevated in ES patients that could possibly be used as biomarker (99). Knoll et al., reported procalcitonin levels 2ng/ml could possibly distinguish patients with ES from patients with bacterimia (100). However, since PCT is also a FDA approved biomarker for sepsis and febrile neutropenic pateints with infections (101, 102), the use of PCT for ES needs to be evaluated in multiple cohorts.

Most of the complications post HCT appears to be as a result of persistent insult to the damaged endothelium throughout HCT procedures. Hence many markers of endothelium damage have been extensively evaluated as potential biomarkers for most HCT complications as well (103, 104).

Challenges in evaluating biomarkers for post-HCT complications

More than a decade of progress in discovering and validating biomarkers for HSCT complications led to incorporating them in clinical trials to verify their impact as a diagnostic, prognostic, or tool for pre-emptive therapy/intervention. For example. Reg3α was shown to distinguish diarrhea due to GI-GVHD from diarrhea due to non-GVHD causes (22). In contrast, Elafin could distinguish skin GVHD from drug hypersensitivity rashes (DHR) (ref). Also, REG3α and elafin were shown to distinguish diarrhea and rashes due to a more systemic disease than GI-GVHD alone (105). Similarly, ST2, TIM3, and IL6 were shown to be diagnostic biomarkers for aGVHD (106). However, these biomarkers' prospective utilization has not yet been achieved. There are more challenges in translating biomarker concentrations toward a possible clinical decision in terms of diagnosis or intervention:

Determination of cut-off values

Plasma biomarker concentrations need a range of cut-off values to make clinical decisions. Different HCT centers have evaluated various biomarkers either singly or as a panel. However, there appear to be no universal cut-off valuesfor different biomarkers, probably due to the methods employed to derive cut-off values. For instance, Hartwell et al. developed an algorithm using logistic regression analysis of biomarker concentrations to derive cut-off values (6). Other groups have used individual biomarker concentrations in respective cohorts to derive cut-off values (4).

Similarly, the association of biomarkers towards specific HSCT outcomes that could not be verified in different cohorts precludes deriving a universal cut-off values. For instance, it was shown that high ST2 levels correlated with steroid-refractory GVHD (4) but was subsequently shown in different cohorts to be associated with six months of non-relapse mortality and not with GVHD (107, 108). Various groups have reported different cut-off values for the same biomarker [For example, ST2: 33.9 ng/ml (107); 740 pg/ml (4); 3230 ng/ml (50), REG3α: 151 ng/ml (22); 1989 pg/ml (50)]. Finally, there always appears an overlap in biomarker concentrations in cohorts with and without HSCT complications impedes a universal cut-off values derivation. Thus, establishing a universal reproducible cut-off values remains a challenge.

Single biomarker vs. panel

Due to the overlap in concentrations of biomarkers in patients with and without HSCT complications, many groups have reported that a single biomarker could be of little value correlating with HSCT outcomes as opposed to a biomarker panel. Elafin was initially discovered to be associated with skin GVHD (48). However, its utility appears very limited owing to the lack of reproducibility (60). The inclusion of elafin to REG3α and ST2 was also shown to be of little value in improving the accuracy of assessing HSCT outcomes (109). On the other hand, biomarkers such as ST2 and REG3α are potentially promising as single biomarkers correlating with therapy-resistant GVHD and GI-GVHD and as panels predicting six months of non-relapse mortality (NRM) (4). A special consideration towards the sensitivity and specificity of biomarkers, either as single or panel, needs to be given to biomarkers' clinical translatability.

Beyond these challenges, the time points for biomarker testing and the frequency of such testing are also not standardized. There is

considerable variation in these parameters in the reported literature so far.

Conclusion

Non-invasive biomarkers have been comprehensively evaluated for detection, diagnosis, and/or prognosis of early complications post-HCT in multiple centers. Various studies have evaluated individual biomarkers alone or as a panel towards GVHD. The past decade of voluminous data has shown that biomarker panels, as opposed to individual biomarkers, are more valuable in diagnosing GVHD or predicting GVHD severity. In this context, MAGIC, a GVHD biomarker panel employing an algorithm using logistic regression, appears to be so promising in terms of its clinical translatability since multiple centers have verified this. On the other hand, biomarkers for other complications, such as SOS, TA-TMA, etc., still need to be confirmed in multiple clinical settings. The association of endothelial damage with post-transplant complications has been a promising addition to the arsenal of biomarkers. However, biomarkers based on endothelial damage are greatly influenced by many factors, such as underlying disease, conditioning regimen, and post-transplant conditions. Nevertheless, multiple studies have progressed well in evaluating endothelial damage biomarkers toward post-HCT complications. EASIX panel to predict SOS severity is the best example.

Prospective studies and clinical trials incorporating biomarker based interventions with clinical endpoints are required to further evaluate the clinical translatability of these biomarkers. In absence of such studies being reported, the clinical translation of biomarkers in HCT is not ready for prime time. The longer turn-around times, variable cut-offs, and assay variabilities also remain as barriers towards practical utility of such biomarkers in HCT for clinical decision making and strategies to circumvent these are needed.

Equally important is understanding the biology of the hitherto validated biomarkers, which will have advantages such as guided pre-emptive therapy, finding novel therapeutic targets for HCT complications, and, more importantly, allowing us to validate if the biomarkers are sensitive and specific.

Progress in biomarker evaluation towards HCT complications is accompanied by challenges such as the derivation of a universal cut-off point, evaluation of individual or panel of biomarkers, and prospective biomarkers assessment. These challenges could be due to differences in techniques used to analyze biomarkers, and serum/plasma sample processing, including dilutions, conditioning regimen intensity, and the source of graft. Nevertheless, recently many studies are moving towards using biomarkers as a guide for preemptive therapy. Thus our knowledge of biomarkers for early complications is ever-expanding, leading to more significant progress in its clinical translatability.

Author contributions

BB, UK, PB, designed the review structure and wrote the manuscript. EM, AAP and SI wrote the manuscript. BG and VM contributed to analysis and review of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Biomarkers for early complications post hematopoietic cell transplantation: Insights and challenges

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Hematopoietic cell transplantation is an established curative treatment option for various hematological malignant, and non-malignant diseases. However, the success of HCT is still limited by life-threatening early complications post-HCT, such as Graft Versus Host Disease (GVHD), Sinusoidal Obstruction Syndrome (SOS), and transplant-associated microangiopathy, to name a few. A decade of research in the discovery and validation of novel blood-based biomarkers aims to manage these early complications by using them for diagnosis or prognosis. Advances in this field have also led to predictive biomarkers to identify patients' likelihood of response to therapy. Although biomarkers have been extensively evaluated for different complications, these are yet to be used in routine clinical practice. This review provides a detailed summary of various biomarkers for individual early complications post-HCT, their discovery, validation, ongoing clinical trials, and their limitations. Furthermore, this review also provides insights into the biology of biomarkers and the challenge of obtaining a universal cut-off value for biomarkers.

KEYWORDS
biomarker, GVHD, endothelial, SOS, HSCT

Introduction

Hematopoietic cell transplantation (HCT) from matched related or unrelated donors to recipients with various hematological disease conditions has become a widely accepted curative treatment of choice. Especially with malignant hematological diseases, the graft versus tumor/leukemia effect (GVT) is a beneficial phenomenon expected to improve the outcome of the procedure. However, a similar effect where graft acting against the recipient's cells, such as graft

versus host disease (GVHD), leads to an undesirable outcome. Graft versus host disease (GVHD) still remains a predominant cause of morbidity and mortality in patients following HCT. Clinically GVHD may present as acute (aGVHD) or chronic (cGVHD) based on the symptoms and time of their presentation. The classical pathway of occurrence of GVHD includes damage of the target organs such as skin, eye, gastrointestinal (GI) tract, liver, or lung, followed by the release of a storm of cytokines, which increases the chance of the donor's immunocompetent cells to recognize the host's alloantigens (1). More than half of HCT patients develop GVHD. Although GVHD is treated by several immunosuppressive agents, responsiveness to these agents, GVHD related morbidity and mortality are still concerns that affect HCT outcomes greatly. In addition to GVHD, other serious complications include hepatic or pulmonary sinusoidal obstruction syndrome (SOS), opportunistic infections (bacterial, viral & fungal), and multiorgan damage. Attempts to improve HCT outcomes include predicting patients who are at high risk of developing post HCT complications, predicting their responsiveness to treatment and early diagnosis of these complications. Composite biomarkers of prognostic values have been recently used in confirming the diagnosis of some of these complications (2, 3).

Excluding the known likely causal factors for some of the adverse effects (such as the donor status, age, comorbidity, sex mismatch between donor and recipient, conditioning, and post-HCT immunosuppressive drug levels), various centers performing allogeneic HCT are concentrating on finding efficient, reliable and robust markers from biological fluids for informative, early detection or differential diagnosis of these complications to optimize the treatment as well as improving the outcome (4–6). Many have successfully reported a variety of blood plasma, serum, and fecal biomarkers, while only a handful of these is repeatedly tested and validated and likely to be used as a biomarker routinely (7). The biomarkers from these sources may be soluble factors, cellular markers, or genetic markers. While many candidate biomarkers from plasma were evaluated and verified in independent cohorts, multi-center clinical trials are still needed to validate their clinical applicability. Similarly cell-free DNA have also been recently evaluated for identifying an array of post-HCT complications including aGVHD, relapse, infection, engraftment failure and chimerism status with an objective of employing a single test/technique for elucidating a comprehensive panel of post-HCT complications (8).

However, one of the major limitations of these biomarker studies is the varying cut-off value as a reference to predict or diagnose these complications between centers. Moreover, and not all biomarkers are referenced across the normal cut-off values between healthy individuals and patients undergoing transplantation. Often these biomarkers are tested between HCT patients with and without complications. This review provides insights into the biological significance of biomarkers, their discovery and validation for HCT complications, challenges in quantification or techniques, and lack of universal target cut-offs.

The biological significance of biomarkers

Plasma biomarkers have been extensively evaluated for complications post HCT, since classic clinical risk scores such as HCT related co-morbidity index often fail to predict, diagnose or prognose such complications. The ultimate aim of plasma biomarker evaluation is its clinical translatability in predicting HCT complications, their severity and their response to treatment. On the other hand understanding the biology of these biomarkers would also pave way for developing more rational and effective treatment strategies for HCT complications. However, literature on biology of the biomarkers for HCT complications appear scanty. While increasing evidences suggest endothelial injury as a common cause for most HCT complications, a complete understanding is still lacking. Here we review the biology of a few biomarkers which are extensively evaluated for multiple overlapping complications.

ST2

The suppression of tumorigenicity 2 (ST2) is a receptor belonging to the interleukin (IL)-1 family and binds specifically to IL-33. ST2 is present in two isoforms: a transmembrane form and a soluble form. The membrane-bound ST2 receptor is expressed on various hematopoietic cells such as T helper 2 (Th2) cells, natural killer (NK) cells, mast cells, antigen-presenting cells, and regulatory T cells (Tregs) (9, 10). The IL33/ST2 complex signaling in these cell types has been observed to have proinflammatory and anti-inflammatory responses depending on the disease type (11–13). During acute GVHD, a surge in IL33 has been observed both in the clinical scenario and in mice models of alloHCT. The mucosal barrier tissues, such as the skin, gastrointestinal tract, and liver, have been significant sources of IL33. During the alloHCT conditioning regimen, damage to these tissues increases IL33 production/release that drives donor Th1 cells expansion leading to inflammatory phenotype and further tissue injury. Recently, it was demonstrated that IL-33 acts directly on donor T cells and increases Tbet expression leading to enhanced Th1 cell polarization and expansion. However, despite these observations of elevated IL-33, this could not be used as a specific biomarker for aGVHD due to its pleiotropic effects.

The soluble ST2 (sST2) receptors are expressed in endothelial cells, epithelial cells, fibroblasts, and T cells (14). The soluble ST2 act as decoy receptors, sequestering free IL-33, thereby preventing IL-33-mediated proinflammatory actions (15, 16). Thus, sST2 was generally considered to negatively regulate IL-33 function (Figure 1). However, this contradicts the association of elevated sST2 with GVHD severity in patients. A possible explanation given by earlier studies was that the release of sST2 in the serum occurs very late in the inflammatory response resulting in the inability of sST2 to sequester circulating IL33 (17).

Zhang J et al. demonstrated in a minor mismatch GVHD model and xenograft GVHD model that sST2 was secreted by intestinal stromal cells, endothelial cells, and alloreactive T cells. More importantly, as GVHD progresses, it was shown that pathogenic T cells (Th17 and Tc17) secrete more sST2 and express less mST2, thereby correlating elevated plasma sST2 levels during alloreactivity. Transient blockade of sST2 during GVHD increased Th2 transcription factor GATA3 and cytokine IL-4, improving Th2 phenotype, which protects against severe GVHD (18).

An overall picture of the ST2/IL33 axis in a severe GVHD context remains elusive. Whether sST2 is involved in the pathophysiology of

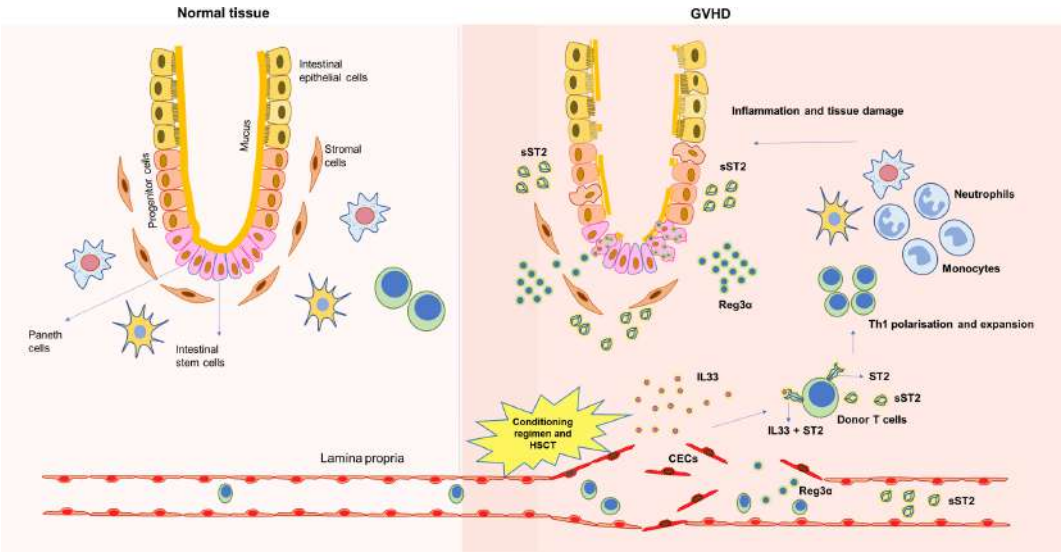

FIGURE 1
Underlying tissue damage during GVHD and release of soluble biomarkers. HSCT transplant procedures, including conditioning regimen, damage underlying endothelium, inflames the tissue and releases soluble factors that could be used as biomarkers during GVHD.

GVHD or it is just a circulating biomarker indicating GVHD severity remains to be clarified.

Reg3α

Regenerating islet-derived -3 α (Reg3α) alpha is one of the antimicrobial peptides secreted by Paneth cells of the gastrointestinal tract and is a C-type lectin having bactericidal actions on most gram-positive bacteria (19). The crypts' innate lymphoid cells 3 (ILC3) secrete IL22, which induces Paneth cells to secrete Reg3α (Figure 1) (20). During HSCT, the crypt cells, including the Paneth cells, are damaged; hence, their numbers are inversely associated with GVHD severity (21). GVHD-induced damage to the gastrointestinal crypt and intestinal mucosa decreases IL22 production and releases antimicrobial peptides stored in these cryptic cells into the bloodstream. Thus, the increased plasma level of Reg3α was strongly associated with GI-GVHD enabling their use as a biomarker (22). It was also observed *in-vivo* in mice models of GVHD that the progression of GVHD suppresses Reg3γ (mouse homolog of human Reg3α) in the GI tract, further worsening GVHD. However, administration of IL22 has been shown to protect the crypt from damage, thereby preventing Reg3γ from being released into circulation. Mechanistically it was demonstrated that Reg3γ functions as an anti-apoptotic protein for intestinal stem cells (ISCs) and Paneth cells (23). Thus, Reg3α has the dual role of being an antimicrobial peptide as well as a survival signal preventing apoptosis of ISCs and Paneth cells.

While Il22 from host cells was recognized to promote intestinal stem cell survival and suppress GI-GVHD (24), a few studies have also shown that IL22 from donor cells augments GI-GVHD (25, 26). In a mouse model of steroid-refractory GVHD, by Song Q. et al. demonstrated that IL22 was produced by donor Th/Tc22 cells, leading to excess production of Reg3γ. However, such excess Reg3γ was shown to result in dysbiosis and worsening of GVHD. Thus, REG3γ could be a therapeutic target for treating steroid-refractory GVHD (27). Hence, whether Reg3alpha is a therapeutic target or a biomarker remains an enigma.

TIM3

T cell immunoglobulin and mucin domain 3 (TIM3) is a transmembrane receptor protein expressed on interferon γ producing T cells, Tregs, myeloid cells, natural killer cells, and mast cells (28). The primary function of TIM3 is to inhibit Th1 responses and cytokine expressions. Hence, its dysregulation correlates with most autoimmune diseases, such as multiple sclerosis (29) and type I diabetes (30). Increased expression of TIM3 has been observed in solid tumors such as lung cancer, gastric cancer, colon cancer, etc., and their high expression levels were associated with low overall survival (31).

Elevated levels of TIM3 in the plasma of patients with GVHD (32) and osteosarcoma (33) have been observed, facilitating their use as potential biomarkers. However, the mechanism of soluble TIM3 release remains an enigma. It could be a splice variant, a metalloproteinase-dependent cleaved product, or a soluble fragment from apoptotic cells. While soluble TIM3 was found to express as a splice variant in mice splenocytes (34), their existence in humans is still debatable.

The mechanistic understanding of TIM3's action in aGVHD remains incomplete and is not explored much. Oikawa et al. demonstrated in a murine model of GVHD that TIM3 plays a crucial role in the activation of CD8+ T cells, which are the primary effectors in target organ destruction in aGVHD. Two weeks post-transplantation, the CD8+ T cells in the spleen and liver of GVHD mice showed enhanced TIM3 and interferon γ (IFNγ) expression. Moreover, the CD8+T cell infiltration was dominant in

the liver of GVHD mice (35). However, the exact mechanism of TIM3 induction in these cells and their shedding into peripheral circulation remains unclear.

Elafin

Elafin is a biomarker associated with the diagnosis and prognosis of skin GVHD. It is an epithelial protein secreted by keratinocytes in response to inflammatory cytokines. Hence elafin's expression is higher in the inflamed epidermis and absent/low in the normal epidermis. It is a peptidase inhibitor-3 or skin-derived anti-leukoprotease (SKALP) with antimicrobial activity and priming innate immune responses (36). It was observed that when induced by GVHD mediating inflammatory cytokines, human keratinocytes express elafin significantly (37, 38). However, the mechanism by which elafin from keratinocytes is released into circulation during GVHD remains unclear.

Biomarkers for acute GVHD

Biomarkers for acute GVHD have been extensively evaluated over the past decade in multiple HCT centers worldwide. These range from plasma, cellular, genetic and in a few cases, a combination. Biomarkers for aGVHD have been measured pre-HCT to personalize GVHD prophylaxis, post-HCT before or at the onset of GVHD, to confirm the diagnosis of GVHD or after treatment to predict treatment response.

Biomarkers measured pre-transplant for modulating GVHD prophylaxis

CD86 is the ligand for costimulatory (CD28) and coinhibitory (CTLA-4) molecules. Karaban et al. reported that the recipients' CTLA-4 CT60GA[GG] genotype, myeloablative conditioning regimen, and use of an unrelated donor were independent predictors of acute GVHD (39). Also, the same group has shown that donor and recipient CTLA-4 mRNA and recipient membrane protein expression measured before transplantation are prognostic for acute GVHD (40). Later they also reported a lack of association of CD86 gene polymorphisms with GVHD. However, they noted a gene-gene interaction wherein patients with a specific CD86 genotype and a CTLA-4 genotype was associated with an increased risk of aGVHD. With a combination of specific donor CD86 genotype and recipient CTLA-4 genotype there was an elevated GVHD risk (41).

In a study exploring the role of donor genetic variations in glucocorticoid pathway on steroid responsiveness of GVHD, although donor SNPs in *ZAP70* and *DUSP1* genes were associated with response, these were not statistically significant on adjustment for multiple testing (42). Cytokine biomarkers – TLR4 and TNFR1 are significantly increased in steroid-refractory acute GVHD compared to those with steroid-responsive GVHD (43).

DNAX accessory molecule-1 (DNAM-1, or CD226) is a leukocyte adhesion molecule constitutively expressed on most CD4+ T cells, CD8+ T cells, natural killer (NK) cells, and monocytes. A retrospective study from Japan showed that higher soluble DNAM-1 measured between day -7 to day 0 of an allotransplant was predictive of a higher risk of acute GVHD. Using a cut-off of 30pM for soluble DNAM-1, the sensitivity for predicting acute GVHD was 43.8%, while the specificity was 82.6% (44). Serum IL-6 levels measured pre-conditioning and one week after transplant were predictive of acute GVHD and transplant-related mortality (45), although, the pleiotropic nature of IL-6 may be a concern. Specific donor graft characteristics like an elevated proportion of T cells with low CD127 and high PD-1 expression have been associated with subsequent acute GVHD (46).

Biomarkers measured post-transplant before or at GVHD onset

Specific patterns of immune reconstitution following transplantation, such as increased CD8+ T cells (both naïve and memory) in the early post-transplant period (on day 15), have been associated with development of subsequent acute GVHD (47).

Elevated plasma REG3α measured at the onset of GVHD predicted non-response to treatment at 4 weeks and also 1 year non-relapse mortality (22). Elevated plasma elafin at the onset of skin GVHD is associated with higher maximum grade of GVHD and also non-relapse mortality (48). Also, elevated ST2 predicts steroid resistance in acute GVHD and non-relapse mortality (4). In non-myeloablative transplants, elevated plasma ST2, REG3α, and elafin measured early post-transplant were predictive of acute GVHD (49). Similarly, in T-replete haplotransplants, elevated plasma ST2 and REG3α measured early post-transplant were predictive of acute GVHD and non-relapse mortality (50). In patients undergoing matched donor T deplete transplantation using anti-thymocyte globulin or alemtuzumab, a biomarker panel including HGF, elafin, sIL-2Rα, sTNFR1, and REG3α was predictive of GVHD and its severity (51).

Lower serum sIL-27Rα at the time of neutrophil engraftment is predictive of acute GVHD and has been shown to correlate with other serum GVHD biomarkers (52). Elevated plasma levels of sIL2-Rα and TIM-3 in the early post-transplant period predicted increased transplant-related mortality and acute GVHD (53).

Similarly, expression patterns of genes and a few microRNAs have also been evaluated as biomarkers post HCT. Transcripts levels of *FOXP3*, *ICOS*, *CD52*, and *CASP1* genes involved in alloreactive immune responses and immune cell interactions were predictive of acute GVHD using a personalized modeling-based gene selection (PMGS) method (54). A risk score developed using metabolite and transcriptome analysis incorporating 5 metabolite markers from glycerophospholipid metabolism was predictive of acute GVHD (55). miR-155 and miR-146a measured in target tissues at the time of GVHD onset and measured in extracellular vesicles in serum and urine in the early post-transplant period before GVHD onset have been predictive of acute GVHD (56).

Biomarkers measured at symptom onset for supporting the diagnosis of GVHD (elafin, calprotectin)

Fecal, and not serum calprotectin is a biomarker for acute gut GVHD and can potentially help diagnose gut GVHD (57). Also, low

tissue amphiregulin expression on immunohistochemistry has been reported in 74% of patients with acute gut GVHD and might aid in diagnosis without classic apoptotic changes (58). Similarly, tissue, and not plasma, elafin on immunohistochemistry can aid in diagnosing acute GVHD involving the skin (59, 60).

Analysis of exosomal miRNA expression using quantitative RT-PCR on plasma samples showed that miR-128 was elevated in late-onset GVHD and is a promising diagnostic marker of late-onset GVHD (61). However, the turnaround times for these biomarkers may limit their practical utility.

Biomarkers measured at the onset of GVHD and after treatment of GVHD for potentially predicting response

The Mount Sinai Acute GVHD International Consortium (MAGIC) algorithm probability score (MAP score) based on plasma ST2 and REG3α is a response biomarker for acute GVHD. After four weeks of therapy, it was shown to predict non-relapse mortality better than the change in clinical symptoms. The MAP score was predictive of non-relapse mortality within every clinical grade of acute GVHD (62). The MAP score has been shown to be helpful when measured at day 28 along with the disease risk index could also identify patients at high relapse risk and low non-relapse mortality risk who can potentially benefit from strategies to enhance the graft versus leukemia effect for relapse prevention (63). Rising REG3α following treatment for GVHD using a novel combination of upfront steroids+ruxolitinib was shown to be a predictor of refractory GVHD (64). However, there is no prospective clinical study on biomarker-based intervention for adding second-line therapy for acute GVHD. A list of various biomarkers measured at different stages during HCT procedure and/or at GVHD onset, with potential clinical values that could help in prediction, diagnosis or prognosis for acute GVHD is summarized in Table 1.

Biomarker guided pre-emptive therapy for GVHD

Initial discovery and validation of ST2 as a biomarker for aGVHD also led to studies investigating inhibitors for ST2 in animal models (65). While this is still in progress, biomarker evaluation has progressed towards guided therapy/intervention for aGVHD with already existing anti-GVHD strategies. Gergoudis et al. have recruited patients at high risk for developing steroid-refractory GVHD (SR-GVHD) based on the MAGIC algorithm probability (MAP) scores on days 7 and 14 post-HSCT. These patients were then treated with alpha-1 anti-trypsin (AAT), a serine protease inhibitor with proven activity against GVHD. Although AAT treatment was well tolerated, the incidence of SR-GVHD was not lowered (66). Nevertheless, the power of biomarker-based SR-GVHD prediction could not be undermined. Instead, such studies pave the way for investigating more treatment options. A recent study involving a prospective phase 2 trial stratified patients based on sIL-2Rα and IL-15 levels. High-risk patients (sIL-2Rα 4500 ng/L or IL-15 31 ng/L) were treated with rabbit anti-thymocyte globulin (ATG) 3 mg/kg on day 8 post-transplant and were compared with controls who had the biomarkers measured but did not participate in this interventional trial. A reduction in GVHD was observed in these patients compared to high-risk controls who did not receive ATG (Hazards ratio of 0.48), signifying the feasibility and effectiveness of such an approach (67).

Biomarkers for sinusoidal obstruction syndrome

Sinusoidal Obstruction Syndrome (SOS), previously, known as veno-occlusive disease, is a severe complication post HSCT affecting liver sinusoidal endothelial cells. About 13 to 20% of allogeneic HSCT recipients develop SOS and the severe form of SOS is associated with multiorgan failure and mortality (68).

Typically, SOS has been observed between one to three weeks post-HSCT. Often clinically indistinguishable from other causes of weight gain, ascites, abdominal pain, and jaundice.

Factors such as conditioning regimen drugs or radiation, releasing cytokines from injured tissues, and the endogenous microbial substances that cross the compromised mucosal barriers lead to the activation of sinusoidal endothelial cells. Sustained activation can progress to endothelium damage (69). The sinusoidal endothelial cells swell and round, forming gaps in the sinusoidal barrier. These alterations facilitate the egress of leucocytes, RBCs, and cellular debris into the perisinusoidal space beneath the endothelial cells and disrupt the endothelial lining leading to sinusoidal embolisms and obstruction of the sinusoidal flow, liver dysfunction, ascites ultimately leading to multiorgan failure (70, 71).

Some reliable markers of endothelial activation and damage are soluble cellular adhesion molecules (sVCAM1, sICAM1, and sP-selectin), coagulation factors (Von Willebrand factor (VWF), thrombomodulin (TM) and plasminogen activator type-1 (PAI-1)) (Table 2).

The microenvironment of the endothelium is significantly altered in patients who undergo allo-HCT. Allo-HCT patients who develop SOS have a significant increase in both VWF and TM levels (69, 84). Furthermore, in patients receiving both tacrolimus and sirolimus as GVHD prophylaxis, levels of VWF and TM (together with ICAM-1 and E-selectin level) serve as SOS predictive biomarkers one-week post HCT (69). Two weeks post- HCT, plasma levels of REG3α, sVCAM1, sICAM1, and TIM3 are shown to be consistently elevated in patients who developed SOS (80). P-selectin levels are shown to be selectively higher in patients who develop severe SOS and elevated circulating levels of PAI-1 allow differential diagnosis between SOS and GVHD, as patients with SOS show elevated PAI-1 but not those with GVHD (81, 85, 86).

In a recent study, a composite diagnostic panel of three biomarkers: L-Ficolin, hyaluronic acid (HA), and VCAM-1, was reported to detect patients at high risk of SOS as early as the first day after HCT, even before clinical manifestation of SOS (82). Additionally, it was proposed that the biomarker panel ST2, ANG2, L-Ficolin, HA, and VCAM-1 could be helpful in the diagnosis of SOS (82). Inflammatory cytokines such as IL2, IL6, IL33, IFNγ, and TNFα are mediators of EC activation and damage. Both TNFα and soluble IL2 receptor α (sIL2Rα) are shown to be elevated during GVHD and SOS (90, 93, 94).

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TABLE 1 List of various types of biomarkers with clinical values that could help in the prediction, diagnosis, or prognosis for Acute GVHD.

S.No	Biomarker	Type	Biological Specimen(s)	Detection Method(s)	Biomarker level(s)	Clinical Value	Ref(s)
1	CD86/CTLA4 polymorphisms	Immunological	DNA	Genotyping	Polymorphism	Predictive	(12)
2	DNAM-1/CD226	Immunological	Serum	ELISA	Increased levels	Predictive	(9)
3	IL-6	Immunological	Serum	ELISA	Increased levels	Predictive	(13)
4	CD127/PD-1	Immunological	Peripheral Blood	Flow cytometry	High frequency of PD-1$^+$ T cells and low frequency of CD127$^+$ T cells in donor graft associated with grades II-III aGVHD	Predictive	(14)
5	*FOXP3* and *ICOS*	Immunological	Peripheral Blood	RT-PCR	Low levels associated with aGVHD Increasing levels correlate with response to anti-GVHD therapy	Diagnostic /Prognostic	(21)
6	PAF, LysoPC, PE, PC, and LysoPE	Metabolic (Biochemical)	Plasma	LC-MS	aGVHD risk score developed	Predictive /Prognostic	(22)
7	sIL2Ra	Immunological	Plasma/Serum	ELISA	Decreased levels	Prognostic	(23)
8	miR-146a and miR-155	MicroRNA	Plasma/Serum	RT-PCR	Increased expression	Diagnostic /Prognostic	(24)
9	Calprotectin	Immunological	Serum	ELISA	Increased levels	Diagnostic /Prognostic	(26)
10	Amphiregulin	Immunological	Serum	ELISA	Decreased levels	Diagnostic /Prognostic	(27)
11	Exosomal miR-128	MicroRNA	Plasma	RT-PCR	Increased expression	Diagnostic Biomarker for Late-Onset aGVHD	(30)
12	Elafin	Epidermal	Tissue	Immunohistochemistry	Increased expression	Diagnostic	(28, 29)
13	Donor *ZAP70* and *DUSPI* SNPs	Biochemical	DNA	Genotyping	Polymorphism	Prognostic	(31)
14	TLR4 and TNFR1	Immunological	Serum	ELISA	Increased levels	Prognostic	(32)
15	ST2, REG3α and Elafin	Immunological	Plasma	ELISA	Increased levels	Predictive/ Prognostic (Non-myeloablative HCT setting)	(17)
16	REG3α and Elafin	Immunological	Plasma	ELISA	Increased levels	Predictive/ Prognostic (Myeloablative HCT setting)	(16)
17	ST2 and REG3α	Immunological	Plasma	ELISA	Increased levels	Predictive/ Prognostic (Haploidentical HCT setting)	(18)
18	HGF, Elafin, sIL-2Rα, sTNFR1, and REG3α	Immunological	Plasma	ELISA	Increased levels	Predictive/ Prognostic (Matched T-cell deplete HCT setting)	(19)

CD, Cluster of Differentiation; CTL14, cytotoxic T-lymphocyte–associated antigen 4; HGF, Hepatocyte growth factor; miRNAs, microRNAs; DNAM1, DNAX accessory molecule-1; IL-6, Interleukin-6; PD-1, Programmed Cell Death Protein 1; FOXP3, forkhead box P3; ICOS, Inducible T-cell COStimulator; PAF, Platelet-activating factor; LysoPC, Lysophosphatidylcholines; LysoPE, Lysophosphatidylethanolamine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; LC-MS, Liquid Chromatography-Mass Spectrometry; ZAP70, Zeta Chain Of T Cell Receptor Associated Protein Kinase 70; DUSP1, Dual specificity protein phosphatase 1; TLR4, Toll-like receptor 4; TNFR1, Tumor necrosis factor receptor 1; ST2, soluble suppressor of tumorigenicity 2; REG3a, regenerating islet-derived protein 3a; sIL2Rα, soluble interleukin-2 receptor alpha-chain; ELISA, Enzyme-linked immunosorbent assay; RT-PCR, Reverse transcription–polymerase chain reaction.

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TABLE 2 List of various types of biomarkers with clinical values that could help in the prediction, diagnosis, or prognosis for SOS.

S.No	Biomarker	Type	Biological Specimen(s)	Detection Method(s)	Biomarker level(s)	ClinicalValue	Ref(s)
1	Ferritin	Biochemical	Serum	Biochemical tests	Increased levels	Predictive	(72)
2	Uric Acid	Biochemical	Serum	Biochemical tests	Increased levels	Predictive	(73)
3	Liver Profiling	Physiological	Liver	MRI	Increased iron overload	Predictive/ Diagnostic	(74)
4	HGF	Immunological	Serum	Immunoassay	Increased levels	Predictive	(75)
5	GSTs GSTA1 GSTM1	Genetic	DNA	Genotyping	Polymorphism	Predictive	(76, 77)
6	MTHFR	Genetic	DNA	Genotyping	Polymorphism	Predictive	(78)
7	HPSE	Genetic	DNA	Genotyping	Polymorphism	Predictive	(79)
8	sICAM-1	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Predictive	(69, 80–82)
9	sVCAM-1	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Diagnostic	(80)
10	sE-Selectin	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Predictive	(69)
11	sP-Selectin	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Predictive	(81, 83)
12	VWF	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic	(69, 84)
13	TM	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic	(69, 84)
14	PAI-1	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Diagnostic	(84–87)
15	VEGF	Endothelial	Plasma/Serum	ELISA	Increased levels	Predictive	(88)
16	ANG2	Endothelial	Plasma/Serum	ELISA	Increased levels	Prognostic/ Diagnostic	(82)
18	miRNAs	MicroRNA's	Plasma/Serum	RT-PCR/Micro-seq Microarray	miRNA dependent	Prognostic	(89)
19	TNFa	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic	(90)
20	ST2	Immunological	Plasma/Serum	ELISA	Increased levels	Diagnostic	(82)
21	REG3a	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic	(80)
22	TIM3	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic	(80)
23	HA	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic/ Diagnostic	(82)
24	L-Ficolin	Immunological	Plasma/Serum	ELISA	Decreased levels	Diagnostic	(82)
25	sIL2Ra	Immunological	Plasma/Serum	ELISA	Increased levels	Prognostic	(90)
26	IGF-1	Immunological	Plasma	Immunoassay (Chemiluminescence)	Decreased levels	Predictive	(91)
27	EASIX	Biochemical	Panel (Serum/Blood)	Biochemical tests	Increased levels	Diagnostic	(92)

SOS, sinusoidal obstruction syndrome; HGF, Hepatocyte growth factor; GST, Glutathione S-transferases; MTHFR, Methylenetetrahydrofolate Reductase; HPSE, Heparanase; sICAM-1, soluble Intercellular CAM protein 1; sVCAM-1, soluble vascular CAM protein; VWF, Von Willebrand factor; TM, thrombomodulin; PAI-1, plasminogen activator type-1; VEGF, vascular endothelial growth factor; ANG2, Angiopoietin2; EV, extracellular vesicles; miRNAs, microRNAs; TNFa, tumor necrosis factor alpha; ST2, soluble suppressor of tumorigenicity 2; REG3a, regenerating islet-derived protein 3a; TIM3, T-cell immunoglobulin and mucin domain-containing protein 3; HA, hyaluronic acid; sIL2Ra, soluble interleukin-2 receptor alpha-chain; EASIX, Endothelial Activation, and stress index panel; IGF1, Insulin-like growth factor 1; ELISA, Enzyme-linked immunosorbent assay; RT-PCR, Reverse transcription–polymerase chain reaction; micro-seq, miRNAs sequencing.

Biomarkers for other early complications post-HCT

Promising results from the studies evaluating biomarkers for GVHD and SOS have also led to the identification of similar plasma biomarkers for other early HSCT complications, such as transplant-associated- thrombotic microangiopathy (TA-TMA) and engraftment syndrome (ES). TA-TMA is characterized by occlusion and disruption of microcirculation as a result of micro-thrombi deposition. It is believed that the disruption of microcirculation results from endothelial dysfunction. Lia G et al. reviewed that the endothelial dysfunction could be due to persistent insult to the endothelium caused throughout the HSCT procedure, starting from the conditioning regimen and subsequently through calcineurin inhibitors (95).

While there are multiple causes for endothelial injury, neutrophil extrusion traps (NETs) also appear to be one component evaluated in the TA-TMA context. A significantly elevated level of NETS, within the first 4 weeks post-HSCT, has been reported to be associated with an increased risk of TA-TMA (96). In contrast, the same study could not find a possible association of thrombomodulin (expressed by endothelial cells and serves as a cofactor for thrombin) with the occurrence of TA-TMA, indicating challenges in understanding the pathophysiology of TA-TMA. Interestingly, elevated ST2 levels on day 14 post-HSCT was also reported to be associated with TA-TMA. The clinical overlap between GVHD and TA-TMA occurrence and endothelial injury as a common factor for both conditions, indicates that ST2 could also be a possible biomarker for TA-TMA (97). A recent study by Okamura H et al. has shown that elevated levels of complement factor Ba on day 7 post-HSCT significantly predicted TA-TMA (98).

Similarly, the symptoms of engraftment syndrome (ES) post HCT appears overlapping with that of either GVHD or with infections. Biomarkers that could help in the early differential diagnosis of ES from other conditions with overlapping symptoms could improve HCT outcomes. Procalcitonin (PCT), a hormokine has been reported to be elevated in ES patients that could possibly be used as biomarker (99). Knoll et al., reported procalcitonin levels 2ng/ml could possibly distinguish patients with ES from patients with bacterimia (100). However, since PCT is also a FDA approved biomarker for sepsis and febrile neutropenic pateints with infections (101, 102), the use of PCT for ES needs to be evaluated in multiple cohorts.

Most of the complications post HCT appears to be as a result of persistent insult to the damaged endothelium throughout HCT procedures. Hence many markers of endothelium damage have been extensively evaluated as potential biomarkers for most HCT complications as well (103, 104).

Challenges in evaluating biomarkers for post-HCT complications

More than a decade of progress in discovering and validating biomarkers for HSCT complications led to incorporating them in clinical trials to verify their impact as a diagnostic, prognostic, or tool for pre-emptive therapy/intervention. For example. Reg3α was shown to distinguish diarrhea due to GI-GVHD from diarrhea due to non-GVHD causes (22). In contrast, Elafin could distinguish skin GVHD from drug hypersensitivity rashes (DHR) (ref). Also, REG3α and elafin were shown to distinguish diarrhea and rashes due to a more systemic disease than GI-GVHD alone (105). Similarly, ST2, TIM3, and IL6 were shown to be diagnostic biomarkers for aGVHD (106). However, these biomarkers' prospective utilization has not yet been achieved. There are more challenges in translating biomarker concentrations toward a possible clinical decision in terms of diagnosis or intervention:

Determination of cut-off values

Plasma biomarker concentrations need a range of cut-off values to make clinical decisions. Different HCT centers have evaluated various biomarkers either singly or as a panel. However, there appear to be no universal cut-off valuesfor different biomarkers, probably due to the methods employed to derive cut-off values. For instance, Hartwell et al. developed an algorithm using logistic regression analysis of biomarker concentrations to derive cut-off values (6). Other groups have used individual biomarker concentrations in respective cohorts to derive cut-off values (4).

Similarly, the association of biomarkers towards specific HSCT outcomes that could not be verified in different cohorts precludes deriving a universal cut-off values. For instance, it was shown that high ST2 levels correlated with steroid-refractory GVHD (4) but was subsequently shown in different cohorts to be associated with six months of non-relapse mortality and not with GVHD (107, 108). Various groups have reported different cut-off values for the same biomarker [For example, ST2: 33.9 ng/ml (107); 740 pg/ml (4); 3230 ng/ml (50), REG3α: 151 ng/ml (22); 1989 pg/ml (50)]. Finally, there always appears an overlap in biomarker concentrations in cohorts with and without HSCT complications impedes a universal cut-off values derivation. Thus, establishing a universal reproducible cut-off values remains a challenge.

Single biomarker vs. panel

Due to the overlap in concentrations of biomarkers in patients with and without HSCT complications, many groups have reported that a single biomarker could be of little value correlating with HSCT outcomes as opposed to a biomarker panel. Elafin was initially discovered to be associated with skin GVHD (48). However, its utility appears very limited owing to the lack of reproducibility (60). The inclusion of elafin to REG3α and ST2 was also shown to be of little value in improving the accuracy of assessing HSCT outcomes (109). On the other hand, biomarkers such as ST2 and REG3α are potentially promising as single biomarkers correlating with therapy-resistant GVHD and GI-GVHD and as panels predicting six months of non-relapse mortality (NRM) (4). A special consideration towards the sensitivity and specificity of biomarkers, either as single or panel, needs to be given to biomarkers' clinical translatability.

Beyond these challenges, the time points for biomarker testing and the frequency of such testing are also not standardized. There is

considerable variation in these parameters in the reported literature so far.

Conclusion

Non-invasive biomarkers have been comprehensively evaluated for detection, diagnosis, and/or prognosis of early complications post-HCT in multiple centers. Various studies have evaluated individual biomarkers alone or as a panel towards GVHD. The past decade of voluminous data has shown that biomarker panels, as opposed to individual biomarkers, are more valuable in diagnosing GVHD or predicting GVHD severity. In this context, MAGIC, a GVHD biomarker panel employing an algorithm using logistic regression, appears to be so promising in terms of its clinical translatability since multiple centers have verified this. On the other hand, biomarkers for other complications, such as SOS, TA-TMA, etc., still need to be confirmed in multiple clinical settings. The association of endothelial damage with post-transplant complications has been a promising addition to the arsenal of biomarkers. However, biomarkers based on endothelial damage are greatly influenced by many factors, such as underlying disease, conditioning regimen, and post-transplant conditions. Nevertheless, multiple studies have progressed well in evaluating endothelial damage biomarkers toward post-HCT complications. EASIX panel to predict SOS severity is the best example.

Prospective studies and clinical trials incorporating biomarker based interventions with clinical endpoints are required to further evaluate the clinical translatability of these biomarkers. In absence of such studies being reported, the clinical translation of biomarkers in HCT is not ready for prime time. The longer turn-around times, variable cut-offs, and assay variabilities also remain as barriers towards practical utility of such biomarkers in HCT for clinical decision making and strategies to circumvent these are needed.

Equally important is understanding the biology of the hitherto validated biomarkers, which will have advantages such as guided pre-emptive therapy, finding novel therapeutic targets for HCT complications, and, more importantly, allowing us to validate if the biomarkers are sensitive and specific.

Progress in biomarker evaluation towards HCT complications is accompanied by challenges such as the derivation of a universal cut-off point, evaluation of individual or panel of biomarkers, and prospective biomarkers assessment. These challenges could be due to differences in techniques used to analyze biomarkers, and serum/plasma sample processing, including dilutions, conditioning regimen intensity, and the source of graft. Nevertheless, recently many studies are moving towards using biomarkers as a guide for preemptive therapy. Thus our knowledge of biomarkers for early complications is ever-expanding, leading to more significant progress in its clinical translatability.

Author contributions

BB, UK, PB, designed the review structure and wrote the manuscript. EM, AAP and SI wrote the manuscript. BG and VM contributed to analysis and review of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Areas of Interest

Clinical Haematology, Conditioning Regimens, Stem cell Transplantation, Transplantation Biology, Pharmacokinetics, and Pharmacogenetics

Work Experience

Presently working as a graduate student at the department of haematology, Christian Medical College, Vellore, India (December 2016 till present).

Academic Qualifications

- D.B.T. –J.R.F. 2016 qualified.
- CSIR-UGC NET (L.S.) 2016 qualified.
- M.Sc. Biotechnology from Amity University, U.P. – 87.2%
- B.Sc. Biotechnology from P.S.G College of Arts and Science, Coimbatore (Bharathiar University), India - (2009-2012) – 77%
- Intermediate from Lisieux Matric Higher Secondary School, Coimbatore, India (2009) - 81%
- High school from Lisieux Matric Higher Secondary School, Coimbatore, India (2007) - 81%

Additional Qualifications (Projects)

- Six months project on "Cross talk between tumor and stroma- Role of Cancer-Associated Fibroblasts in breast cancer progression" at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India. (December 2013- June 2014).
- Summer internship on "Molecular Biotechnology & Bioinformatics" at International Institute of Information Technology, Pune, India. (2011).
- Winter Training on "Screening of Transgenic plants using PCR" at Genohelix Labs, Bangalore, India (2010).
- Training on "Instrumentation methods and chemical analysis" at the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore, India (2010).

Workshops and Conferences

- Presented poster on "Glutathione S transferase gene promoter polymorphism (*GSTA1*B*) influences haematopoietic cell transplantation (HCT) outcome in patients with β thalassemia receiving treosulfan/fludarabine/thiotepa regimen" at "2023 Tandem Meetings of ASTCT and CIBMTR" held on February 15 -19, 2023, at Orlando, U.S.A.
- Presented poster on " Early Immune Reconstitution following Treosulfan-based Reduced toxicity conditioning in patients undergoing allogeneic transplantation" at "2023 Tandem Meetings of ASTCT and CIBMTR" held on February 15 -19, 2023, at Orlando, U.S.A.

- Presented poster on " Early Immune Reconstitution following Treosulfan-based Reduced toxicity conditioning in patients undergoing allogeneic transplantation" at "2023 Tandem Meetings of ASTCT and CIBMTR" held on February 15 -19, 2023, at Orlando, U.S.A.
- Presented Poster on "High Melphalan systemic exposure is associated with poor event-free survival after autologous stem cell transplantation (ASCT) in patients with Multiple Myeloma" at the Indian Myeloma Congress 2023, Bangalore, India (13th-15th January 2023).
- Presented poster on "Population pharmacokinetics of mycophenolic acid in Haploidentical transplant recipients receiving Post-transplant Cyclophosphamide based GvHD prophylaxis" at 64th ASH Annual Meeting, New Orleans, U.S.A. (December 10-13, 2022).
- Presented poster on" Lower Treosulfan Systemic Exposure Predicts Graft Rejection in Patients with Beta Thalassemia Major Undergoing Allogeneic Hematopoietic Cell Transplantation" at "2022 Tandem Meetings of ASTCT and CIBMTR" held on April 23-26, 2022, at Salt Palace Convention Center, Salt Lake City, Utah, U.S.A.
- Oral presentation on "Low erythrocyte thiopurine nucleotide levels predict relapse in patients with Acute Lymphoblastic Leukemia on 6-mp maintenance therapy" at Haematocon (virtual) (November 10-13, 2021).
- Oral presentation on "Low erythrocyte thiopurine nucleotide levels predict relapse in patients with Acute Lymphoblastic Leukemia on 6-mp maintenance therapy" at Annual Research Day, C.M.C. Vellore, India (October 21-22, 2021).
- Oral presentation on "NT5E gene polymorphisms impacts improved HSCT outcome in patients with β-thalassemia (Oral-761)" at 25th annual meeting of APBMT (virtual).
- Presented two posters on "Treosulfan Metabolite (S, S-EBDM) Pharmacokinetics Influences Regimen Related Toxicity in Patients with Beta Thalassaemia Major Undergoing HSCT" and on "A 5'UTR polymorphism in NT5E gene but not Fludarabine Pharmacokinetics influences HSCT outcome in Acute Myeloid Leukaemia" at International Symposium on Bone Marrow Failure (IBMFS), Vellore, India (3-4 February 2020)
- Presented poster on "Treosulfan Metabolite (S, S-EBDM) Pharmacokinetics Influences Regimen Related Toxicity in Patients with Beta Thalassaemia Major Undergoing HSCT" at 61ST A.S.H. Annual Meeting, Florida, U.S.A. (December 6-10, 2019)
- Presented poster on "A 5'UTR polymorphism in NT5E gene but not Fludarabine Pharmacokinetics influences HSCT outcome in Acute Myeloid Leukaemia" at Annual Research Day, C.M.C. Vellore, India (November 20-22, 2019).
- Presented poster on "A 5'UTR polymorphism in NT5E gene but not Fludarabine Pharmacokinetics influences HSCT outcome in Acute Myeloid Leukaemia" at Haematology Cancer Consortium (H.C.C.), Kochi, India. (Aug 16-18, 2019)
- Presented poster on "Pharmacokinetics and efficacy of Generic Melphalan are comparable to innovator formulation in patients with multiple myeloma undergoing Autologous Stem Cell Transplantation "at "Highlights of T.C.T. Meetings 2019" meetings (April 5-7, 2019) at Chennai, India.
- Presented poster on "Pharmacokinetics and efficacy of Generic Melphalan are comparable to innovator formulation in patients with multiple myeloma undergoing Autologous Stem Cell Transplantation " at "T.C.T. Meetings 2019" meetings (February 20 – 24, 2019), Houston, Texas, U.S.A.

- Oral presentation on "Targeted IV Busulfan-Based Conditioning Regimen in patients with Acute Myelogenous Leukemia Undergoing Allogeneic Hematopoietic Stem Cell Transplantation – a single centre experience" at Haematocon 2018 (October), Kochi, India.
- Presented poster on "Pharmacokinetics of melphalan in ASCT in myeloma" at Haematocon 2018 (October), Kochi, India.
- Oral presentation on "Pharmacokinetics of melphalan in ASCT in myeloma" at Myeloma Meet 2018 (September) at Kolkata hosted by T.M.C., Kolkata, India.
- Presented poster on "Genetic Variants in Drug Metabolizing and Transporter Genes Explain Variability in fludarabine and treosulfan Pharmacokinetics in Patients Undergoing HSCT" at "Highlights of BMT TANDEM 2018" meetings (April 7 – 8, 2018) New Delhi, India.
- Attended 2nd National Symposium on Therapeutic Drug Monitoring (NSTDM) at C.M.C., Vellore, India (July 2017).
- Attended 33rd Annual Convention of Indian Association for Cancer Research (IACR), from February 13 to 15, 2014, with a broad theme "Discovery, Innovation, and Translation in Cancer Research" at Kollam, Kerala, India.
- Presented poster on "Biosensors and medical applications" at National Conference on "Current trends in Advanced Biomedical Technology" at Nehru Arts and science college, Coimbatore, India. (2011).

Publications:

- Kulkarni UP*, **Pai AA***, Kavitha ML, Selvarajan S, Lionel S, Devasia AJ, Korula A, Fouzia NA, Sindhuvi E, Abraham A, Srivastava A, Mathews V, George B, Balasubramanian P. Endothelial Activation and Stress Index-Measured Pretransplantation Predicts Transplantation-Related Mortality in Patients with Thalassemia Major Undergoing Transplantation with Thiotepa, Treosulfan, and Fludarabine Conditioning. Transplant Cell Ther. 2022 Jul;28(7):356.e1-356.e6. DOI: 10.1016/j.jtct.2022.05.001. Epub 2022 May 9. PMID: 35550442. https://doi.org/10.7554/eLife.65421
- **Pai AA,** Mohan A, Benjamin E.S.B., Illangeswaran RSS, Xavier Raj I, Janet NB, Arunachalam AK, Kavitha ML, Kulkarni U, Devasia AJ, Fouzia NA, Abraham A, Srivastava A, George B, Mathews V, Korula A, Balasubramanian P. NUDT15 c.415C>T Polymorphism Predicts 6-MP Induced Early Myelotoxicity in Patients with Acute Lymphoblastic Leukemia Undergoing Maintenance Therapy. Pharmgenomics Pers Med. 2021 Oct 2;14:1303-1313. Doi: 10.2147/PGPM.S325813. PMID: 34629890; PMCID: PMC8495143**.**
- **Pai AA**, Devasia AJ, Panetta JC, Mani S, Stallon Illangeswaran RS, Mohanan E, Balakrishnan B, Lakshmi KM, Kulkarni U, Aboobacker FN, Korula A, Abraham A, Srivastava A, Mathews V, George B, Balasubramanian P. Pharmacokinetics and Efficacy of Generic Melphalan Is Comparable to Innovator Formulation in Patients With Multiple Myeloma Undergoing Autologous Stem Cell Transplantation. Clin Lymphoma Myeloma Leuk. 2020 Feb;20(2):130-135.e1.
- **Pai AA**, Panetta JC, Mohanan EP, et al. Treosulfan Metabolite (S, S-EBDM) Pharmacokinetics Influences Regimen Related Toxicity in Patients with Beta Thalassaemia Major Undergoing HSCT. Blood (2019) 134 (Supplement_1): 1977.
- **Pai AA**, Mohanan E, Balakrishnan B, et al. Genetic Variants in Drug Metabolizing and Transporter Genes Explain Variability in Fludarabine Pharmacokinetics in

Patients Undergoing HSCT. doi:10.1016/j.bbmt.2017.12.623.

Achievements

- **ASTCT award 2022 and 2023**
- **ASH Abstract achievement awards** - 2019 and 2022
- Won second prize for oral presentation at **Haematocon 2021.**
- **Graduation Topper (B.Sc. Biotechnology)** of P.S.G. College of Arts and Science, Coimbatore.
- General Proficiency in French (School level and College level)
- Certificate course **in Developmental psychology.** (2011)

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I hereby declare that the above details are accurate to my consent.

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17-07-2023