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OUTCOME AFTER ALLOGENEIC STEM CELL TRANSPLANTATION WITH SPECIAL REFERENCE TO NON-MALIGNANT DISORDERS, CHIMERISM AND GRAFT CELL COMPOSITION

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Outcome after allogeneic stem cell transplantation with special reference to non-malignant disorders, chimerism and graft cell composition

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my
family

ABSTRACT

The outcome for patients undergoing allogeneic stem cell transplantation (HSCT), a treatment for several severe malignant and non-malignant disorders with hematopoietic origin, is a balancing act between beneficiary effects and risk factors. Treatment success is still limited by excessive immune response, such as graft-versus-host–disease (GVHD) and graft failure (GF), or the lack thereof as in malignancy relapse or severe infections. The main focus of this thesis is to evaluate treatment outcome of different patient groups and evaluate the effect of routine testing for these patients.

In 2004, the decision to use an unrelated donor (URD) rather than a HLA-identical sibling for patients with a non-malignant disorder was, due to a higher risk for complications, more debatable than today. In **Paper 1**, we retrospectively analysed the outcome for 25 patients with non-malignant disorders who underwent HSCT with a unrelated HLA-A,B DRβ1-matched donor. All patients received anti-thymocyte globulin (ATG). Only 2 patients rejected their graft and the cumulative incidence of acute (only grade I and II were diagnosed) and chronic GVHD was 24%, and 21%, respectively. Also, the overall survival (OS) was 84% indicating that the results were comparable to patients undergoing a HSCT with a HLA-identical donor.

Chimerism is a PCR-based technique used to determine the genotypic cell origin of post transplantation hematopoiesis for the detection of graft failure (GF) and relapse. Post HSCT, we can find either a mix of donor and recipient, i.e. mixed chimerism (MC) or only cells of donor origin i.e. donor chimerism (DC). In **Paper 2**, we retrospectively evaluated 58 patients (64 transplants) with non-malignant disorders and studied the outcome in relation to chimerism status and, in addition, how high MC was clinically managed (to avoid GF). We found MC in 64% of the transplants. Donor chimeric (DC) was more common after myeloablative conditioning (MAC) than after reduced conditioning ($p=0.04$), and patients with DC had a higher incidence of acute GVHD grade II–III ($p=0.002$) compared to patients with MC. Owing to high MC, no conclusive treatment was established, but patients with GF who underwent a re-HSCT did well.

Outcome for adult patients after HSCT have improved. In **Paper 3**, we compared outcome after HSCT for pediatric patients between time periods 1992-2002 (P1) and 2003 to 2013 (P2). The most significant changes during P2 compared with P1 were a decrease in MAC protocols, altered GVHD prophylaxis and more eligible patients had a non-malignant diagnoses ($p=0.002$). Results showed more acute GVHD, less transplanted mortality (TRM) but relapse rate was unchanged. In all, 3-year overall survival (OS) improved from 58% in P1 to 78% in P2 ($p<0.001$).

Lastly, pursuing the quest for relevant factors influencing patient outcome, its known that donor grafts entail not only stem cells (CD34+) but also potentially active lymphocytes whos

numbers can differ depending on the donor and donor site. In **Paper 4**, we hypothesized that these donor graft lymphocyte sub-sets could influence patient outcome. We collected and evaluated routine flow cytometry donor graft data (i.e. CD34+, CD3+, CD19+, CD4+, CD8+, CD3-CD56+CD16+, CD4+CD127lowCD25high) for 299 patients with malignant diseases who underwent HSCT between 2006 and 2013 with peripheral blood stem cell grafts (we analysed unrelated separated from sibling donors). The multivariate analysis showed in the unrelated-group an association between a high CD8+ graft dose and decreased risk for relapse (p=0.006) and in the sibling group a high CD19+ graft dose was associated with increased risk for transplanted related mortality (p=0.036) and acute GVHD (p=0.003).

SAMMANFATTNING

Vid allogen (individ-olik) hematologisk stamcellstransplantation (HSCT), kommer främst patienter med maligna blodsjukdomar i fråga, men även de med allvarliga icke-maligna tillstånd såsom ärftliga benmärgsdefekter, metabola sjukdomar, svåra immunbrister och sjukdomar i de röda blodkropparna. Bot möjliggörs eftersom givarens stamceller ger upphov till nya celler som direkt eller indirekt botar sjukdomen, alternativt utplånar maligniteten. Givaren (donatorn) är antingen ett vävnads(HLA-)identiskt syskon eller mer ofta en välmatchad (nära vävnadsidentisk eller HLA-matchad) obesläktad donator från något av de frivilliga donatorregistren såsom det svenska Tobiasregistret. Stamceller erhålles antingen från donatorns benmärg, perifert blod eller navelsträngsblod. De överförs till patienten efter en s.k. förbehandling (konditionering) med cellgifter och ibland radioaktiv strålning. Denna är antingen kraftfull, då mottagarens (recipientens) benmärg helt slås ut, s.k. myeloablativ konditionering, eller en mildare form s.k. reducerad konditionering. För att undvika komplikationer såsom avstötning (rejektion) av stamcellsenheten (graftet) eller att donatorn alltför kraftfullt ska reagera på recipientens vävnader, s.k. graft–versus-host-disease (GVHD) krävs immunsuppression efter HSCT. Sedan starten för HSCT i Sverige på 1970-talet har många förbättringar skett, men alltjämt återstår problem för patienterna i form av återfall i grundsjukdomen, allvarlig GVHD, rejektion samt allvarliga infektioner.

Avhandlingens syfte är att leda till bättre resultat för patienter som genomgår HSCT genom att utforska olika faktorer som kan styra det kliniska förloppet.

Första arbetet är publicerat 2004 (Allogeneic stem cell transplantation for non-malignant disorders using matched unrelated donors. BBMT, 10:877-882). Historisk sett har man tvekat inför att använda en icke HLA-lik givare till en patient med en icke-malign sjukdom eftersom de löper större risk för komplikationer bl.a. GVHD, något som de till skillnad från patienter med en malign sjukdom, inte är betjänta av. Vi gjorde en sammanställning av de 25 patienter med en icke-malign sjukdom som hade genomgått HSCT med obesläktad HLA-lik givare på Karolinska Huddinge och fann bl.a. att resultaten var likvärdiga med dem som hade en HLA-identisk givare. Eftersom de flesta patienter saknar en HLA-lik givare, betyder bl.a vår studie att dessa patienter i större utsträckning kan komma i fråga för en HSCT.

Andra arbetet är publicerat 2009 (Allogeneic hematopoietic SCT in patients with non-malignant disease, and importance of chimerism. BMT 2009:44,757-763). Efter en HSCT existerar celler från 2 olika individer i patienten (donator och recipient), även kallad chimerism. I efterförloppet är det värdefullt att veta förhållandet mellan celler av recipient- respektive donatorsursprung dvs om en patient har celler från både recipient och donator, sk mixed chimerism (MC) eller bara celler med donatorsursprung, donatorchimär (DC). Metoden har använts för att försöka upptäcka återfall hos patienter med maligna sjukdomar,

så att behandling mot de maligna cellerna, såsom utsättning av immunsuppression och vidare infusion av donatorns lymfocyter snabbt kan inledas. Vi ville se om tekniken hade en plats i uppföljningen av patienter med icke-maligna sjukdomar, d.v.s. om chimerismutseendet eventuellt kunde förutsäga komplikationer såsom avstötning av graftet och/eller GVHD. Vi analyserade chimerismresultat hos 58 patienter med icke-maligna sjukdomar och korrelerade resultaten med hur det hade gått för dessa patienter. Vi fann mer MC hos patienter som erhållit reducerad konditionering och att de patienter som fick GVHD oftare var DC efter HSCT. Av de 24 patienter med hög MC (stor andel egna celler) återfanns samtliga de patienter som sedermera rejekterade sitt graft. Den kliniska handläggningen av patienter med hög MC varierade, men att för de patienter som stötte bort sitt graft var det framgångsrikt med en ny HSCT.

Tredje arbetet är publicerat 2016 (Improved overall survival for pediatric patients undergoing allogeneic stem cell transplantation – A comparison of the last two decades. Pediatric Transplantation. 2016 Aug;20(5):667-74.) Detta är en jämförande studie av pediatriska patienter som genomgått HSCT mellan 1992 och 2002 (188 barn) respektive mellan 2003 och 2013 (201 barn) på Karolinska Huddinge. Eftersom såväl vår egna som internationella data talar för att vuxna patienter klarar sig bättre efter en HSCT på senare år ville vi se om det även gällde barn som genomgått HSCT hos oss. Resultaten från vår studie visar att flera förändringar har skett i den senare tidsperioden. Fler erhåller transplantationer p.g.a icke-maligna sjukdomar, fler får reducerad konditionering och fler barn erhåller transplantationer med stamceller från navelsträngsblod som stamcellskälla än föregående årtionde. I den senare perioden ökar risken för att få akut GVHD, ingen förbättring när det gäller återfallsfrekvensen men risken att drabbas av transplantationsrelaterad dödlighet är klart mindre. Sammantaget ger det bättre överlevnad för de barn som är transplanterade på senare år. Detta kan betyda att HSCT blir tillgänglig som ett behandlingsalternativ för fler barn.

Fjärde arbetet (The importance of graft cell composition in outcome after peripheral stem cell transplantation in patients with malignant disease) handlar om 'graft composition' dvs cellsammansättningen hos givarens graft. Sedan tidigare är det känt att det innehåller förutom stamcellerna (CD34+) även andra immunkompetenta (d.v.s. celler förmögna att påverka mottagaren varför de kan vara av betydelse för utfallet för patienten) celler, såsom CD19+(B-celler), CD3+(T-celler), CD3-CD56+CD16+(NK-celler), CD4+CD127^{low}CD25^{high} (regulatoriska T-celler), CD4+(T-hjälparceller) och CD8+ (cytotoxiska T-celler). Dessa celler räknas med hjälp av flödescytometri innan transplantationen. Vi vet sedan tidigare att dessa cellnivåer kan skilja sig beroende på om givaren är besläktad eller obesläktad eller vilken stamcellskälla man använder. Ibland skördas graftet på ett annat nationellt eller internationellt center, och transporten däremellan har också visat sig påverka dessa cellnivåer. Vi ville därför studera om antal och inbördes relation av dessa lymfocyter har betydelse för hur det senare går för patienterna transplanterade på Huddinge. Vi analyserade patienter med maligna sjukdomar som hade genomgått stamcellstransplantation mellan 2006 och 2013 med givare som lämnat perifera stamceller. De som fått celler ifrån ett syskon hade ökad risk för att få

GVHD om graftet hade höga antal CD19+ celler. Om graftet innehöll låga nivåer av CD8+ celler från en obesläktad givare löpte patienterna större risk för återfall. Detta är en liten studie, men det skulle kunna innebära en möjlighet att manipulera graftet innan det överförs till patienten. Alternativt kan en sådan kunskap leda till, som för de patienter som erhåller graft med låga nivåer av CD8+ celler, att man övervakar patienten noga och exempelvis, för att stärka givarcellernas verkan mot patientens elakartade blodsjukdom, sätter ut immunsuppressionen tidigt efter HSCT.

LIST OF SCIENTIFIC PAPERS

I. *Allogenic stem cell transplantation for nonmalignant disorders using matched unrelated donors.* **Svenberg P**, Remberger M, Svennilson J, Mattsson J, Leblanc K, Gustafsson B, Aschan J, Barkholt L, Winiarski J, Ljungman P, Ringdén O.
Biology of Blood Marrow Transplantation 2004 Dec;10(12):877-82.

II. *Allogeneic hematopoietic SCT in patients with non-malignant diseases, and importance of chimerism.* **Svenberg P***,* Mattsson J, Ringdén O, Uzunel M.
Bone Marrow Transplantation 2009 Dec;44(11):757-63.

III. *Improved overall survival for pediatric patients undergoing allogeneic hematopoietic stem cell transplantation - A comparison of the last two decades.* **Svenberg P**, Remberger M, Uzunel M, Mattsson J, Gustafsson B, Fjaertoft G, Sundin M, Winiarski J, Ringdén O.
Pediatric Transplantion. 2016 Aug;20(5):667-74.

IV. *The importance of graft cell composition to outcome after allogeneic stem cell transplantation in patients with malignant disease.* **Svenberg. P**, Wang. T, Uhlin. M, Watz E, Remberger, M, Ringden O Uzunel O. Submitted

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LIST OF ABBREVIATIONS

AA	aplastic anemia
APC	antigen presenting cell
ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukemia
ATG	anti-thymocyte globulin
Bu	busulfan
BM	bone marrow
BMF	bone marow failor
CAR	chimeric antigen receptor
CB	cord blood
CD	cluster of differentiation
CMV	cytomegalovirus
CR1	first complete remission
CyA	cyclosporine A
Cy	cyclophosphamide
DC	donor chimerism
DLI	donor lymphocyte infusion
EBV	ebstein-barr virus
Flu	fludarabine
FHL/HLH	familial/ hemophagocytic lymphohistiocytosis
G-CSF	granulocyte colony stimulating factor
GF	graft failure
GVHD	graft-versus-host disease
GVL/GVT	graft-versus-leukemia/graft-versus-tumour
Gy	Gray
HLA	human leukocyte antigen
HSCT	allogeneic hematopoietic stem cell transplantation
IBFM	inherited bone marrow failure

IEM	inborn errors of metabolism
IST	immunosuppressive treatment
KIR	killer-cell immunoglobulin-like receptor
MAC	myeloablative conditioning
MC	mixed chimerism
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
MPS	mucopolysaccharidosis
MRD	minimal residual disease
Mtx	methotrexate
NK	natural killer
OS	overall survival
PID	primary immunodeficiencies
PBSC	peripheral blood stem cell
PTLD	post-transplant lymphoproliferative disorder
RIC	reduced intensity conditioning
SCA	sickle cell anemia
SCID	severe combined immunodeficiency
Sib	sibling donors
Sir	sirolimus
SNP	single-nucleotide polymophism
STR	short tandem repeats
Tac	tacrolimus
TAM	transplant-associated microangiopathy
TBI	total body irradiation
TCR	T-cell receptor
TKI	tyrosine kinase inhibitor
TRM	transplant related mortality
URD	unrelated donor
VNTR	variable number of tandem repeats
VOD/SOS	veno-occlusive disease/sinusoidal obstructive syndrome

1 INTRODUCTION

1.1 HEMATOPOIESIS

In humans, the hematopoietic system give rise to $1x10^{16}$ granulocytes, erythrocytes and thrombocytes during a 70-year lifespan combined(*1*). In perspective, this is the equivalent of ten times the weight of an adult. The ability of self-renewal while maintaining its undifferentiated state and the capacity to differentiate into specialized cells types is widely considered to be the definition of a stem cell.

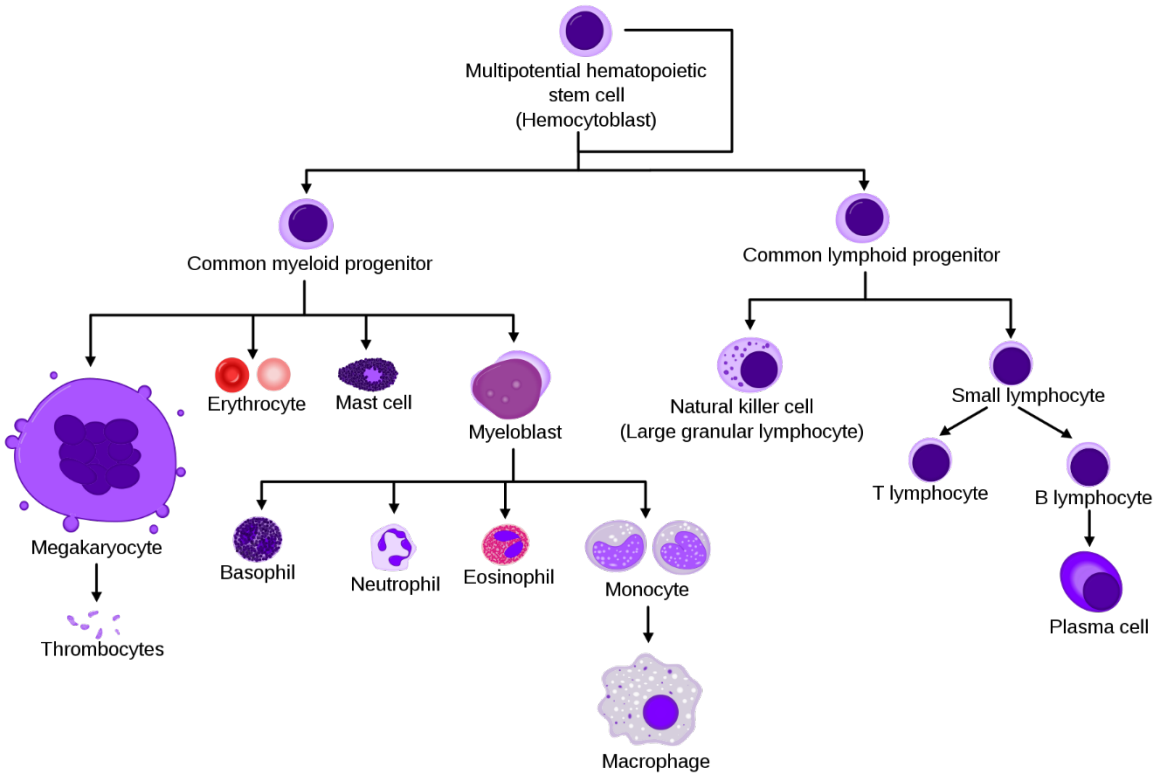

Figure 1. Gross scheme of hematopoesis.

The multipotent hematopoietic stem cell (HSC) differentiate in either a myeloid (erythrocytes, polymorphic granulocytes, monocytes and platelets) lineage or a lymphoid (T- and B-lymphocytes and plasma cells) lineage via a series of progenitor steps in the blood, **Figure 1**(*2*). During early development, primitive hematopoesis is mainly focused on erythrocyte formation delivering oxygen to the rapidly growing embryo. Due to lack of renewal capacity, the primitive hematopoiesis is transitory and replaced by the more definitive hematopoiesis. During this time the HSC is formed in the embryo aorta-gonad-mesonephros but subsequently migrates to the liver, the main organ for hematopoiesis in weeks 6-24 of gestation. Hereon after, the bone marrow is gradually becoming the dominating site for hematopoesis(*3,4*).

1.2 THE IMMUNE SYSTEM – THE SHORT VERSION

To protect the host by distinguishing between self and non-self is the major task for the immune system. Microbial intruders face an intricate system harboring mechanisms for preventing entry, allowing recognition and elimination of the intruding pathogen. Also, the immune system surveys human cell division and potentially malignant cells are recognized and dispersed(*5*).

The multipotent HSCs give rise to the cells responsible for the immune response, **Figure 1**, which is generally divided in the innate and the adaptive immune system, **Figure 2**.

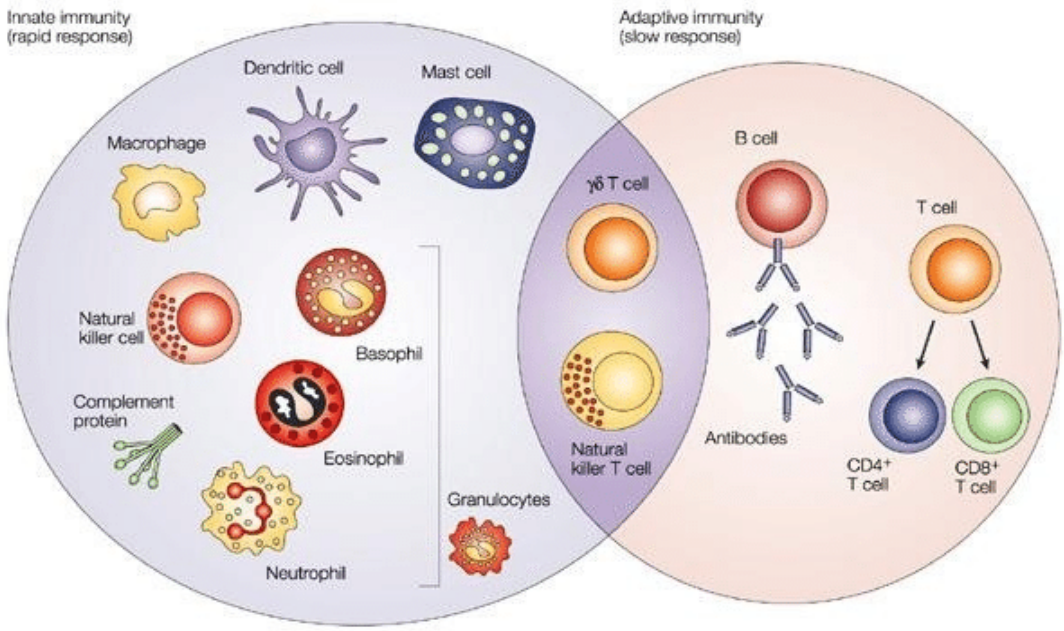

Figure 2. **The innate and adaptive immune response.** Dranoff et al.(*6*)

The innate system is found in all types of plants and animals. Due to its rapid response, it is the first line of microbial deference and acting in numerous ways. It entails physical (skin and mucosa) and chemical (histamine, leukotrienes etc.) barriers. It recruits immune cells, i.e. granulocytes, monocytes and mast cells to the site of infection through chemokine and cytokine signaling. Due to conserved structures on bacterias, e.g. lipopolysaccharides (LPS) and some viruses-infected cells, the innate response recognizes and removes unwanted intruders from various tissues. The complement system is a biochemical cascade helping the immune system to clear pathogens by e.g. marking pathogens for destructions. Since the

innate system reacts to different pathogens in a similar manner it is sometimes referred to as non-specific.

In difference to the innate system, the key feature of the adaptive immune system is to react to a specific pathogen and its capability to prepare for a re-infection, i.e. eliciting an immunological memory. The main characters of the adaptive immune system are B- and T-lymphocytes. Due to somatic rearrangement, they have unique B- or T-cell receptors for each lymphocyte enabling them to have a large number of diversity for pathogen (non-self) recognition. There are two major subtypes of T- lymphocytes: cytotoxic T-cells (CD8+) and T –helper cells (CD4+). To elicit a response the pathogen needs to be presented to a T lymphocyte through (a self-receptor) the human leukocyte antigen (HLA, se section 1.4) of a cell. Endogenous pathogens are presented through HLA class I, found in all nucleated cells, to CD8+ cells, and CD4+ cells recognize exogenous pathogens when presented by HLA class II which are found only on antigen presenting cells (APC) e.g. macrophages and dendritic cells, **Figure 3**. These professional APC's have a sentinel function, thus after engulfing a pathogen they respond by producing inflammatory cytokines and migrating to a lymph node and there present the antigen to awaiting lymphocytes. Importantly, in order to prevent an excessive immune response the system has also immunosuppressive features, often carried out by a small fraction of T-lymphocytes, i.e. regulatory T-cells. In order for cells to communicate and facilitate the immune response, small polypeptides called cytokines i.e. interleukins, interferons and chemokines mediate the process(*7*). B-lymphocytes identify a pathogen when antibodies on its surface bind to a specific antigen. This leads to, via the assistance of T-helper cells, maturation of the same B-cell into a plasma cell, generating multiple production of the initial recognizing antibody.

Bridging over the innate and adaptive immune systems are specific leukocytes named γδT-cells and natural killer (NK)-cells. Hence they both have properties aligning either with the adaptive or innate immune system. The γδT-cells have a T-cell receptor and a possibility to develop a memory phenotype (adaptive immune properties) but also a rapid non-specific response to molecules produced by microbes(*7*). The NK-cells solves the problem with distinguishing between self and non-self by killing virus- or malignant cells who has altered the structure of the cell surface major histocompatibility complex i.e. 'missing -self'(*8*).

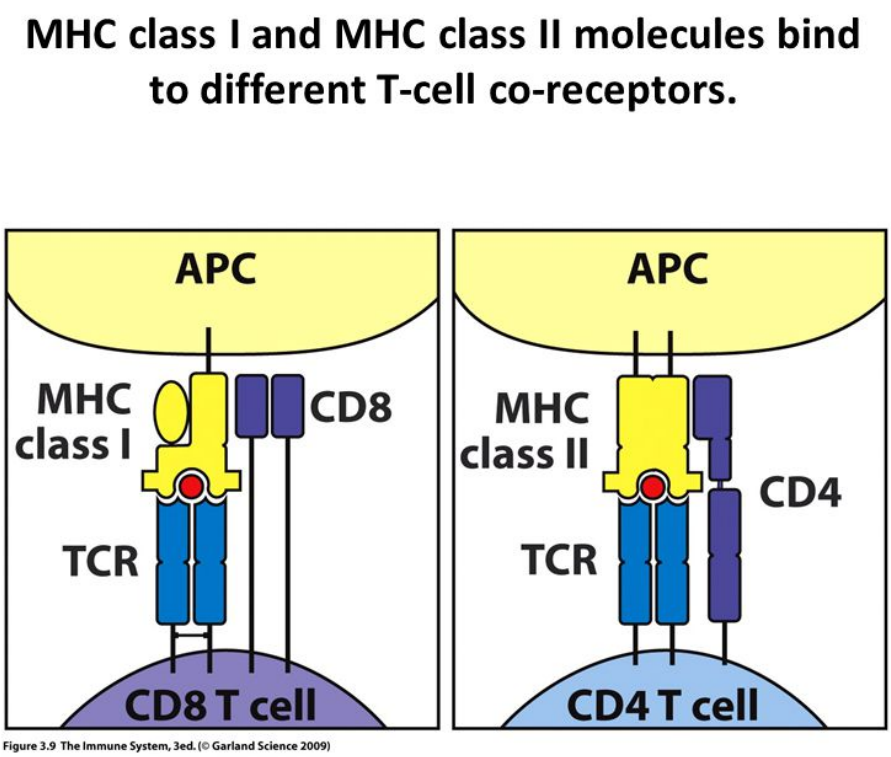

Figure 3. APC, antigen-presenting cell; TCR, T-cell receptor; MHC, major histocompatibility complex. Reprinted from THE IMMUNE SYSTEM, Third Edition by Peter Parham © 2009 by Garland Science, Taylor & Francis Group, LLC. Used with permission of the publisher, W. W. Norton & Company, Inc. All rights reserved.

1.3 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION – A BRIEF BACKGROUND

It is well conceived that the starting point for modern allogeneic HSCT began in the shadow of World War II and the continuing threat of nuclear warfare. Since burn wounds and hematological aplasia is a major effect of high dose radiation, much of the pioneer work was focused on hematological recovery and survival of skin grafts. Jacobson et al(*9*) showed that autologous hematological recovery was possible if the spleen was shielded during lethal radiation and Lorenz et al(*10*) achieved the same results infusing bone marrow in radiated mice. Soon after, Main and co- workers(*11*) began revealing the field of immunological tolerance when infusion of allogeneic bone marrow in mice promoted skin graft survival from the same donor. Some years earlier Nobel laureate Snell et al discovered that differences in genes, named major histocompatibility complex (MHC), caused tissue rejection on a cellular level in a mouse model(*12*).

This caught the interest of oncologist as an optional treatment for patient with hematological diseases with dismal prognosis, leading to irradiation of the patients' malfunctioning bone marrow and the replacement of marrow cells from a healthy and genetically dissimilar donor i.e. HSCT. Most important, the first syngeneic (twin-sibling) HSCT was performed by Nobel laureate Dr. E. Donnall Thomas in 1957(*13*). The following years were burdened by unsuccessful clinical outcome due to relapse, engraftment failure, infections and secondary disease, also known as graft versus host disease (GVHD)(*13,14*), up to a point when prominent hematologists abandoned the idea of treating patients with a HSCT.

However, in continuation of the MHC- work of Snell performed years earlier, work by Storb et al. showed that dog leukocyte antigen (DLA) matched canine pairs had superior survival than DLA-mismatched pairs(*15*). This contributed to the unraveling of human leucocyte antigen (HLA) and from there on the stage was then set for HLA-matched but genetically dissimilar (allogeneic) HSCT in the 1970s(*16*). Since then, allogeneic HSCT has step by step become safer and more available. Due to the lack of HLA-identical sibling donors for a majority of patients, HSCT with unrelated HLA-matched donors began in the 1980s(*17*) and years later voluntary donor registries (e.g. The Tobias registry in Sweden) evolved leading to more available donors. The treatment prior to HSCT i.e. conditioning included initially total body irradiation (TBI) leading to total ablation of the bone marrow, i.e. myeloablative conditioning (MAC). In order to reduce side effect of TBI and improve graft survival, alkylating agents were introduced i.e. cyclophosphamide (Cy)(*18*) and then later Busulfan (Bu). For older patients and those with co-morbidities, the pre-treatment, MAC, was even more reduced in the 1990s with the introduction of reduced intensified conditioning (RIC)(*19*). Also, rather than disease control being managed by MAC, malignancy eradication was found to rely on donor alloactive T-cells, i.e graft-versus-leukemia effect (GVL)(*20*). To avoid GVHD, different immunosuppressive drugs and combinations have been tried, but the initial combination of cyclosporine A (CyA) and methotrexate (Mtx) is still widely used(*21*). In the 1990s alternative donor source such as peripheral blood stem cells (PBSC)(*22*) alleviated the procedure of stem cell donation for donors and is now more common than bone marrow (BM) as stem cell source from HLA identical siblings(*23*) and unrelated donors (URD)(*24*). Also, alternative donors with more HLA-disparity i.e., haploidentical donors(*25,26*) followed by the introduction of cord blood(*27*), have evolved as a safer alternative for patients lacking a more matched donor, contributing to an increase in HSCT numbers(*28-30*). Cellular therapy in HSCT has evolved since it was shown that donor lymphocytes could hamper a pending disease relapse(*31*), and engineered lymphocytes receptor cells which combine antigen binding (to a malignant cell) and T-cell activation

(leading to disposal of malignant cell) i.e. CAR-T cell(*32*). Cells of other origins such as mesenchymal stromal cells and fetal membrane cells have been introduced in order to overcome side effects e.g., GVHD(*33,34*)

1.4 TRANSPLANTATION IMMUNOLOGY

Since the discovery of the HLA-antigen, the effects of matching donor and recipients have revealed more aspects of alloimmunity i.e. donor response to recipient antigens.

More than 200 genes constitute the MHC-complex (the genes are named MHC, the protein molecules are more consistently called HLA-antigen) and it resides on the short arm of chromosome 6. There are three types, i.e. classes, of MHC genes. MHC Class I encodes for the alpha-chain of classical (HLA-A,-B and -C) and non-classical (HLA-D,-E, -F, -G,-H)(*35*). In the Class II region, HLA-DR, -DP and DQ are heterodimers encoded by a co-localizing alfa- and a beta-chain (as earlier stated, these encounter engulfed exogenous proteins and present the combination of self (HLA) and non-self (antigen) to the T-helper (CD4+) cells in order to ensure an immune response, **Figure 3**). Class III do not present antigens, they are involved in immunity by expressing complement proteins and cytokines such as TNFα(*35*). Furthermore, apart from these genes being multi-geneic (several loci in one individual) and characterized by a high degree of allelic polymorphism (several isoforms of the same gene), each parent provides a haplotype (a linked set of MHC- genes) to each offspring, which are co-dominantly expressed (both parental alleles of the gene are expressed at the same time), **Figure 4**. This creates a vast HLA-disparity and helps in host protection against intruding pathogens while being a hurdle for successful HSCT. The degree of HLA-mismatch between recipient and donor determines the risk for allogeneic HSCT-related severe outcomes i.e. graft failure (GF) or GVHD but also contributes positively with the GVL effect in some settings. However, even in a fully matched donor-recipient transplant, other antigens presented by HLA can affect HSCT outcome. The best described are the minor histocompatibility antigens (mHAg), polymorphic genes in non-HLA-regions reflecting differences in individuals(*36*). Also, ABO-blood group incompatibility is not a contraindication for HSCT but a potential factor for adverse events, such as hemolysis, delayed engraftment and GVHD(*37*). Much of the recent success in HSCT treatment is due to understanding the HLA-system and the development of HLA typing techniques(*38*).

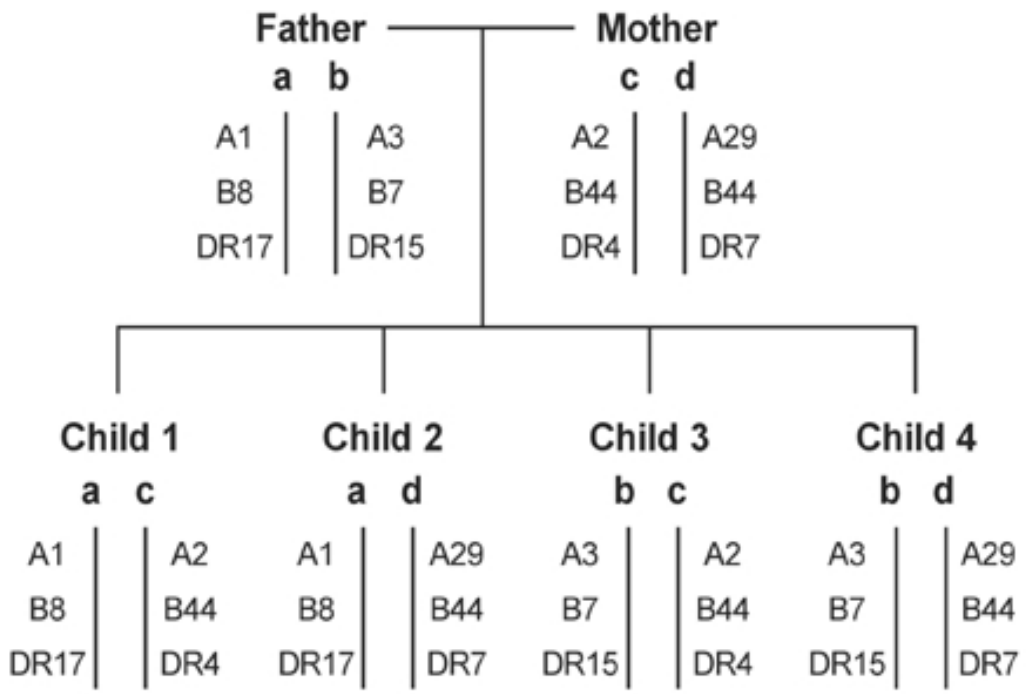

Figure 4. Mendelian inheritance of HLA haplotypes demonstrated in a family. Choo, S.Y (*39*).

1.5 INDICATIONS FOR HSCT

As stated earlier, HSCT is an established procedure used primarily in the treatment of malignant and non-malignant diseases of hematological origin. Based on donor origin, HSCT can be *syngeneic*, when stem cells comes from a genetically identical donor i.e. monozygotic twin, *autologous* when the patient's own stem cells are infused often after high-dose chemotherapy, or *allogeneic* with a genetically different donor. This thesis is focused on allogeneic HSCT, in this thesis abbreviated HSCT.

Historically, HSCT was restricted to patients with acute leukemia, aplastic anemia and severe combined immunodeficiencies. As HLA typing and supportive care has advanced, the HSCT process is now considered safer and is available to a larger group of patients. Today, both the European Group for Blood and Marrow Transplantation (EBMT) and the American Society for Blood and Marrow Transplantation (ASBMT) together with Center for International Blood and Marrow Transplantation Research (CIBMTR) regularly publish updates of treatment indications(*40,41*).

Prior to accepting a patient fulfilling an indication for HSCT, the risk for adverse outcomes needs to be assessed in each patient. A score is assigned from 0 (best) to 7 (worst) in an

additive way(*42*). Five factors are taken into account: patient age, stage of the disease, time from diagnosis, donor type and donor-recipient gender combination(*43*). In addition, factors like Karnofsky performance score(*44*) ≤80 (or Lansky score(*45*) in children ≤16 years), the patient's cytomegalovirus (CMV) serostatus, iron overload and co-morbidities are taken into account. Their impact is not uniform and depends on the sum of risks. This means, for example, that survival is worse for CMV seropositive patients compared to CMV negative patients in low- but not in high-risk patients(*46*).

In 2016, out of the more than 16000 HSCTs performed in Europe, the most common indication was myeloid (56%) and lymphoid (31%) malignancies followed by non-malignant diseases (12%)(*41*). Over the years, indications for HSCT have altered why the EBMT gives continuing updated advice about HSCT indications and their settings, i.e disease, status and recommendable donor. Thus, different combinations of diagnoses and donor sources have been classified as 'standard of care', 'developmental', or 'generally not recommended'. Here is an overview of the diagnoses most frequently considered for HSCT, with focus on those covered in the thesis. It is generally accepted that patients are divided in two groups; those with malignant and non-malignant disorders.

1.6 MALIGNANT DISORDERS

1.6.1 Myeloid malignancies

Adult acute myeloid leukemia (AML) is a particularly heterogeneous, clonal disease where patients have a considerable risk for relapse despite remission after induction treatment(*47*). For patients in first complete remission (CR1), the post-induction treatment can include HSCT or an autologous HSCT (in adults) depending primarily on leukemic cell molecular aberrations and treatment response(*48*). For pediatric AML, despite broad overlap with adult AML, there are differences in disease development, diagnostics criteria and treatment. For children with favorable prognostic markers e.g. lacking the FLT3-internal tandem duplication without NPM1 mutation and responding well, cure rates with conventional chemotherapy are about 70%. Therefore in the Nordic countries, HSCT is standard of care only in high-risk pediatric patients or if relapse occurs(*49, 50*).

The reciprocal translocation of genetic material from chromosomes 9 and 22 is called Philadelphia (Ph^+) chromosome. This causes a fusion gene named BCR-ABL resulting in a tyrosine kinase signaling protein that leads to abnormal cell division. Chronic myelogenous leukemia (CML) is virtually always Ph+. The introduction of tyrosine kinase inhibitors (TKI)

in the late 1990s has today become the first line treatment in chronic phase for adults and pediatric CML-patients(*51,52*). However, HSCT is the only curative treatment and unresponsiveness to TKI treatment or advanced toxicity (in children) makes these patients eligible for HSCT if a suitable donor is available.

For adult patients with myelodysplastic syndromes (MDS), HSCT is considered to be standard of care particularly if treatment is offered before disease progression(*53*). In the rare cases of pediatric MDS or juvenile myelomonocyte leukemia (JMML), HSCT with a sibling- or a well-matched donor is now standard of care(*54*).

Due to the lack of consistent survival benefit for patients with standard risk multiple myeloma (MM), HSCT should only be considered for patients with high-risk MM with poor long-term prognosis when the risk of disease progression outweighs the transplant-related risks(*55*).

1.6.2 Lymphoid malignancies

In recent updates, the most common diagnoses candidates for allogeneic HSCT in all ages was acute lymphatic leukemia (ALL) and non-Hodgkin lymphomas (NHL)(*41*). In young adults with ALL, treatment has been influenced by more aggressive pediatric protocols reducing the need for HSCT. However, considering adult ALL is burdened by more poor risk cytogenetics, HSCT is the standard of care in high-risk patients in CR1 and beyond(*56*). In contrast, pediatric ALL has a longtime overall patient survival close to 90% why HSCT is limited to a small high-risk group i.e. poor responders and persistent minimal residual disease (MRD)(*57*).

Adult and pediatric patients with Hodgkin and NHL, initially have a good prognosis. Furthermore, in a relapse situation, most patients with Hodgkin are considered for autologous HSCT. Hence, HSCT is now only a clinical option for patients with advanced relapse (*58, 59*).

HSCT for solid tumors is beyond the scope of this thesis.

1.7 NON-MALIGNANT DISORDERS

As a group, patients with non-malignant disorders are vastly heterogeneous(*60*). The rationale for HSCT ranges from implementing a new hematopoietic system in patients with aplastic

anemia(*61*), immunodeficiencies(*62*) or enzyme replacement in patients with storage diseases or inborn errors of metabolism(*63-65*) or correcting the erythropoiesis in patients with hemoglobinopathies(*66,67*). In addition, the decision to undergo a HSCT is based on the disease impact on duration and/or quality of life carefully weighing the benefits compared to the risks of a HSCT. This is different from patients with a malignant condition and why HSCT procedures with minimal risk for severe side effects i.e. GVHD and infections are wanted. Also, as these diagnoses are often of autosomal recessive inheritance, siblings may be affected excluding them as donors.

Aplastic anemia (AA) is a rare bone marrow disorder in both adults and children with acquired and less common constitutional etiology. The disease is defined by a pronounced pancytopenia, i.e. low marrow cellularity <25%, low reticulocytes (<1%) and low thrombocytes ($<20\times10^9$/l) in peripheral blood. When peripheral blood neutrophil granulocytes are below $0{,}5\times10^9$/l it is called severe AA (SAA) and the term very severe AA (VSAA) is used when peripheral blood neutrophil granulocytes are $<0{,}2\times10^9$/l the. HSCT is considered standard of care for patients younger than 50 years with VSAA with a HLA-identical sibling or a well-matched URD. Older patients might benefit more from up-front eltrombopag or immunosuppressive treatment (IST) i.e., anti-thymocyte globuline (ATG) or CyA(*68*).

Patients with inherited bone marrow failures syndromes (IBMF) are usually identified when they develop hematologic complications such as severe bone marrow failure, MDS, or AML. They also often have specific birth defects or other physical abnormalities that suggest a syndrome. Treatment with HSCT will not correct the underlying disease nor their co-existing extra-medullary organ defects but will improve the bone marrow failure of the patient(*69*). Fanconi anemia (FA) is caused by impaired response to DNA damage are also an indication for HSCT with special considerations to the conditioning regimen due to high risk for acute toxicity as well as late effects with MAC(*69*). Also, the optimal time for HSCT is probably prior to transfusion dependency and androgen treatment(*70*). Much more rarely eligible for HSCT is Dyskeratosis congenita (DKC), a condition caused by defect telomere maintenance. However, TRM together with late onset of pulmonary- and liver fibrosis is a concern, why special considerations have to be taken with reference to donor selection, patient current status and conditioning regimen(*71*).

Severe primary immunodeficiencies (PID) are characterized by profound effects on the patient's innate and/or adaptive immune system leading to a gradual increase in susceptibility to infections. The diagnosis of severe combined immune deficiency (SCID) is considered an

emergency for infants and HSCT should be offered as soon as possible after diagnosis (*72*). Also in patients with non-SCID, such as those with pure T-cell immunodeficiency, phagocytic disorders (Kostmanns syndrome or other severe congenital neutropenias and Chronic granulomatous disease) and haemophagocytic syndromes, are subject to HSCT(*73*).

Patients with inborn errors of metabolism (IEM) entail a plethora of rare storage diseases. Inherited defects in either lysosomal (the waste disposal of the cell) enzymes or peroxisomal (degrades long fatty acids) function affect several organ functions among which neurological and cognitive impairment are most devastating for children. Therefore, treatment recommendations are based on the disorder, its phenotype including age onset and rate of progression in combination with the risk for HSCT taken the donor availability into account. Most commonly linked to HSCT are the mucopolysaccharidoses (MPS), a subset of lysosomal diseases patients where glucosaminoglycans are not degraded due to a missing/defect enzyme. This leads to an accumulation of glucosaminoglycans i.e. heparan and dermatan sulfate in the bones and connective tissue resulting in nerve compression(*63*).

Patients with Hurlers syndrome (MPS type IH) have insufficient levels of α-L iduronidase and, since it is produced in cells with hematopoietic origin, HSCT is now standard of care (after enzyme replacement therapy which has been available since 2003)(*65,74*).

The hemoglobinopathies consists of the thalassemias and sickle cell disease (SCD). The former are disorders which affect the synthesis of one or more hemoglobin chains resulting, in severe cases, in transfusion-dependent anemia and inflicting iron overload over time. They are divided in α- and β-thalassemias and in the latter undergo HSCT is standard of care after risk assessment (age, liver size, liver histology or problems with iron chelation) if an HLA-matched sibling donor is available. SCD is caused by substitution of valine by glutamic acid in position 6 of the β-globin gene on chromosome 11. This leads to a defective form of hemoglobin (HbS) resulting in anemia with vaso-occlusive pathology. For SCD patients, HSCT after a severe event of vasculopathy is considered standard of care in presence of an HLA-identical sibling donor. There is an ongoing discussion of how to pinpoint patients with a high risk for SCD sequelae i.e neurologic involvement, chronic debilitating pain or recurrent acute chest syndrome, inability to tolerate transfusions, and progressive organ damage to whom HSCT with a URD donor would be an advantage (*75,76*).

HSCT for autoimmune disorders is beyond the scope of this thesis.

1.8 DONOR SELECTION AND STEM CELL SOURCE

In the beginning of the HSCT-era, an HLA-matched sibling donor was the only thinkable option. This is still the preferred source of donor but is only available in about 30% of patients. The remaining 70% of patients receive stem cells from URD's, haploidentical donors or an umbilical cord blood (CB) unit(*77*). In addition, the advancement of tissue-typing techniques means that the majority of HSCT donors today are URD, often selected from one of the voluntary donor registries around the world(*41,78,79*). Because a vast majority of donors in the registries are of western European ancestry, the probability to find a matched URD is 30-70%, depending on the patient's ethnicity and frequency of the HLA-genotype in the donor registries(*77*). A high resolution 10/10 match (HLA-A, -B, -C, -DRB1, -DQB1, i.e. 2 alleles/gene totaling 10 traits) is considered the gold standard although a 9/10 match is acceptable as a ¨well-matched¨ donor if the mismatch is in DQB1(*80*) or an HLA C mismatch(*81*). Consequently, improvements in HLA-typing has led to outcome improvements so that matched URD is now comparable with matched sibling donors used for patients with malignancies(*82-84*) and non-malignant disorders(*85, 86*).

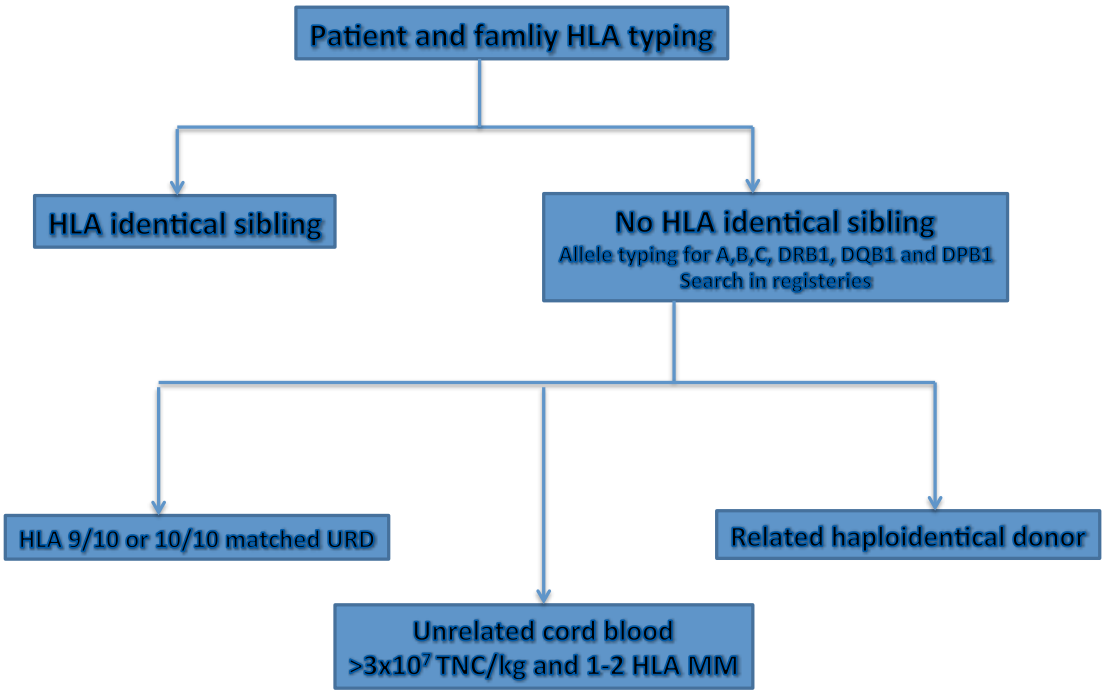

Figure 5. Algorithm for donor selection.

In lack of a sibling or a matched URD, an unrelated CB or a related haploidentical donor may be considered, **Figure 5.** CB units have a lower number of mature, potentially alloactive T-cells and a reason for why lower levels of HLA-compatibility are accepted(*87*). However, more than 2/6 mismatched genes (HLA -A, -B and DRB1 allele typing) are associated with adverse outcomes(*88*). CB has a lower risk of transmitting infection, i.e. cytomegalovirus (CMV) and it is ready for immediate use. The most important variable linked to outcome is the cell dose (2,5-3×10^7 nucleated cells/kg recipient) to overcome slower engraftment and prolonged immune reconstitution(*87*). A comparative analysis has shown similar outcomes for CB-HSCT compared to patients with acute leukemia who received graft from a matched related donor(*89*). A recent report of HSCT activity in Europe revealed increasing use of haploidentical HSCT (haplo-HSCT)(*41*). Most patients have a related haploidentical donor but historically these have been associated with a substantially higher risk for GVHD and graft rejection unless the graft was T-cell depleted(*90*). Nowadays, T-cell depletion is either achieved by αβ(+) T cell depletion(*91*) or post-HSCT administration of cyclophosphamide (Cy). Post–transplant administration of Cy 3 days after stem cell infusion have shown acceptable levels of GVHD and GF(*26*). Rapidly proliferating alloreactive donor T-cells are eliminated by Cy, whereas other non-alloreactive T-cells are spared, promoting immune tolerance and preserving antimicrobial immunity(*92*). As in the case of CB-HSCT, some studies have shown promising outcome when comparing haplo-HSCT with a matched URD or a HLA-identical sibling donor (*93*) also for AML patients with active disease(*94*).

Stem cells can be retrieved from BM, PBSC or from a CB unit, each with advantages and disadvantages(*95*). The collection of donor PBSC is preceded by administration of granulocyte stimulating factor (G-CSF), which mobilizes stem cells from the BM to the peripheral blood prior to harvesting by apheresis. In recent years, PBSC are most commonly used as donor source in adult patients and a large Cochrane review showed similar overall survival when comparing PBSC with BM grafts(*96*). However, it is well-known that PBSC increases the risk for chronic GVHD (*97, 98*) and therefore BM remain the graft of choice in patients with non-malignant disorders and pediatric patients(*99*).

1.9 CONDITIONING REGIMEN AND IMMUNOSUPPRESSION

The cytoreductive treatment of patients prior to HSCT is known as the conditioning. Historically, the purpose has been to create space for donor hematopoietic progenitor cells

and suppress host factors to avoid graft rejection i.e. immunosuppression and disease eradication(*100*). The two modalities most commonly used nowadays are MAC and RIC(*101*).

The chemotherapy intensity of MAC leads to severe pancytopenia (anemia, leukopenia and thrombocytopenia) without ability of autologous recovery. Over the years, a frequently used regimen included Cy and total body irradiation (TBI) (*102*). Since then, modifications have been made. TBI is more selectively used in lymphoid hematological malignancies (e.g. together with Vp-16, etoposide, for pediatric ALL patients) due to its immunosuppressive properties and activity against malignancies including chemo-resistant disease and penetration in sanctuary sites. TBI has been replaced with Bu avoiding long-term TBI side effects such as cataract, pulmonary toxicities, secondary malignancies and, particularly for pediatric patients, growth retardation(*103*). Considering that Bu increases the risk of SOS, sinusoidal obstructive syndrome (earlier named veno-occlusive disease, VOD(*104*)), blood level monitoring of Bu has improved results(*105*) and the combination Bu/Cy is comparable with outcome after using Cy/TBI(*104, 106*). Due to substantial TRM with Bu/Cy, a randomized comparison with Flu/Bu has been showed to be advantageous and is now considered standard of care in adult patients with acute myeloid leukemia(*107*).

The rationale for RIC is that less cytoreductive treatment not only reduces toxicity, enabling HSCT for more aged and co-morbid patients, but also leaves eradication of the remaining malignancy to donor effector cells i.e. graft-versus-leukemia/graft-versus-tumour effect (GVL/GVT). After the introduction of fludarabine (Flu) based RIC in the 1990's(*19*) there has been a vide expansion of different protocols, also introducing other agents such as treosulfan, clofarabine or thiotepa (*100, 101*). Similar outcomes are reported between RIC and MAC conditioning in patients with myeloid malignancies, although the increased relapse rate is compensated for less transplant related mortality in RIC patients (*108-110*).

Thus, in relation to diagnosis, age, available donor and co-morbidity of the patient, the choice of conditioning regimen is currently a risk/benefit discussion among treating physicians.

Furthermore, since T-cells are major mediators in both wanted (immune reconstitution and GVL/GVT) and unwanted (GF and GVHD) effects, additive measures to the cytoreductive pre-treatment can be taken striving for separating the two entities(*111*). Total T-cell depletion of donor graft results in lower incidence of GVHD but increased risk for malignant disease recurrence, GF and delayed immune reconstitution(*112,113*). Therefore, selective depletion of the more GVHD-reactive αβ T-cells while keeping the γδ T-cells (responsible for immune

reconstitution and, together with KIR/HLA mismatched NK cells, malignancy surveillance) has shown promising results(*114*). In some centers ATG, i.e., in vivo T-cell depletion, is used in patients with an URD donor to reduce T-cell alloreactivity, leading to lower incidence of GVHD(*115,116*) but with potentially adverse effects such as cytokine release syndrome, virus reactivation, and in patients receiving higher doses, increased risk for malignant relapse(*117,118*).

After HSCT, the most commonly used immunosuppressive regimen is CyA in combination with Mtx, from the Seattle group (*21*). Other calcineurin inhibitors (CI) such as tacrolimus (Tac) can be used in monotherapy or in combination with Mtx(*119*). Tac has replaced CyA in some center due to a randomised trial which showed that tacrolimus was linked to less severe GVHD. Sirolimus (Sir) is another option (*120*) in combination with tacrolimus(*121*) or mycophenolate mofetil (MMF)(*122*). Albeit comparative studies between Tac/Sir and CyA/Mtx(*123*), Tac/Sir and Tac/Mtx(*124*) or MMF + CI and Mtx +CI(*125*), no optimal combination has shown superior impact on survival compared to the classical Seattle protocol. Due to development of immune tolerance between recipient and donor, immunosuppression is discontinued after 3-6 month for patients with malignancies and after 1 to 2 years for non-malignant disorders (as stated previously, no beneficial effect of GVHD).

1.10 COMPLICATIONS

Treatment with HSCT remains hazardous, mainly offered to patients with a severe condition. A way to measure HSCT treatment success is the incidence of different complications, e.g. GVHD, GF, malignant relapse, infections and poor immune reconstitution.

Days after HSCT, early complications due to the cell destruction caused by conditioning treatment is a major concern for patient mortality and morbidity. Hemorrhagic cystitis (HC) is an inflammation of the urinary bladder mucosa with symptoms ranging from painless haematuria (grade I) to severe hemorrhage of the urinary tract (grade IV). The incidence ranges from 5-25%, risk factors are URD and MAC(*126*) and for those with late onset acute GVHD and viral infections (*127*). Preventive measures include uromitexan and treatment include hyperhydration sometimes combined with bladder irrigation(*128*).

The SOS/VOD of the liver develops usually within a month after HSCT, has an incidence of 5% and is characterized by hepatomegaly, ascites, hyperbilirubinemia and abdominal pain

according to the Baltimore or modified Seattle criteria(*129*). However, these criteria lack the ability of early identification and severity assessment of SOS, why revised criteria have recently been published by the EBMT(*130*). Risk factors are earlier treatment with the CD33 antibody gemtuzumab ozogamicin(*131*) or alkylating agent Bu(*132*). Prevention with ursodeoxycholic acid (a hydrophilic bile acid) (*133, 134*) and defibrotide (a mixture of single-stranded oligonucleotides)(*135*) has been successful. Furthermore, transplant-associated microangiopathy (TAM) is characterized by the presence of schistocytes (fragmented red blood cells), thrombocytopenia, fever and renal impairment(*136*). Switching the patient's current immunosuppressive drug to an alternative one may resolve mild to moderate cases, while plasma exchange or blocking the complement system with eculizumab is considered for more severe conditions(*137*).

The combination of cytoreductive pre-treatment, ongoing immunosuppressive drugs and pending engraftment exposes patients to infections. This is a major concern for clinicians as 20% of patients who die within the first 100 days after HSCT is due to infections(*79*). Immune recovery is often divided into three phases, pre-engraftment (days one to +30), post-engraftment (days +30 to +100) and late phase. During each phase, patients are more prone to be affected by different microbial pathogens(*138*), **Figure 6.**

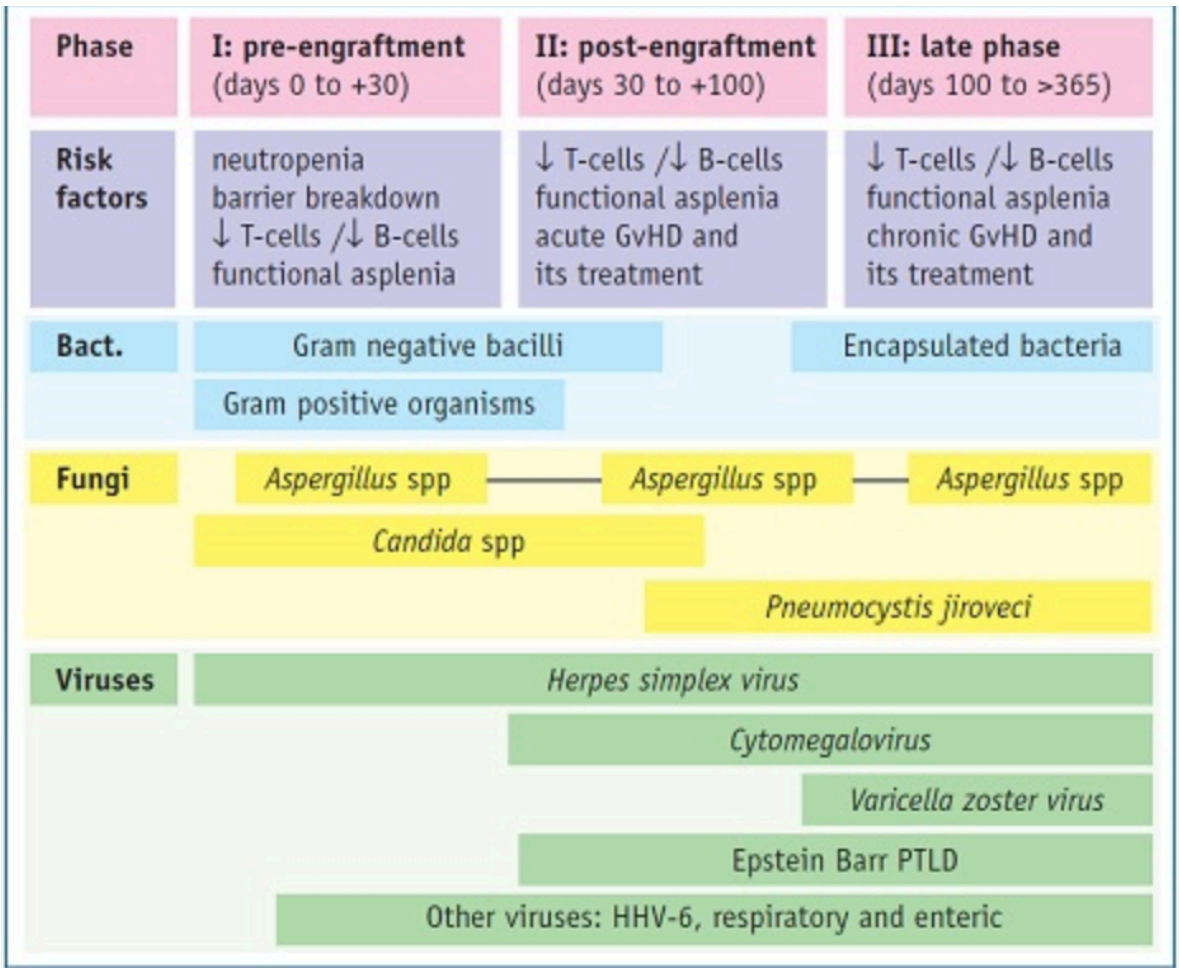

Figure 6. Phases of opportunistic infections after HSCT. With permission fom the editors of the 6th edition of ESH-EBMT Handbook, adapted from Tomblyn et al.(*138*).

In the first phase, neutropenia and mucosal damage leave the patient susceptible to infectious agents similar to those after a course of chemotherapy in patients with hematological malignancies. Depending on the conditioning regimen and stem cell source, this phase has a duration of 5 to 30 days. During this time, gram-positive bacterial infections are most frequent although gram-negative ones can be more severe(*139,140*). Due to antibiotic prophylaxis, gram-negative bacterial infections can be reduced(*141*). Also, prophylactic strategies for fungal infection i.e. Candida spp. with fluconazole are undertaken by some centers(*142*).

Infections during the early post–engraftment phase are due to an immature donor-derived adaptive immune system. CMV reactivates in almost all patients but only one third develop symptoms, due to preemptive treatment strategies based on routine PCR-based monitoring(*143-145*). Epstein-Barr Virus (EBV) may induce post-transplant

lymphoproliferative disorder (PTLD). Treatment consists of rapid tapering of immunosuppressive drugs but rituximab and lymphoma-like treatment may be necessary(*146*). However, adoptive immunotherapy with EBV-specific cytotoxic T-cells have also been shown to be successful(*147*). Moreover, adoptive strategies for treatment of adenovirus, a condition more prone to pediatric patients, have shown effect in a multicenter trial(*148*) To note, acute GVHD and treatment thereof is often associated with viral reactivation and fungal infections during this phase.

In the late phase beyond the first hundred days, cellular and humoral immunity mature and the risk for infections is tightly linked to the presence and severity of GVHD. Pneumocystis jiroveci is, due to co-trimoxazole prophylaxis, rarely seen, but may occur in patients with low CD4+ T-cell counts in combination with early termination of co-trimoxazole(*149*). Pediatric patients with persistent hypogammaglobulinemia, IgG <4g/l, is treated with immunoglobulin(*150*). In addition, as the patients are deprived of their specific immunity, the patients undergo a schedule for active immunization post-HSCT(*151*).

1.11 GRAFT-VERSUS-HOST DISEASE.

A leading cause of HSCT treatment failure is GVHD and it entails the attack of donor cells on different recipient tissues. GVHD is usually divided into the acute and chronic form, traditionally separated by time of occurrence (before or after 100 days post-HSCT) however with a major overlap.

Acute GVHD may be initiated during the conditioning regimen of the recipient when host tissues are damaged. This leads to inflammation and immune response by alloreactive donor T-cells recognizing major (and minor) histocompatibility antigens of host tissues leading to activation of inflammatory effector cells which induce symptoms in skin, gastrointestinal tract and liver(*152*). To treat the patient and assess outcome, acute GVHD is graded within each organ system weighed together to a functional grade between I and IV, with an incidence of 40% for grades II-IV. For malignant diseases, in some centers grade I is not treated at all whereas the prognosis for patients with grade IV is very poor(*153*).

HLA-disparity between recipient and donor, unrelated donors and TBI-included conditioning regimen are risk factors for acute GVHD(*154,155*). The standard treatment for acute GVHD is high dose corticosteroids but there is no treatment consensus for steroid-refractory acute

GVHD(*156*). However, novel treatment approaches with different cellular therapy i.e. mesenchymal stromal and fetal membrane cells and the anti-inflammatory properties of the JAK1/2 inhibitor have shown promising results in patients with steroid-refractory GVHD(*34, 157,158*).

In contrast to acute GVHD, the symptoms of chronic GVHD are more similar to patients with autoimmune diseases such as Sjögrens syndrome and scleroderma i.e. oral xerostomia, dermatitis and keratoconjunctivitis. Chronic GVHD is a major concern for long-term survivors with an incidence of 30-50%(*159, 160*). Due to better thymus recovery, the incidence of chronic GVHD in children is lower(*161*). Although the etiology of chronic GVHD is less known, alloreactive lymphocytes are considered to play a major role. The risk factors include prior acute GVHD, PBSC as stem cell source, older age and female donor to male recipient(*154*) and treatment overlaps the treatment for acute GVHD(*156*).

1.12 RELAPSE AND GRAFT VERSUS LEUKEMIA.

The leukemic relapse incidence after HSCT is a major challenge, causing more than half of the late (>100 days) deaths occurring post-HSCT(*79*).

Over the years, GVHD has been linked to the alloimmune reaction targeting the malignancy i.e. GVL/GVL effect(*162, 163*). Thus, differences in HLA and T-lymphocyte levels not only promote GVHD but also GVL. Firstly, higher incidence of relapse occurred in syngeneic compared to sibling HSCT in both mice and humans (*162, 164*). Secondly, relapse increased when depleting T-cells from donor grafts as GVHD-prophylaxis (*113*). Kolb et al have presented proof of GVL when treating relapsing CML patients after HSCT with infusing of donor lymphocytes(*31,165*). To minimize GVHD and maximize the GVL-effect, several paths have been explored in recent years. The role of NK-cells in malignancy control due to killer cell immunoglobulin–like receptor (KIR)-ligand mismatches (the ligand is present on donor hematopoietic cells and absent on recipient tumor cells) has been explored(*166,167*). Graft manipulation has also emerged in recent years. Depletion of the alloreactive TCRαβ T-lymphocytes from their TCRγδ relatives has gained attention, in particular for haplo-recipients(*114*). Due to similar recognition of malignant cells as NK cells, the TCRγδ T-cells graft maintains the GVL effect (and immune responses toward microbial agents) while posing less risk for GVHD (*168,169*). Another cellular strategy for patients with leukemic relapse, e.g. with B-cell malignancies, involves the use of synthetic engineered chimeric antigen receptor T cells (CAR-T cells), targeting leukemic cell surface molecules. These

receptors are introduced to the cells using a viral vector and do not require APC's for antigen presentation nor are they HLA-restricted to evoke a response(*32*). Different strategies to increase the present immune response toward malignant recurrence have been tried, most recently in the shape of cytokine, Il-15, administration to patients with hematological relapse(*170*).

1.13 GRAFT FAILURE.

While GVHD and GVL occur in the direction of donor against recipient, the reverse situation of an immune reaction of the recipient against donor stem cells results in GF. GF can be either primary (neutrophil granulocytes $<0,5\times10^9$/l by day 28 post-HSCT in the absence of infection or relapse) or secondary (loss of initial engraftment and recurrence of neutrophils $<0,5\times10^9$/l)(*171*). Also, poor graft function, another entity associated with poor outcome(*172*) defined as bi-lineage cytopenia of donor origin. GF can be detected by chimerism analysis (see later section) and patients at risk are mainly those with HLA disparity, alternative donors, low stem cell dose and patients with a non-malignant disorder(*173*). Treatments for GF depend on HSCT indication but G-CSF-treatment, DLI, re-HSCT with original donor or re-HSCT with a new donor with prior conditioning with chemotherapy have been tried(*172*). For patients with poor graft function, a boost of CD34+ cells from the original donor without prior chemotherapy is a safe alternative(*174*).

1.14 SUPPORTIVE CARE

The Joint Accreditation Committee ISCT-Europe & EBMT (JACIE) is an European official accreditation body in the field of HSCT and cellular therapy. It promotes high-quality patient care and medical and laboratory practice through a profession-led, voluntary accreditation scheme. At Huddinge University hospital, a JACIE-accredited center, the cell handling and HSCT-patient care are in accordance with JACIE standards(*175,176*). Supportive care comprises all treatments given to prevent, control, or relieve complications and side effects in the HSCT process, i.e. nutrition and management of nausea, oral mucositis, alternate causal factors of pain and handling of central venous devices. Gut decontamination with oral ciprofloxacin and fluconazole during neutropenia (absolute neutrophil count ANC $<0.5\times10^9$/l) with fluconazole administration continues for three months after HSCT. Patients who are herpes simplex virus positive by ELISA receive acyclovir. To avoid varicella zoster reactivation acyclovir administration one year after HSCT has been shown to be effective,

even in patients receiving RIC(*177*). The risk of pneumocystis jiroveci infection is minimized with co-trimoxazole prophylaxis when ANC > 0.5×10^9/l(*138*) for a duration of six month. Recommended prophylaxis for patients with GVHD under steroid treatment to avoid fungal infections is posaconazole(*178*). In addition, regular CMV(*179*)- and EBV(*180*)-PCR monitoring for infection for at least 3 months after HSCT and readily preemptive treatment decreases the risk for disease. Less liver toxicity and GVHD is achieved with ursodeoxycholic acid(*134,181*) from conditioning until three months after HSCT.

1.15 POST- HSCT MONITORING

The phenomenon of chimera is defined by the co-existence of cells from two different origins and the genetic difference between the recipient and donor is the prerequisite for molecular chimerism monitoring after HSCT(*182*). Chimerism testing analyses the relation between donor and recipient in different hematopoietic cell lineages e.g. T-cells (CD3+), B-cells (CD19+), NK-cells (CD56+), myeloid cells (CD33+) and hematopoietic progenitor stem cells(CD34+)(*182,183*). Modern chimerism techniques are PCR-based, amplifying different polymorphic sequences of the genome to separate recipient and donor with a sensitivity of 0.01-1% depending on the method used(*183,184*). The term donor chimerism (DC) is used when >95% of the analysed cells are of donor origin. Mixed chimerism (MC) describes a state when cells are 5-95% of donor origin. GF is usually defined as having >95% of cells of recipient origin(*185*). Of note, DC and MC were defined differently in paper II presented in this thesis to make it comparable to a previous study(*186*).

The original intent of HSCT is for the donor-derived hematopoietic system to fully replace the recipient one, i.e., full DC. Chimerism analysis showing increasing numbers of recipient-derived cells may be signs of GF in patients with aplastic anemia(*185,187,188*) and relapse in leukemia(*189,190*). Furthermore, DC of T-cell subsets shortly after HSCT is associated with an increased risk for acute GVHD(*191,192*). However, since the introduction of RIC-regimens, patients with MC are more likely to occur(*193*) and a state of stable MC can be associated with tolerance rather than a pending adverse advent i.e. GF or relapse(*194*). In HSCT performed for non-malignant disease such as Hurlers disease, enzyme production can be improved with only 10-20% of MC(*195*). For both malignant and non-malignant diseases, MC dynamics is monitored over time since late GF and relapses do occur(*196,197*). Furthermore, chimerism analysis can help to evaluate the effect of DLI (i.e. lowering high recipient MC with donor-derived T-cells) or be a useful tool to decide to provide prophylactic

DLI i.e. to augment the GVL-effect in case of increasing MRD or in the situation of incipient GF(*198-200*).

Although useful for engraftment monitoring, chimerism analysis does not specifically detect leukemia cells. For more sensitive detection of cells that cause disease, analysis of leukemia specific markers are needed. Numerous techniques have evolved over the years in order to identify the presence of small numbers of malignant cells expressing molecular markers of disease more sensitive than microscopic pathology examination, namely minimal residual disease (MRD). Nowadays, the most commonly used method of MRD- detection is by flow cytometry, monitoring the patients with a leukemia-associated immunophenotype (LAIP)(*201*). In addition, increased sensitivity but more labor intensive are molecular detection of MRD. For example, in patients with CML, MRD quantification can be performed by detection of BCR-ABL transcripts(*202*). Other disease- and patient-specific methods using PCR have been used and shown to be of importance in detecting MRD(*203, 204*). However, MRD diagnostics is still lacing a combined sensitive, fast and standardized method why high-throughput sequencing and next-generation (multidimensional) flow cytometry, both evaluating millions of sequences or cells, respectively is under scrutiny(*205*).

1.16 BASIC BIOSTATISTICS

Survival analysis is a model for time until a certain 'event', most often death. Ideally, in survival studies, all patients would enter at the same time, undergo HSCT at the same time and remain in the study until the outcome of interest was achieved. However, patients undergo HSCT at different time points and some patient's data are incomplete, either due to not having presented the observed event yet, or are lost to follow up i.e. censored data. Statistical accommodations in spite of these condition are the backbone of survival analysis(*206*).

Primary events post-HSCT such as engraftment, acute or chronic GVHD, relapse or death can be used to calculate different outcomes, e.g., TRM – the probability of dying without disease relapse, acute GVHD – the probability of developing acute GVHD (also, grading of severity), OS - the probability of overall survival irrespective of disease state etc, several of which are used in this thesis. Importantly, there are two categories of probability curves. Increasing probability curves over time involve acute and chronic GVHD, TRM and relapse and survival events show curves with decreasing probability.

The most frequently used survival curve is the Kaplan-Meier curve measuring the survival time from a certain date to the date of death or another significant event. Two outcomes are possible; either the patients have the event outcome (e.g. death) or they do not (censored patients). The curve is an estimated probability of survival. In addition, sometimes a comparison between two survival curves is wanted why mathematically ¨weighing¨ the two cohorts tests if a comparison can be made using, for example, a log-rank test(*207,208*). When data supposedly is not normally distributed, a non-parametric test for comparing outcome between two groups the Mann -Whitney U-test (engraftment) or

Furthermore, a competing risk is an event that poses a problem in survival studies. A patient can either; a) have relapse, b) have no relapse and is alive, or c) no relapse and is dead. Here death becomes a competing risk for relapse probability and a more suitable analysis for this is a cumulative incidence curve(*209*).

Moreover, when wanting to find out if one, out of many, possible prognostic variables is relevant for outcome in a specific patient cohort, a multivariate analysis is performed with a proportional hazard regression analysis described by Cox(*207*). In addition, having adjusted for factors with known impact on outcome, variables previously unknown can be found significant.

Finally, as previously stated, competing risks is often a reality in HSCT-studies and when the intent is to estimate the occurrence of one cause, e.g. relapse, but other events, such as TRM, can compete and need to be adjusted for, a proportional hazard for the subdistribution of competing risk must be employed (*210*).

2 AIMS

The general aim for this thesis was to improve knowledge and understanding for certain subsets of patients undergoing HSCT. The questions were raised from daily clinical practice with adult and pediatric patients in combination with existing routine laboratory testing. This thesis aims to more closely understand important clinical concerns stated below.

- What is the outcome for patients with a non-malignant disorder who undergo HSCT with a matched URD? Is it comparable with the outcome for HLA-identical sibling donors, and if so can explanatory factors be identified?

- Can chimerism be of importance in predicting outcome, i.e. acute GVHD and rejection for patients with non-malignant diseases? How are patients with high recipient mixed chimerism responding to different immunomodelatory treatments?

- Has outcome for pediatric patients who undergo HSCT improved over the years? If so, can explanatory factors be identified and if not, what should be the focus of future improvements?

- Are donor graft lymphocyte sub sets numbers important to outcome after PBSCT in patients with malignant diseases?

3 PATIENTS AND METHODS

3.1 CHIMERISM ANALYSIS

Different techniques have been used in paper II. The technique is based on finding highly polymorphic gene sequences both in the recipient and in the donor to enable separation. The markers used are variable numbers of tandem repeats (VNTR; minisatellites), short tandem repeats (STR; microsatellites) and single nucleotide polymorphism (SNP). The VNTR and STR analyses are based on PCR amplification followed by separation with gel(*191*) and capillary electrophoresis, respectively(*184*). SNP analysis is based on quantitative real-time PCR(*184*). Nowadays, STR analysis is the most commonly used method for quantitative chimerism assessment after HSCT(*183*). VNTR and STR analyses have a sensitivity of 1-5% to detect the minor cell population while real-time quantitative PCR analysis of SNP markers can reach down to 0.01%. Prior to HSCT peripheral blood samples are collected from the donor-recipient pair to identify a robust marker. DNA from donor and recipient pre-transplantation samples was extracted using standard protocols (Qiagen, Hilden, Germany). Chimerism data were collected 1, 3, 6 and 12 months after HSCT and at the last follow-up. In patients with high MC, see paper II, all available data were collected post-HSCT. In order to enhance sensitivity, lineage-specific chimerism was performed. Selection of T cells (CD3+), B cells (CD19+) and myeloid cells (CD13+, CD33+ or CD45+ cells) from peripheral blood were done using immunomagnetic beads (Dynal, Oslo, Norway).

Hereafter, sample separation markers were PCR amplified and subsequently quantified with fluorescence detection applying capillary electrophoresis(*184*).

3.2 FLOW CYTOMETRY

In paper IV, flow cytometry was used to assess the graft content of CD34+ cells and non-CD34+ cells i.e., CD3+ (T-cells), CD19+ (B-cells), CD4+(T-helper cells), CD8+(Cytotoxic T-cells), CD3-CD56+CD16+ (NK-cells), CD4+CD127lowCD25high (regulatory T-cells) cells. This is done routinely at the CCC, Karolinska University Hospital, Huddinge prior to HSCT. Flow cytometry is based on the use of marker-specific antibodies labeled with to fluorochromes. Cell suspension incubated with the antibodies are then run on a flow cytometer i.e. a type of dye, a molecule that is excited by light at a certain wavelength and emits this energy at another wavelength, a process called fluorescence. By letting a laser beam hit the fluorochrome in a controlled fashion, the emitted fluorescence can be detected.

This beam is also scattered around the cell allowing assessment of cellular characteristics. In addition, the cells are passed through the laser beam in a single-cell line enabling analysis of one cell at a time and with multiple channels of fluorescence, the complexity of each cell can be analysed(*211, 212*). The exact flow cytometry protocol used for paper IV has been described previously(*213*).

Ethics

The studies were approved by the Regional Ethical Review Board in Stockholm, DNR 63/96, 425/97 and 103/03.

3.3 PATIENTS

	Paper 1		Paper 2	Paper 3		Paper 4	
Factor	**SAA**	**IEM**		**1992-2002**	**2003-2013**	**Sibling**	**URD**
N=	12	13	58	188	201	97	202
Age	15 (1-39)	1 (0.5-39)	7 (0–64)	8 (0-17)	8 (0-17)	49 (15-71)	52 (4-71)
Gender (M/F)	4/8	10/3	29/29	105/83	132/69	53/44	127/75
Diagnosis:							
aLeukemia	-	-	0	104	72	50 (52%)	111 (55%)
cLeukemia	-	-	0	0	0	13 (13%)	17 (8%)
Lymphoma	-	-	0	0	0	10 (10%)	21 (10%)
MDS/MPN	-	-	0	14	20	17 (18%)	46 (23%)
Other malignant	-	-	0	13	16	7 (7%)	7 (3%)
Non-malignant	12	13	58	57 (30%)	93 (46%)	0	0
Late stage (>(CR1/CP1)	-	-	-	90	73	54 (56%)	115 (57%)
Donor age	41 (21-44)	32 (23-47)	29 (0-50)	28 (0-56)	24 (0-54)	48 (17-71)	29 (18-54)
Donor:							
MRD	0	0	19	77 (41%)	60 (30%)	97	-
URD	12	12	35	82 (44%)	92 (46%)	-	202
Mismatched	0	1	10	29 (15%)	49 (24%)	0	0

SC source:							
BM/PBSC/CB	12/0/0	8/5/0	44/14/6	150/35/3	140/31/30	0/97/0	0/202/0
TNC dose	4.6 (0.6-8.3)	5.7 (1.5-59.3)	3.7 (0.03-81.39	3.5 (0.03-80.0)	4.7 (0.3-81.3)	13.4 (5.0-36.4)	10.9 (4.3-36.9)
Conditioning:							
RIC/MAC	7/5	0/13	32/28	178/10	126/75	47/50	85/117
TBI-based	9	1	5	108	53	31 (32%)	59 (29%)
Chem-based	3	12	53	80	148	66 (68%)	143 (71%)
ATG	12	13	61	132 (70%)	157 (78%)	0	197 (98%)
GVHD prophylaxis:							
CyA+Mtx	7	11	32	158 (84%)	123 (61%)	74 (76%)	148 (73%)
Tac+Sir	3	0	17	0	36	22 (23%)	50 (25%)
Other	1	3	17	0	42	1 (1%)	4 (2%)
Follow-up (years)	2.1 (0.8-10.4)	3.7 (1.5-11.1)	4.3 (0.4–10.6)	16.8 (12.0-22.8)	5.5 (1.0-11.9)	7.4 (3.0-10.5)	5.6 (2.7-10.4)

SAA, severe aplastic anemia; IEM, inborn errors of metabolism; URD, unrelated donor; aLeukemia, acute leukemia; cLeukemia, chronic leukemia; MDS, myelodysplastic syndrome, MPN, myeloproliferative neoplasm; MRD, matched related donor; BM, bone marrow; PBSC, peripheral blood stem cells; CB, cord blood; TNC, total neutrophil count; RIC, reduced intensity conditioning; MAC myeloablative conditioning; TBI, total body irradiation; Chem, chemotherapy; CvA, cyklosporin A; Mtx, methotrexate; Tac, tacrolimus; Sir, sirolimus,

4 RESULTS AND DISCUSSION

4.1 PATIENTS WITH NON-MALIGNANT DISORDERS WITH UNRELATED DONORS – PAPER 1.

Diseases like non-malignant disorders are not only rare but also difficult to categorize. In the first paper, we separated the 12 patients with bone marrow failures from the other 13 patients with non-malignant disorders and named them IEM (inborn errors of metabolism). However, only eight of 13 patients in fact had IEM. The other five patients (four with FHL and one SCID) were originally classified as primary immunodeficiencies.

In summary, an encouraging OS of 84% was observed in the whole cohort considering that other groups had reported worse outcome (between 35-55%)(*214-216*), although these studies did include more patients with mismatched grafts. Also taken to consideration, these twenty-five patients underwent HSCT between 1993 and 2003 at our center, during which supportive care and HLA-matching were not as developed as in more recent years(*217, 218*) (see also paper III).

This is a heterogenic, small and retrospective study and any conclusions need to be verified in further studies. However, a rare diagnosis is not an exclusion criteria for survival data analysis.

BMF patients were separated from IEM patients based on the presumption that prior immunosuppressive treatment and heavy transfusion could reflect outcome. However, due to low numbers, statistical analysis of cumulative incidence of acute and chronic GVHD was performed on the group as a whole.

Conditioning was heterogeneous in the SAA group due to the lack of international consensus. SAA patients mainly received 50mg/kg Cy for four consecutive days together with various fractionation and doses of TBI and Fludarabine and all patients received rabbit ATG (Thymoglobulin; IMTIX, Sangstat-Lyon, France) with a median total dose of 9.0 mg/kg (range 2.9 and 12.8 mg/kg). All SAA patients successfully engrafted and the recommendation from this paper was similar to current recommendations, consisting of four days of Flu, lower Cy dose (50mg/kg) and 2Gy TBI(*219, 220*). The cumulative incidence of acute GVHD grade II-IV and chronic GVHD was 24% and 21%, respectively but not more than grade II (acute GVHD) and mild (chronic GVHD). However, GVHD is unwanted for patients with non-malignant disorders and 4/6 patients who developed acute GVHD had received G-CSF. Since the association of G-CSF with increased incidence of GVHD was established (*221*), G-CSF

is no longer a part of supportive care regime. Furthermore, five patients were given PBSC, and since this is also associated to chronic GVHD(*97-99*), these patients are now preferably offered BM as stem cell source. In addition, all patients received ATG in a median dose of 9mg/kg to reduce incidence of GF and GVHD(*115*). However, out of the 4 patients who succumbed, 3/4 deaths were due to infections, which raised question regarding the ATG dose. The dose of ATG when TBI is included in the regimen has since been reduced to 5-7,5mg/kg (*117, 219*). Also, different in vivo T-cells depletions have been tried(*222*). In order to further decrease GVHD we tried different prophylaxis against GVHD. CyA + MMF was given to four patients and Sir + Tac to five patients. However, later data have shown no superior effect of these two combinations to the classical CyA and four doses of Mtx (*121, 125*).

With the high OS of 83 % for BMF patients and no observed GF, we considered earlier HSCT with a matched URD instead of upfront IST. This is supported by current data suggesting that outcome using a matched URD is similar to patients receiving a graft from a HLA-identical donor(*218*). In addition, pediatric patients do better after an upfront matched URD than after a failed IS treatment and subsequently undergoing a matched URD HSCT(*223,224*).

For the IEM-group, all but one received Bu + Cy as conditioning regimen. Most patients were below the age of six, a risk factor for VOD/SOS(*129*), and one patient succumbed to VOD/SOS. The current data and recommendations have since diverged for the different diseases in this group. For Hurlers disease, enzyme replacement therapy has become available and recent data show superior survival for MAC and CB as stem cell source, if lacking a suitable sibling (*74*). In contrast, to obtain better OS for hemophagocytosis syndromes, a fludarabine-based RIC regimen has shown promising results(*225*). For patients with MLD and ALD, HSCT is rarely an option nowadays due to promising gene-therapy results(*226, 227*) but indication for HSCT do exist(*228*).

To conclude, this paper suggested in 2004 that for pediatric patients with SAA; bone marrow instead of PBSC as stem cell source, no G-CSF post HSCT as supportive care, conditioning with CY+Flu+TBI in combination with ATG and considering earlier HSCT when a suitable HLA-matched identical sibling is not available. These recommendations are in line with current practice(*229, 230*).

4.2 PATIENTS WITH NON-MALIGNANT DISORDERS WITH REGARD TO CHIMERISM – PAPER 2.

Since chimerism analysis was first evaluated to detect malignant relapse(*231, 232*) and GF (*189, 233*), more knowledge about chimerism status in RIC patients(*233,234*) and its relation to GVHD(*191*) has accumulated. We wanted to evaluate our cohort of non-malignant patients to determine whether MC can predict GF or GVHD and, in a situation with high MC, what are the current measures used to prevent GF?

The methods used for separating donor and recipient cells evolved during the course of this study. When the study began, VNTR was used(*235*) which was replaced by STR in 2003. As a complement and with much higher sensitivity, quantitative single nucleotide polymorphism was introduced in 2005(*184, 236*). However, to date, efforts in Europe are made to harmonize chimerism sampling (*183*) using foremost microsatellites (STR) as a separation marker. Also, the current definitions of MC (5-95% of donor cell origin) and DC (i.e. >95% donor origin) differed from our definitions (MC =1-99% of donor origin, DC>99% of donor origin). Because of the sensitivity of STR (1%) and a previous study, this definition was chosen(*186*). Furthermore, we define a patient as having high degree of MC if two consecutive samples were >30% of recipient origin. The patient chimerism samples were collected at 1, 3, 6 and 12 months post-HSCT. Chimerism results in different cell lineages were not presented separately because of the fact that they were mostly coherent after HSCT. In a minority of cases, in which lineage chimerism values were different, the T-cell or myeloid lineage with the highest recipient levels defined the chimerism status. In addition, no specific lineage dominated the high MC group. This contradicts other studies pinpointing an increase in NK- and T-cells for both early and late relapses(*233, 237*).

Patients who developed DC were strongly associated with development of acute GVHD, (GVHD grade II-III, OR 6.1, 95%CI 1.7-22.6, p=0.007). Also, DC was more frequent in patients receiving MAC compared to RIC (50% and 22% respectively p=0.036), and since rejection was similarly distributed between RIC and MAC (of the patients who rejected the first time), a RIC for these patients might be more suitable. Interestingly, there was a similar incidence of MC and DC in patients who developed chronic GVHD. This could be due to small patient numbers or that DC is not required for chronic GVHD, indicating a subset of alloreactive T- and perhaps B-cells for patients with MC and chronic GVHD.

Mixed chimerism was detected in 64% of the transplants. Factors linked to developing MC were mismatched grafts and patients who had received RIC as conditioning regimen (78%

compared to 50% for MAC, p=0.036), also previously described by others. Since PBSC donor grafts contain higher T-cell numbers increasing the risk for chronic GVHD(*98, 238*), although small numbers, we found no difference in the frequency of MC after using BM compared to the use of PBSC. In contrast, all eight transplants with CB developed MC (three stable and five high MC, for definition se paper II) leading us to believe that HLA disparity and low graft cell dose are factors associated with MC development. However, a subsequent retrospective study of CB-HSCT done at our center showed that most of these patients developed DC (*239*).

All nine HSCTs resulting in GF and five out of six deaths were in the high MC group (24 patients). We analyzed the clinical actions taken to avoid rejection and management consisted of changing immunosuppression and DLI administration, as previously described(*240*). Interestingly, MC status improved (i.e. moved toward DC) after increasing immunosuppression (in 2 patients) or decreasing immunosuppression (in 3 patients). In patients with malignancy, when an increase in MC status occurs, immunosuppression is usually tapered to strengthen the donor- versus- recipient T-cell response. Here, the rationale to increase the immunosuppression was to hamper the thriving recipient cells in combination with reluctance so taper immunosuppression due to the risk for GVHD. Calcineurin inhibitors form complexes with intracellular binding proteins which subsequently inhibit cytokine production, thereby inhibiting further lymphocyte stimulation(*241*). Putatively, calcineurine inhibitors may have different affinity to these binding sites creating a difference in response between individuals. Hence, a dose alteration may affect the donor lymphocyte more than the recipient lymphocytes.

In seven out of 17 transplants, MC was decreased or continuously stable after administration of DLI. No patient developed cytopenia and only two out of 15 patients developed mild acute GVHD, a commonly reported side effect of DLI(*199, 200*). However, three deaths occurred in this group (PTLD, a secondary malignancy and mors subita) indicating that these patients are still at risk for other adverse events despite improvement of MC. Moreover, in 10 patients, DLI had no effect on MC and five of these patients subsequently rejected their grafts. In patients with malignancies, the prognosis of DLI outcome depends on recipient cell load and the discontinuation of immunosuppression. Other groups have suggested using a low dose of DLI in patients with non-malignant disease when MC increases above 30% of recipient cells (*240*). Thus, the poor outcome for our patients was probably due to DLI administration when MC was too high (median of 65%) in addition to continuation of immunosuppression.

Out of 8 patients who ultimately rejected their first grafts (one patient rejected the second graft), 6 underwent a second HSCT, of which 5 were in remission at the time of follow-up. Reduced conditioning was used and half of the patients were re-transplanted with a new donor. Although only a few patients were re-transplanted in our study, a recent single center report evaluated the outcome after a second HSCT in 30 patients with non-malignant disorders with a 5-year OS of 53% and event-free survival of 47%(*242*). These data suggest that even if rejection occurs, the outcome after a second transplantation is encouraging. However, the clinical situation for a patient with MPS I-H with graft rejection is quite different from a patient with hemoglobinopathy underlying the need to carefully evaluate risks and benefits of a re-HSCT.

Furthermore, three patients had MC< 20% of donor cell origin but remained in remission at the time of follow-up. A boy with FHL without any sign of hemophagocytosis and with immunosuppression tapered two years prior to follow-up. A girl with Hurlers syndrome (MP1-H) had undergone a second HSCT due to GF and subsequently increased to MC <20% donor. However, although her urine-glycosaminoglycan was slightly elevated, her enzyme levels (leukocyte-α-iduronidase) were normal and defined as in remission. Finally, a boy with aspartylglucosaminuri (AGU) had a slow MC-increase in T- and myeloid lineages three years after tapering the immunosuppression. At the time of follow-up, disease markers were normal and defined as in remission. These three patients complicate the ability to define pre-emptive measures when there is high MC as it is unknown which of the patients will ultimately reject(*243*). However, these findings show that MC as low as 10-20% of donor origin may be sufficient to restore or maintain disease control in certain non-malignant disorders(*196, 244*).

Like Paper I, this was a small retrospective study analyzing a vastly heterogeneous cohort of patients, with a short follow-up time, preventing any firm conclusion. The OS of 87% was encouraging but these patients clearly have severe events after HSCT why a event-free survival analysis would have brought additional value. Also, this study lacks any firm conclusion as to how to conduct pre-emptive measurements for patients with high MC.

4.3 COMPARING OUTCOME BETWEEN TWO DECADES FOR PEDIATRIC PATIENTS UNDERGOING HSCT – PAPER 3.

We wanted to evaluate the evolution of outcome in pediatric patient outcome undergoing HSCT during two-time intervals (1992-2002 – P1 and 2003-2013 - P2) and draw out influencing factors. In line with previous and contemporary studies (*217,245,246*) the most important finding was an improved OS in patients transplanted in P2 (78% compared to 58% in P1, p<0.001).

Between the two-time periods, we found several changes with potential impact on outcome. Conditioning regimens including TBI were replaced in P2 by combining different chemotherapeutic drugs (p<0.001). The alkylating agent Bu most often replaced TBI, aiming to reduce radiation side effects such as cataract, growth retardation and infertility but also improving event free survival particularly in patients with matched URD (*247*). In addition, Bu can be dose-optimized due to measurable pharmacokinetics further reducing toxicity(*105, 248,249*). In later years, RIC frequency increased (p<0.001), also as has been reported by other centers (*250-252*). Flu and Bu are more commonly used, but the definition of RIC has been debated (*101,244*). The increase in RIC is most probably explained by the fact that RIC has become the first choice of conditioning regimen for many non-malignant disorders, i.e. Flu-based conditioning for hemophagocytic lymphohistiocytosis (*225*). In P2, we also found the use of more mismatched donors, probably entirely explained by the increased use of CB as stem cell source. This also explains the later neutrophil engraftment in P2 compared to P1 (HR=1.21, 95% CI=1.09–1.34; p< 0.001). Due to expanding CB banks and awareness of HLA-matching, CB use has increased worldwide (*89,253*). The use of PBSC as donor source was unchanged in P2 (35) compared to P1 (31). As BM is the recommended source of stem cells for pediatric patients, the donor choosing PBSC can explain the fact that PBSC was unchanged in recent years.

Furthermore, the increased usage of RIC-protocols and mismatched donors might reflect on the incidence of GF in P2. Even though there was an increase in GF during P2 compared to P1 (14,4% and 4,8%, respectively p=0.01), no increased risk appeared in the corrected multivariate analysis. More patients were diagnosed with acute GVHD grade II-IV in P2 compared to P1 (HR=1.77, 95%CI=1.43–2.66; p=0.003). Furthermore, the incidence of acute GVHD increased both in patients with nonmalignant disorders and in patients with malignant diseases from 18% to 30%, respectively, in P1 to 24% and 47% in P2. Although P2 contained more RIC, and median stem cell dose was lower, explanatory factors could be the increased

usage of mismatched grafts in combination with more commonly usage of GVHD-prophylactic combinations other than Mtx and CyA. However, the latter did not emerge in the multivariate analysis, why we speculated whether the increase of acute GVHD could be due to more aggressive diagnostics, i.e. readily performing colonoscopy in patients with suspected acute GVHD of the gastrointestinal tract. The use of mismatched donors was more commonly found in P2 compare to P1 (p=0.01). However, this cohort consisted of more CB-donors who, at least in a single CB-setting, have been found to be associated with lower incidence of acute GVHD, due to higher graft numbers of naïve T-cells subsets (*253,254*). More over, the increase in acute GVHD in P2 did not affect improved OS, indicating improved treatment for acute GVHD. In contrast to recent findings(*255*), we found a lower incidence of chronic GVHD in P2 (15%) compared to P1 (32%, p<0.001) but still, it was associated to using PBSC as graft source. This finding is also encouraging due to the observation of increase in acute GVHD in P2, a known risk factor for chronic GVHD(*163*). However, we speculated weather this lower incidence would negatively impact relapse rate. Resent studies show both that chronic GVHD is associated with GVL only in CML patients(*256*), a rare diagnosis in pediatrics, while others have shown an association between chronic GVHD and GVL only in the first hundred days post-HSCT(*255*). We found no significant change in relapse-rate between the two time-periods overall, but, we did observe that patients with chronic GVHD surviving more than a hundred days were associated with less relapse (HR=0.29, 95%CI=0.15–0.57; p< 0.001), indicating an unwanted decrease in chronic GVHD in P2. In caution, this may be due to our follow-up time, missing late relapses. Several groups have also observed that relapse incidence has not decreased over time (*253,257*), flagging this as a prime object for future improvements.

In contrast, TRM i.e., non-relapse mortality between the two time periods improved significantly. Improvements in supportive care(*144,258-261*), treatment of infections and GVHD are thought to contribute. In addition, improved high resolution HLA-typing resulting in better matching of donor/recipient-pairs. This has also been unequivocally shown for most patients groups (*253,262,263*). Hence, trends in performing HSCT with mismatched-donors, increasing RIC regimens and more non-malignant diseases resulted in reduced mortality. OS improved for all patients, but for patients with non-malignant disorders undergoing HSCT with a matched URD, 5-year OS increased from 72% in P1 to 93% in P2 (p=0.02). This is a major contributor to our improved overall survival in the pediatric cohort.

4.4 THE IMPORTANCE OF GRAFT LYMPHOCYTE SUB SETS TO OUTCOME – PAPER 4.

The original aim was to conduct a large, single cohort study to analyze grafts of all patients transplanted since 1998. However, since factors influential to outcome i.e, diagnosis, HLA-matching, donor source, conditioning regimens and immunosuppression has changed over the years, we decided to narrow the cohort in terms of time interval, diagnosis and stem cell source, selecting only patients with malignant diseases undergoing HSCT between 2006 and 2013 with PBSC as stem cell source. In addition, we divided the cohort in URD and HLA-matched sibling donors (Sib) hypothesizing that ATG administration (given to all but five patients with URD grafts) and graft transportation (most URD donors were collected from external centers) and handling might influence donor graft cell subsets. As primary endpoint we chose GVHD, relapse incidence, TRM and OS.

The study included cell subsets routinely analyzed at our center prior to HSCT. Lymphocyte subsets included cells known for their importance in alloreactivity and immune reconstitution i.e. T-cells (CD3+), T-helper cells (CD3+CD4+), T-cytotoxic cells (CD3+CD8+), NK-cells (CD3-CD56+CD16+) and B-cells (CD19+)(*152,264-268*). T-regulatory cells are usually identified by expression of intracellular Foxp3, however, our CD3+CD4+CD127^{low}CD25^{high} marker is a known surrogate(*269*). Routine analysis of CD3+CD4+CD127^{low}CD25^{high} was started later at our lab which was a contributing explanation to the higher incidence of missing data.

We found no difference in OS between the URD and Sib donor group. Prior studies show an inferior OS with CD34+ cells $<4\times10^6$/kg(*42*), a level which both of our groups attained. Lymphocytes were significantly lower in the URD group compared to the Sib group probably due to graft handling and transportation as described by others(*213*). Graft viability was similar between the two groups, 99% and 98%, p<0,001, respectively. Thus, potentially ruling out collecting PBSC from external centers as a factor for graft viability. Other factors such as number of apheresis could also have been influential. Furthermore, the ratio of CD4+/CD3+ and CD4+/CD8+ was similar indicating an alike distribution of those cell subsets between the two groups.

When analyzing the Sib group with multivariate analysis, patients receiving a graft with a higher dose of CD19+ had an increased risk of both acute GVHD (1.09, 1.03-1.15, p=0.003) and TRM (1.09, 1.01-1.17, p=0.036). Others have presented similar findings (*270*), however, B-cell involvement in acute GVHD is less studied than in chronic GVHD(*271*). Clearly, in a

post-conditioning cytokine milieu, as the B-cell becomes an APC it exposes recipient antigen to donor CD4+ cells spurring on T-cell recruitment in second phase of acute GVHD(*272*). B-cell deficient mice show less T-cell response to mHAgs, resulting in less acute GVHD(*273*) which could be mentioned as additional evidence linking B-cells to acute GVHD. Also, reports of encouraging outcome of patients with steroid resistant GVHD with rituximab treatment (*272*) and low incidence of acute GVHD when rituximab was added to RIC in patients with B-cells lymphomas(*274*) adds to proof that B-cells are involved in the pathophysiology of acute GVHD. It is also possible, since all donors were G-CSF-primed, that this resulted in enhanced B-cell activation leading to plasma cell phenotype (CD45RO+,CD19+) and donor antibody production could theoretically be directed to recipient antigens. Manipulation of the graft B-cell content in sibling donors to reduce acute GVHD is easily done i.e. increasing immunosuppression with rituximab, albeit, this does leave a series of potential adverse effects for immune reconstitution(*266*). In contrast, patients receiving sibling donor graft with high levels of CD19+ cells would benefit from increased surveillance of severe acute GVHD. Finally, B-cells have historically been affiliated with chronic GVHD explained by failure to achieve B-cell tolerance (*275*). However, graft CD19+ cells was not found to be significant in the multivariate analysis for chronic GVHD, we found an association between high graft CD4+ dose and increased risk for chronic GVHD. Current evidence for chronic GVHD diagnostic markers based on numerous studies, support an inverse association between donor graft nucleated cell and CD34+ cell dose, plasmacytoid dendritic cells, and NK-cell dose and subsequent risk for chronic GVHD(*276*), why our finding was more probably due to the underpower of this study.

The multivariate analysis also revealed that a high ratio of graft CD4+/CD8+ cells was associated with an increased risk of developing acute GVHD. A similar finding has been reported in haploidentical HSCT(*277*) and a high CD4+/CD8+ ratio early post-HSCT is associated with acute GVHD(*278*). We speculate whether this was the result of high numbers of CD4+ cells or low numbers of CD8+ cells. High numbers of CD4+ cells might imply more immunosuppressant CD4+CD127^{low}CD25^{high} regulatory T-cells somewhat contradicting our findings(*279*). Reports associating low CD8+ cells numbers and acute GVHD are scarce. In contrast, high numbers of CD8+ cells have been found to increase the risk for acute GVHD in patients with malignancies receiving RIC(*280*), while other studies show no such findings(*281,282*).

The univariate analysis showed less relapse in the Sib group compared to URD. This contradicts a current theory that HLA disparity in major and/or minor Ag between recipient and donor benefits URD donors in regards to relapse. However, the probability for relapse has been shown to be similar comparing siblings and URD donors in larger studies (*283,284*) as well for haploidentical donors compared to siblings(*285*) suggesting that HLA disparity is not the key factor for GVL. To note, in addition to the small numbers in our cohort, a major difference between the Sib and URD-group is the exclusion of ATG prior to HSCT in siblings(*117,286*), which also could be a confounding factor.

The most important finding in the URD group multivariate analysis was the association between high CD8+ numbers and low probability of relapse. The CD8+ cell is a cytotoxic effector cell important for GVL, proven in the DLI setting, first published by Kolb et al(*31*). In addition, when we analyzed cell doses in quartiles, only the highest quartile had a significant reduced probability of relapse, indicating that a donor graft with high CD8+ levels is advantageous. This finding was supported by another study, where younger donors were identified with higher CD8+ content(*287,288*). Moreover, donor age in our URD-cohort was significantly lower than in the Sib-group (29y versus 48y, respectively $p>0.001$). Although a younger donor is preferable, donor selection is based on HLA-matching, which makes donor selection based on CD8+ levels imaginary. However, patients who receive a graft with low CD8+ numbers can be increasingly monitored i.e. by MRD methods if suitable and hasty discontinuation of the immunosuppression or DLI administration if available.

In conclusion, there was a correlation between high donor CD8+ cell dose and a low probability of relapse in the URD group while for patients with sibling PBSC donors, a high graft CD19+ cell dose was associated with acute GVHD. However, prospective randomized trials looking at the impact of cell dose on outcomes should be conducted to avoid limitations coming from retrospective analyses, this study included.

Finally, the conflicting results analysing graft cell composition on HSCT outcome could be explained by the insufficient information received from mere numerical graft cell subset analysis. Our group has shown that additional precision in assessing patients outcome i.e., risk for acute GVHD can be attained by pinpointing donor graft cell risk phenotypes i.e., PD-1 T-cells, their allogeneic reactivity in combination with biomarkers in the patients plasma(*289,290*).

5 CONCLUSIONS

- Outcome for patients with a non-malignant disorder treated with HSCT using a well-matched URD, and ATG-included conditioning, is comparable to outcome using a HLA-identical sibling donor.

- For patients with a non-malignant disorder, development of DC after HSCT is associated with a higher incidence of acute GVHD I-III and the development of MC is associated with patients treated with RIC. GF occurs in patients with high MC but the intensity of conditioning regimen do not seem to be crucial.

- No conclusive treatment for patients with high MC was established, but patients with GF eligleble for a second HSCT do well.

- Pediatric patients undergoing HSCT have better OS in recent years due to foremost decreased TRM. However, relapse rate is unchanged.

- Lymphocyte subsets in G-CSF-primed PBSC donor graft given to patients with malignant disorders could be of importance to outcome. Higher graft CD8+ cell dose is associated with lower probability of relapse in patients with URD while for patients with sibling donors, a higher graft CD19+ is associated with more acute GVHD.

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8 ORIGINAL PAPERS