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FROM RISK INDICES TO RECONSTITUTION OF IMMUNITY: STUDIES OF OUTCOME-RELATED FACTORS IN PATIENTS UNDERGOING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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“COUPLE”. To give and to receive is a prerequisite for life.
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From risk indices to reconstitution of immunity: Studies of outcome-related factors in patients undergoing allogeneic hematopoietic stem cell transplantation

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To the patients,

For trust and constant challenges

To my colleagues,

For inspiration and guidance

To my family,

For love and support

ABSTRACT

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potent immunotherapeutic procedure which has the possibility to cure otherwise lethal hematological diseases. Its usability in clinical practice is limited by tangible risks of detrimental transplantation-related complications. Consequently, it is of great importance to adjust eligibility criteria and individualize allogeneic HSCT treatment protocols to maximize the chance of a positive outcome for every single patient.

The general aim of this thesis has been to study influential outcome-related factors in patients undergoing allogeneic HSCT. In the enclosed research papers, specific focus has been directed to improve interpretations of patient comorbidity and hematological indication prior to transplantation (**paper I**), to optimize stem cell dose (**paper II**), the choice of graft-versus-host disease (GVHD) prophylaxis (**paper III**) and to evaluate immune reconstitution after treatment (**paper IV**).

In **scientific paper I**, we performed a retrospective study of 521 consecutive adult allogeneic HSCT-patients transplanted at the Karolinska University Hospital for hematological malignancy from 2000 to 2012 to compare the predictive capacity of the Hematopoietic Cell Transplantation-specific Comorbidity Index (HCT-CI) and the disease risk index (DRI) for transplantation-related mortality (TRM) and overall survival (OS). Both indices could predict OS (with poorer survival in the highest risk groups) but failed to predict differences in TRM. In summary, obtained study data showed that the studied indices should be evaluated according to local data prior to their implementation on individual patients on the single-center level.

For patients with pre-existing comorbidities, the use of reduced intensity conditioning regimens (RIC) has made allogeneic HSCT a valid treatment option. In **scientific paper II**, we sought to determine the optimal hematopoietic stem cell dose (CD34+ cell dose) related to survival in RIC transplantations with peripheral blood stem cell grafts (PBSCT). Using consecutive transplantation registry data from the Center for International Blood and Marrow Transplant Research (CIBMTR), we retrospectively analyzed 1,054 patients with AML or MDS who underwent RIC PBSCT between 2002 and 2011. Grafts from HLA-matched siblings containing $< 4 \times 10^6$ CD34+ cells/kg recipient and grafts from unrelated donors containing $< 6 \times 10^6$ CD34+ cells/kg recipient, were associated with higher overall mortality after transplantation. Consequently, CD34+ cell dose in PBSCT grafts should be kept above these respective thresholds if possible.

In **scientific paper III**, we compared the standard GVHD prophylaxis regimen of cyclosporine and methotrexate (CsA/Mtx, n = 106) with a combination of tacrolimus and sirolimus (Tac/Sir, n = 103) in a prospective randomized trial. Based on previous publications on sirolimus, the hypothesis was that Tac/Sir would lead to less acute GVHD and reduced TRM. Analyses of study data did not show any significant differences in incidence of grades II-IV acute GVHD between the groups (CsA/Mtx: 41%, Tac/Sir: 51%), and data for TRM and OS were similar. In conclusion, the two GVHD prophylaxes provided comparable outcomes in patients after matched related or unrelated allogeneic HSCT, but study data indicated differences in toxicity in certain transplantation settings.

GVHD prophylaxis and conditioning regimens constitute risk factors for prolonged immunodeficiency related to impaired transplantation outcomes. In **scientific paper IV**, we sought to investigate the effects of the two different GVHD prophylaxis protocols (from paper III) on lymphocyte reconstitution and replicative capacity using PCR-derived levels of TREC, KREC and telomere length as proxy markers. Levels of lymphocyte excision circles were not significantly different between the GVHD prophylaxis groups, but patients with TREC or KREC levels above median during study follow-up had reduced TRM and superior OS.

In summary, the results and conclusions presented in this thesis may be useful in the continuous endeavor towards a safer and more individualized allogeneic HSCT procedure for future patients. Either as arguments for adjusted transplantation protocols or as basis for future research hypotheses.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Benmärgen är människans blodbildande organ. Den innehåller blodstamceller som efter utmognad bildar våra blodkroppar. I en frisk benmärg skapas en reglerad mängd av röda blodkroppar (för gasutbyte mellan lungorna och kroppens organ), blodplättar (som kan hejda blödningar), och vita blodkroppar (leukocyter, som utgör en vital del av kroppens immunförsvar).

Sjukdom i benmärgen påverkar blodbildningen och immunförsvaret. För särskilt svåra och livshotande sjukdomstillstånd, exempelvis akuta former av blodcancer (leukemier), myelodysplastiska syndrom (MDS), eller medfödda immunbristtillstånd, kan enda chansen till bot vara att genomgå en transplantation med blodstamceller som doneras från en frisk givare (allogen hematopoietisk stamcellstransplantation, HSCT). Transplantationen medför dock betydande risker för svår sjuklighet och död i olika behandlingskomplikationer. Det finns också en risk att de transplanterade cellerna stöts bort av patientens kvarvarande immunförsvar (avstötning), eller att blodsjukdomen kommer tillbaka trots genomförd transplantation (återfall).

Syftet med stamcellstransplantationen är att ersätta patientens sjuka blodbildning genom att tillföra friska blodstamceller som kan mogna ut i benmärgen och återskapa friska blodkroppar och ett fungerande immunsystem. Inför transplantationen ges cytostatika och eventuellt strålning för att försvaga patientens immunförsvar (för att minska risken för avstötning) och för att döda en så stor del av de sjuka blodcellerna i benmärgen som möjligt. Detta medför att patienten förlorar sitt immunförsvar och blir extremt infektionskänslig. Återbyggnaden av immunförsvaret, så kallad immunrekonstitution, efter transplantationen tar tid (månader till år) och påverkas av en rad olika faktorer (till exempel patientens ålder, medicinering med immunhämmande läkemedel, olika komplikationer till behandlingen, etc.). Bildandet av ett nytt välfungerande immunförsvar kräver att nya immunceller bildas, antingen genom celldelning av de immunceller som medföljer vid transplantationen, eller genom nybildning från donatorns transplanterade blodstamceller.

Eftersom de nya blodcellerna utvecklas från donatorstamceller som till viss del har en annorlunda vävnadstyp (även om sökning sker efter så välmatchade donatorer som möjligt), så kan transplantationen fungera som en effektiv immunologisk terapi mot elakartade blodsjukdomar. Leukocyterna i det nya immunförsvaret kan identifiera och döda blodcancerceller som finns kvar i patienten (transplantat-kontra-tumöreffekt, GVL). Samtidigt finns en risk att det nya immunförsvaret reagerar för kraftigt på patientens vävnader, vilket kan ge upphov till avstötningsreaktioner (transplantat-kontra-värd-sjukdom, GVHD). Dessa reaktioner är svårbehandlade och kan vara dödliga om insatt behandling inte fungerar. Akut GVHD kan utvecklas snabbt efter transplantation och drabbar främst hud, lever och tarmsystemet. Den kroniska formen utvecklas mer långsamt och kan angripa kroppens samtliga organ, med symptom som efterliknar autoimmuna sjukdomstillstånd (stela leder, torra slemhinnor, förhårdnad hud, försämrad lungfunktion, etc.).

I denna avhandling studeras olika faktorer som kan inverka på patientens behandlingsresultat efter allogen HSCT. **I det första arbetet** undersöktes hur väl två olika internationella riskmodeller, som utvecklats för att försöka förutspå utfallet efter en allogen HSCT innan behandlingen genomförs, kunde förutspå transplantationsrelaterad dödlighet (TRM) och överlevnad hos vuxna patienter som genomgått behandling med allogen HSCT vid Karolinska Universitetssjukhuset åren 2000-2012. Den ena modellen (Hematopoietic Cell Transplantation-specific Comorbidity Index, HCT-CI) förutspår risk för TRM genom att patientens samsjuklighet värderas inför transplantation (dvs., patientens övriga sjukdomar utöver blodsjukdomen, t ex leversjukdom eller lungsjukdom). Den andra indexmodellen (Disease Risk Index, DRI) förutspår risk för återfallsrelaterad död efter transplantation utifrån vilken blodsjukdom patienten har. Studien inkluderade 521 patienter och visade att patienterna som enligt modellerna klassificerades till de högsta riskgrupperna för HCT-CI och DRI inte överlevde lika länge efter sina transplantationer som de patienter som hamnade i de lägsta riskgrupperna. Ingen av riskmodellerna kunde dock förutspå skillnader i TRM, vilket till viss del skiljer sig från tidigare rapporter. I studien identifierades också specifika patientgrupper med tydligt skilda utfall efter transplantation trots att de enligt indexmodellerna var i samma riskgrupp. Studieresultaten visade att båda indexmodellerna kan ha ett värde under utredningen inför allogen HSCT, men att det är viktigt att validera dem i sitt eget patientmaterial för att veta hur de bäst ska användas.

För att minska risken för sämre behandlingsresultat hos patienter med hög samsjuklighet eller hög ålder så kan förbehandlingar med lägre intensitet användas inför transplantationen (reducerad konditionering). **I det andra arbetet** undersöktes vilken dos av blodstamceller (CD34+ celler) från donatorn som krävs för att förbättra behandlingsresultat och överlevnad när sådana transplantationer genomförs mot akut myeloisk leukemi och MDS, de vanligaste orsakerna för transplantation hos äldre patienter. Studien genomfördes tillsammans med en amerikansk forskningsorganisation (CIBMTR), till vilken mer än 450 transplantationscentra världen över rapporterar data över genomförda transplantationer. Analys av 1057 transplantationer visade att stamcellsprodukter från vävnadslika syskon som innehöll $< 4 \times 10^6$ CD34+ celler/kg patientvikt kunde kopplas till högre dödlighet efter transplantation, medan nivån för transplantationer från obesläktade givare var $< 6 \times 10^6$ CD34 celler/kg patientvikt. Den huvudsakliga slutsatsen var att målet för donerade blodstamceller således bör sättas högre än dessa gränser.

För att kontrollera det tillväxande immunförsvaret och minska risken för GVHD efter allogen HSCT ges immunhämmande behandling till alla patienter i 3-6 månader, ibland längre (GVHD-profylax). Den vanligaste profylaxbehandlingen har länge varit en kombination av läkemedlen ciklosporin och metotrexat (CsA/Mtx). Under det senaste decenniet har dock nya immunhämmande preparat tagits fram. Kombinationen av läkemedlen takrolimus och sirolimus (Tac/Sir) har varit särskilt intressant eftersom sirolimus i vissa studier visat sig ha effekt mot tumörer och olika

infektioner. För att jämföra den nya läkemedelskombinationen Tac/Sir med standardbehandlingen CsA/Mtx, så genomfördes **i det tredje arbetet** en prospektiv studie inom vilken 209 patienter randomiserades mellan de två olika typerna av GVHD-profylax. Det var första gången dessa två GVHD-profylax jämfördes i en randomiserad studie. Behandlingen visade att båda läkemedelskombinationerna hade liknande behandlingsresultat efter transplantation. Studien visade också att en viss typ konditionering kunde medföra särskilt svåra komplikationer om patienterna fick Tac/Sir som GVHD-profylax.

Det fjärde delarbetet utgick från patienterna i det tredje arbetet, och utgjordes av ett laborativt arbete på blodprover som samlats in under den randomiserade studien. En laborativ metod (PCR) användes för att mäta nivån av markörer för immunrekonstitution i patienterna vid olika tidpunkter efter transplantation. När nya leukocyter bildas skapas specifika restprodukter från cellernas arvsanlag, så kallade excisions-cirklar. Tidigare studier har visat att nivån av dessa återspeglar hur brett och varierat patientens nya immunförsvar är. Resultaten från denna studie visade ingen skillnad i nivåer av excisions-cirklar mellan GVHD-profylaxgrupperna, men däremot visades att de typer av konditionering som innehöll antikroppar mot T lymfocyter gav tydligt sänkta nivåer. Yngre patienter hade högre nivåer, troligen till följd av att brässen (som är viktig för utvecklingen av T-lymfocyter) har en bättre funktion hos yngre än hos äldre eftersom den tillbakabildas under det naturliga åldrandet. Studien visade också att patienter som hade excisions-cirklar under mediannivå efter transplantation hade högre TRM och sämre överlevnad, främst till följd av fler och svårare infektioner. Det kan således finnas stöd för att införa mätning av excisions-cirklar som en del av uppföljningen efter allogen HSCT, för att kunna vidta åtgärder för patienter med låga nivåer (snabbare nedtrappning av den immunhämmande behandlingen, eller förlängd infektionsprofylax).

Sammanfattningsvis presenterar avhandlingen några resultat och slutsatser som kan motivera justeringar av nuvarande behandlingsprotokoll för att göra allogen stamcellstransplantation säkrare. Andra resultat (exempelvis i delarbete IV) bör främst ses som underlag till hypoteser för framtida forskning. Avhandlingens delarbeten styrker uppfattningen att den sammantagna bedömningen inför en allogen stamcellstransplantation är av vikt för att värdera vilka patienter som utifrån risk- och nyttoanalys kan accepteras för transplantation. Transplantationens olika delar (donatorval, konditionering, stamcelldos, uppföljningsprotokoll, etc.) bör individualiseras så mycket som möjligt till patientens förutsättningar. Som man sår, får man skörda.

Strävan att förbättra behandlingen med allogen HSCT kommer tveklöst att fortsätta så länge den kan försvara sin position som den mest effektiva immunterapi som hittills förts in i klinisk rutin. Allt annat vore ett svek, dels mot dem som donerar friska blodstamceller, men främst mot de patienter som väljer att utsätta sig för en av de farligaste medicinska behandlingarna som finns, för att få möjligheten att överleva sina livshotande blodsjukdomar.

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*JT and AG share first authorship for this work.

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CONTENTS

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
ALWP	Acute leukemia working party
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
ATG	Anti-thymocyte globulin
BM	Bone marrow
CD	Cluster of differentiation
CIBMTR	Center for international blood and marrow transplant research
CMV	Cytomegalovirus
CR	Complete remission
CsA	Cyclosporine A
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DFS	Disease-free survival
DLI	Donor lymphocyte infusion
DNA	Deoxyribonucleic acid
DRI	Disease risk index
EBMT	European society for blood and marrow transplantation
G-CSF	Granulocyte colony-stimulating factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRFS	GVHD- and relapse-free survival
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HCT-CI	Hematopoietic cell transplantation-specific comorbidity index
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

KREC	Kappa-deleting recombination excision circle
MAC	Myeloablative conditioning
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MRD	Minimal residual disease
mRNA	Messenger ribonucleic acid
Mtx	Methotrexate
NK cell	Natural killer cell
NOD	Nucleotide-binding oligomerization domain
NRM	Non-relapse mortality
OS	Overall survival
PAMPs	Pathogen-associated molecular patterns
PBSC	Peripheral blood stem cell
PBSCT	Peripheral blood stem cell transplantation
PCR	Polymerase chain reaction
RIC	Reduced intensity conditioning
Sir	Sirolimus
Tac	Tacrolimus
TBI	Total body irradiation
TLR	Toll-like receptor
TMA	Thrombotic microangiopathy
TNC dose	Total nucleated cell dose
TNF	Tumor necrosis factor
TREC	T cell receptor excision circle
TRM	Transplant-related mortality
UCB	Umbilical cord blood
URD	Unrelated donor
US	United States of America
V(D)J genes	Variable (V), diversity (D), joining (J) genes
VOD/SOS	Veno-occlusive disease/Sinusoidal obstruction syndrome

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

1.1.1 A brief overview of immunobiology

All research projects presented in this thesis have been performed in the field of clinical allogeneic hematopoietic stem cell transplantation (HSCT). This introduction to the immune system is consequently limited to primarily cover parts of relevance for the studies presented in papers I-IV and, in some aspects, the other relevant publications listed above. For a more detailed overview of the numerous components and actions of the immune system, references are provided to outstanding reviews in published textbooks (*Murphy et al. 2012, Parham et al. 2015*).

In general terms, immunity is defined as resistance to infectious disease. The human body's ability to respond to exposure by pathogenic microorganisms or substances is a result of investments in tissues, cells, molecules and highly regulated mechanisms dedicated to defense (i.e. the immune system). Directed immune responses have been crucial to human survival since the dawn of existence, and genetic analyses indicate that infection-related mortality have been a component of natural selection during evolution, which has continuously shaped the immune system (*Barreiro et al. 2010*). Due to its ability to distinguish "self" (and/or safe) from "non-self" (and/or potentially dangerous), a healthy immune system is further available to protect the body from other types of diseases aside from infectious agents, such as development of cancer. Transformed cells may be recognized as defect or foreign, resulting in their eradication by immune-mediated mechanisms (*Barnes et al. 1956, Schreiber et al. 2011, Corthay 2014*).

In contrast to these beneficial roles, abnormal or exaggerated immune responses, or response to incorrect or undesirable targets, can mediate damage. Examples include autoimmune diseases, states of chronic inflammation or allergic reactions (*Richardson et al. 2011, de Souza et al. 2016, Sharif et al. 2018*) and different immune responses or complications after allogeneic HSCT.

1.1.2 Innate and adaptive immunity

Immune host defenses in humans are classically grouped under innate and adaptive immune mechanisms. The innate part provides fast actions through relatively non-specific activation, e.g. bacterial and viral DNA/RNA, lipopolysaccharides (LPS) and immune complexes. It consists of physical barriers, phagocytes and cytokine-producing cells (e.g. neutrophils, macrophages and natural killer [NK] cells), proteins of the complement system and antimicrobial peptides. Neutrophils and macrophages express pattern recognition receptors, including Toll-like receptors (TLR) and

nucleotide-binding oligomerization domain (NOD)-like receptors, capable of detecting conserved pathogen-associated molecular patterns (PAMPs) and retinoic acid-inducible gene-I-like receptors (RIG-I) that can recognize certain viruses (*Medzhitov 2007*). Macrophages can be activated via TLRs to produce cytokines, including tumor-necrosis factor alpha (TNF-α), interleukin 1β (IL-1β) and IL-6 which give rise to inflammatory responses. The innate immune system can also react to damage-associated molecular patterns (DAMPs), or endogenous signals from damaged/dying cells (*Green et al. 2009*).

The NK cells of innate immunity can, aside from TLR-mediated activation and induced apoptosis after Fas-ligand/receptor activation, also rapidly kill cells after "missing-self" recognition (*Ljunggren et al. 1990*). Their inhibitory killer-cell Immunoglobulin (Ig)-like receptor (KIR) can identify cells lacking major histocompatibility complex (MHC) class I expression, e.g. certain virus-infected cells or tumor cells (*Karre 2002*), which are not easily detected or eliminated by other immune cells (*Della Chiesa et al. 2014*). NK cells secrete cytokines such as IFN-γ and TNF-α, which act on other immune cells like macrophages and dendritic cells (DC) to enhance immune responses. They can also develop long-lasting and antigen-specific memory to viruses and haptens, which has led to an emerging interest in harnessing NK cells for cancer immunotherapy (*Peng et al. 2017, Li et al. 2018*).

The adaptive immunity provides a more specialized defense against antigens by engagement of cells and receptors selected for their specific reactivity with target antigens; a slower response compared to innate immunity mechanisms. The adaptive immune system is mainly activated by the innate parts through antigen-presenting cells (APC) that can engulf and process protein antigens into peptides and present it in context of MHCs on the cell surface. Adaptive immunity can also be activated by antigens binding directly to unique adaptive immune cell receptors, developed through random gene rearrangements (see below).

T cells recognize antigens in conjunction with MHC glycoproteins (*Zinkernagel et al. 1974*). MHC class I molecules are heterodimers, consisting of an α-chain, forming the peptide-binding cleft, and the supporting protein β2-microglobulin. They are expressed on nucleated cells and present processed peptides derived from proteins in the cell's cytoplasm. Hence, these peptides reflect on-going intra-cellular processes such as intracellular infections (*Trowsdale et al. 2013*). MHC class I molecules bind specifically to T cell receptors (TCR) of CD8+ T lymphocytes (cytotoxic T cells). Once activated, cytotoxic T cells can kill the infected cells to limit disease.

In MHC class II molecules present on professional APCs, the peptide-binding domain is formed by an α- and a β-chain. MHC class II present peptides derived from extracellular proteins to TCRs on CD4+ cells (T helper cells) that produce cytokines and provide co-stimulation to propagate further immune reactions upon activation (*Trowsdale et al. 2013*). Professional APCs include DCs, macrophages and

B lymphocytes whereas non-professional APCs include thymic epithelial cells (which function as APCs in short periods, necessary for production of competent T cells and self-tolerance).

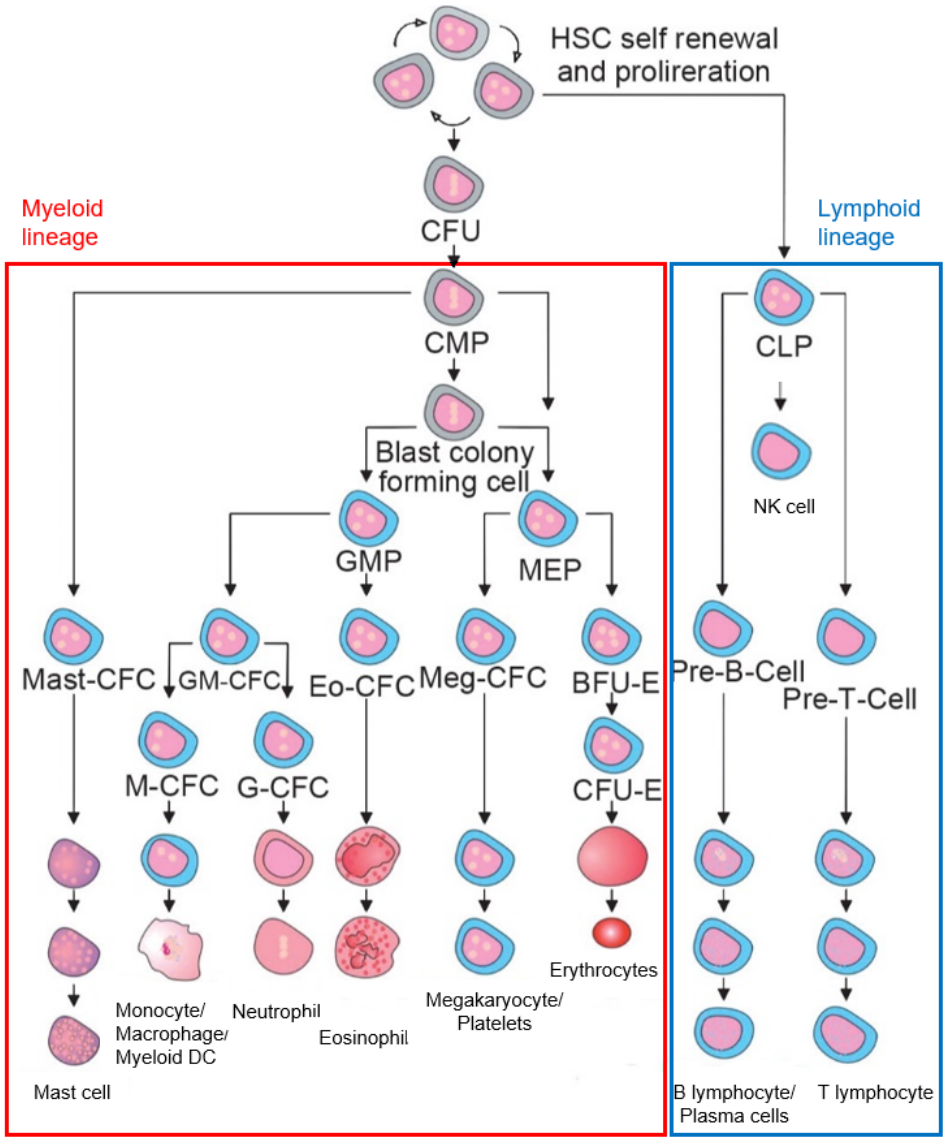
An additional type of antigen-presentation is cross-presentation, where antigens located on the MHC class II pathway can be transferred to the MHC class I pathway of antigen-presentation. This is possible since certain APCs can process and present extracellular antigens with MHC class I molecules to CD8+ cytotoxic T cells. Cross-presentation enables APCs to trigger an MHC class I-dependent immune response without being infected. In the process of "cross-priming", naïve cytotoxic CD8+ T cells are stimulated to become activated cytotoxic CD8+ T cells. Cross-presentation is pivotal for immune reactions against viruses and tumors that do not readily infect APCs, and required to induce cytotoxic immunity by antigen vaccination, including tumor vaccination. The APC with the most efficient ability to cross-present antigens in this setting is the DC (*Melief 2003*).

Dendritic cells are primarily found in peripheral tissues where they are activated upon ingestion of pathogens. They then respond by producing inflammatory cytokines and by migrating to draining lymph nodes to exert antigen-presentation to lymphocytes.

Antigen-specific B cells can recognize a variety of pathogen-associated antigens by their Ig-receptors. Following activation, a modified soluble form of the B cell's Ig is excreted as a circulating antibody, exerting its different effector functions (including activation of the complement system, binding to pathogens to facilitate phagocytosis or blocking essential receptors on pathogen surfaces).

1.1.3 Hematopoiesis

Blood and immune cells are derived from continuous hematopoiesis in the bone marrow (BM). It begins with hematopoietic stem cells (HSC), which can either branch into more differentiated types of hematopoietic cells or divide in self-renewal. Multipotent HSCs give rise to colony forming units, and further differentiation occurs along two destined lineages, the myeloid and the lymphocytic (Figure 1). In healthy hematopoiesis, myeloid-derived cells ultimately give rise to granulocytes, erythrocytes, monocytes/macrophages and megakaryocytes/platelets. Lymphoid-derived cells develops into NK cells, pre-B and pre-T lymphocytes which continue to differentiate into B and T cells with effector functions (*Hoffbrand et al. 2016*).



Abbreviations: HSC, hematopoietic stem cell; CFU, colony forming uints; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor; CFC, colony forming cell; BFU, burst forming unit; CFU, colony forming unit; NK cell, natural killer cell; DC, dendritic cell.

Figure 1: Gross scheme of hematopoiesis.
Figure modified from Firth (*Firth et al. 2012*).

A functional hematopoiesis requires numerous somatic cell divisions, including DNA replication at every mitosis. However, eukaryotic DNA polymerase can add nucleotides only in one direction (5' → 3') resulting in the "end replication problem". The DNA polymerase cannot continue its duplication activity to the very end of the chromosome, which inevitably results in chromosome shortening at each cell division. To prevent loss of important genetic material in this process, telomeres (disposable nucleotide repeats of the sequence AGGGTT) protect the chromosome ends from fusion and de-gradation, maintaining genome stability. The telomere length shortens at each cell division but can be replenished/maintained by telomerase enzymatic activity (*Parham et al. 2015*).

The majority of the hematopoietic cells proliferate and differentiate in the BM, while T cell progenitors migrate from these areas early in their development to the thymus to continue their maturation. This process includes somatic recombination in which each T cell obtains a functional and specific TCR, rendering a naïve T cell with the ability to recognize a specific antigen. Similarly, progenitor B cells undergo somatic recombination in the bone marrow, creating an immature B cell with a unique Ig antibody receptor (*Murphy et al. 2012*).

1.1.3.1 Lymphocyte diversity, V(D)J recombination, and the creation of excision circles

Both B and T lymphocytes have exclusive abilities to create a vast amount of unique antigen-receptors. This is possible because of somatic recombination, the process of rearrangement of the gene segments encoding respective antigen-receptors (Ig and TCR, respectively).

Germ-line DNA of the progenitor B and T cells holds genes coding for multiple variable (V), diversity (D) and joining (J) gene segments separated by introns and placed between the leader (L) and constant (C) regions (*Schatz et al. 2011b*). To enter the first steps of somatic recombination, recombination activating genes (RAG1 and RAG2) create double strand breaks in the lymphocyte DNA (*Ru et al. 2015*), and recombination begins when V(D)J recombinase binds to a recombination signal sequence flanking a coding gene segment.

During the following process of V(D)J random recombination, progenitor B cells first undergo heavy chain locus rearrangements (located on chromosome 14) followed by κ and λ light chain recombination (encoded at chromosome 2 and 22, respectively). Assembly of the heavy chain and a light chain ultimately results in the formation of an IgM surface molecule on the immature B cell.

During formation of the heavy chain, the DNA gene first undergoes J and D rearrangement to bring the C region closer. In the next step, the formed DJ segment binds to the V region. After completion of the V(D)J recombination, the remaining introns are removed by splicing and the mRNA strand coding for the heavy chain protein is formed (the combined LVDJC-regions) (*Jung et al. 2006*).

The precursor B cell also contains genes coding for the light chain, which is a bit different from the heavy chain gene since it does not contain a D region. After J and V recombination, introns are spliced off to form an mRNA strand coding for the light chain protein (the combined LVJC-regions). This is then linked to the heavy chain to form the final Ig structure (*Schatz et al. 2011a*).

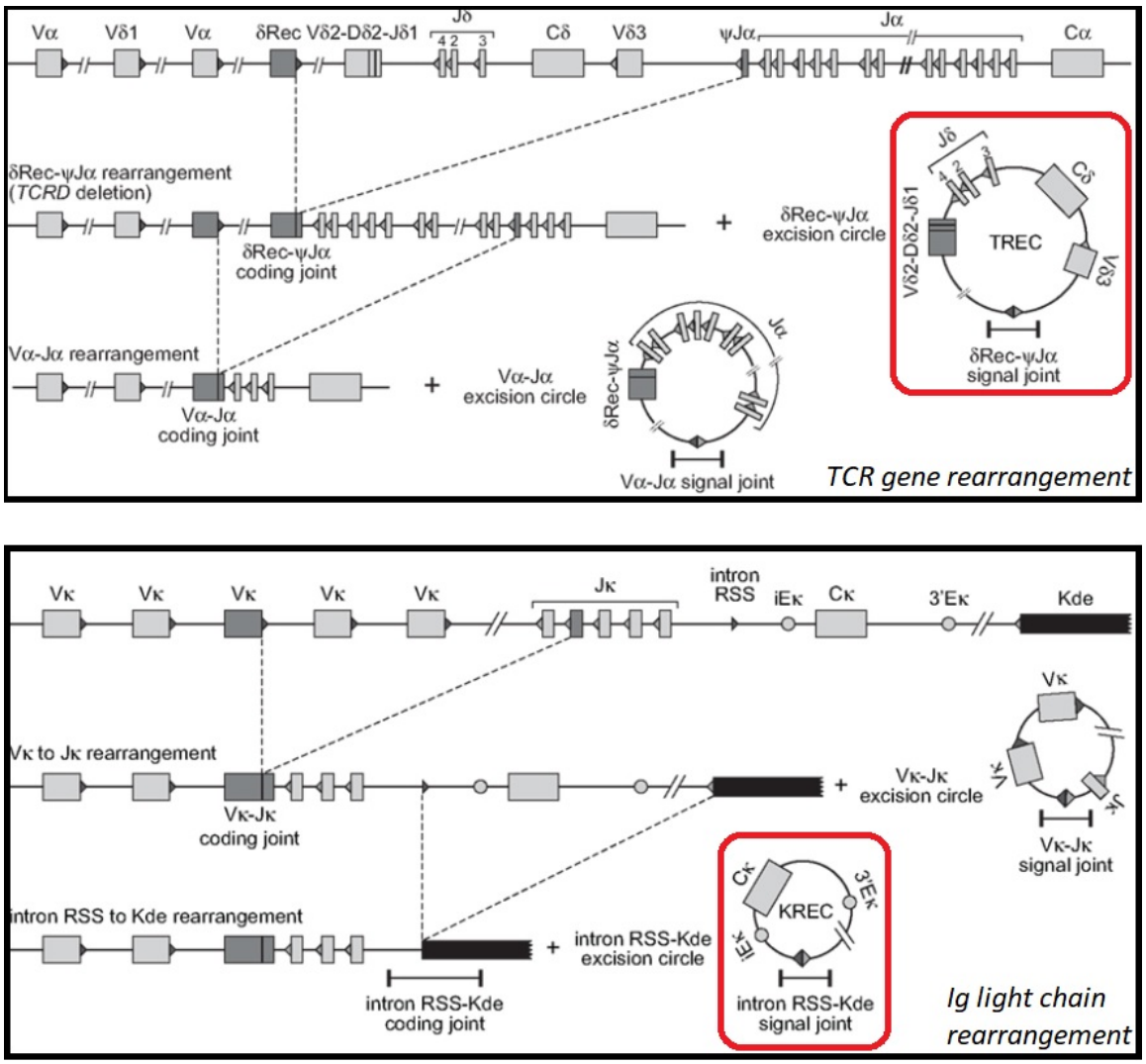
Since the DNA contains a vast amount of different V, D, and J regions, the process described can generate an enormous antibody repertoire. Diversity and specificity can also be further enhanced by addition of random new nucleotides during the recombination process, known as junctional diversity.

By calculation, approximately 3×10^{11} Ig combinations are possible through combinatorial and junctional diversity (*Murphy et al. 2012*).

A similar process of ordered recombination occurs in T cell progenitors after migration to the thymus. The thymocytes start off as double-negative T cell progenitors (CD2+ CD4- CD8-). Proliferation is followed by rearrangement in the δ-, γ- and β-chain genes, which may result in a committed TCRγδ T cell if the receptor formed is functional (*Prinz et al. 2013*). These TCRγδ T cells mature and migrate from the thymus to peripheral tissues. However, most T cell progenitors rearrange the β-chain gene first and assemble a pre-TCR. Signals through this pre-TCR temporarily stop further rearrangements and induce proliferation and expression of CD4 and CD8, after which the CD4+CD8+ double positive lymphocytes resume rearrangement of their α- , γ-, and δ- genes (*Parham et al. 2015*). The assembly of the β- and α- chains results in formation of the TCRαβ, expressed on the majority of T cells.

As mentioned above for B cells, estimates of the theoretical number of different TCRs that could be produced by V(D)J gene rearrangement in the thymus are approximately 1×10^{15} (*Murphy et al. 2012*). In a similar way as described for B lymphocytes' Ig, V(D)J gene rearrangements occur in the TCRδ- and TCRβ-loci prior to splicing, while the TCRα- and TCRγ-segment do not contain a D region and directly couple a V to a J gene segment. The gene segment encoding the δ locus is embedded in the α gene segment. Therefore, during recombination of the α chain, the TCRδ locus is deleted by δRec-ψJα rearrangements in the TCRαβ T cell lineage. This creates a copy of a ligated T cell receptor excision circle (TREC). Correspondingly, during V(D)J recombination of the Ig κ locus in B cells, a VJ joint κ-deleting recombination excision circle (KREC) is formed (Figure 2) (*van Zelm et al. 2011*).

A specific function of these lymphocyte excision circles has not been shown. They are commonly described as by-products without replicative capacity. Consequently, they are diluted during the continuous cell divisions of the lymphocyte, while the corresponding rearrangement product is inherited by all daughter cells (*Livak et al. 1996, van Zelm et al. 2011*). According to these properties, TREC and KREC quantification can be used as proxy biomarkers for *de novo* lymphocyte synthesis and immune reconstitution, for example by polymerase chain reactions (PCR) (*Al-Harthi et al. 2000, Mensen et al. 2013*).



Abbreviations: RSS, recombination signal sequence; TREC, T cell receptor excision circle; KREC, κ-deleting recombination excision circle; TCR, T cell receptor; Ig, immunoglobulin.

Figure 2: Illustration of lymphocyte V(D)J recombination and the formation of TREC and KREC during TCR and Ig light chain rearrangement.
Figure modified from van Zelm (*van Zelm et al. 2011*).

1.1.3.2 T cell subsets

Human T cells can be divided into specific subsets. The major division into CD4+ T helper (Th) cells and CD8+ cytotoxic T cells has been mentioned above. The CD4+ Th cell compartment is diverse and enables development of T cells with optimal response to different pathogens (*Parham et al. 2015*).

Th1 helper cells are generated when naïve T cells are primed in the presence of IL-12 and are typically directed against intracellular pathogens. They can produce IL-2 and IFN-γ. IL-2 has various effects in the T and NK cell compartments (primarily via its direct effect as a T cell growth factor) but can also promote differentiation of immature T cells into regulatory T cells and, hence, prevent autoimmunity. IFN-γ can activate macrophages to destroy cells with intracellular bacteria.

Th2 helper cells are induced when naïve T cells are primed with IL-4. They typically act against extracellular pathogens by immune responses triggering B cells, eosinophils and mast cells.

Th17 helper cells are pro-inflammatory and of importance for maintaining mucosal barriers, contributing to pathogen clearance at these surfaces. Their main effector cytokines are IL-17, which targets immune cells to produce granulocyte-colony stimulating factor (G-CSF), and IL-8, which leads to production and recruitment of neutrophils.

Regulatory T cells are developed in the thymus or peripheral tissues and can exert immunosuppressive action by regulating the activity of other immune responses. They can inhibit the activation of naïve T cells by suppressing induction and proliferation of effector T cells, including maintaining tolerance to self-antigens, either by contact-dependent mechanisms or by cytokine-secretion (IL-10, TGF-β). Survival and function of regulatory T cells require IL-2. They express high levels of the IL-2 receptor on their surface and, hence, reduce the availability of IL-2 for T cells in their vicinity.

1.2 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

1.2.1 General introduction to transplantation and allogeneic HSCT

A successful and safe transplantation procedure, regardless of the tissue or organ transplanted, strives to overcome a number of medical obstacles. The transplanted tissue must retain or restore its physiological function in the transplanted host, the health of the (living) graft donor must be maintained after donation and the recipient's immune system must be prevented from rejecting the graft (*Dupont 1997, Linden 2009*).

Modern allogeneic HSCT treatment has only been possible after decades of immense preclinical and clinical research (*Henig et al. 2014*). The first published BM transfusion in humans was performed in 1939 in a patient with aplastic anemia (*Osgood et al. 1939*). Starting off in various animal models (*Jacobson et al. 1949, Lorenz et al. 1951*), the first allogeneic HSCT was pioneered by Thomas et al, reporting six patients intravenously infused with BM after radiation and chemotherapy in 1957 (*Thomas et al. 1957*). After breakthroughs and implementation of immunological research, including the discovery of the MHC system described above (*Snell et al. 1953, Mann et al. 1969*), the field could move forward after initial dismal clinical results (*Bach et al. 1968, Bortin 1970, Thomas et al. 1975a, Thomas et al. 1975b, Thomas et al. 1977*).

As a result of these (non-exhaustive) milestones and continuous developments, allogeneic HSCT has been established as a curative treatment for a broad spectrum of diseases, predominantly hematologic malignancies, immunodeficiencies and inborn errors of metabolism (*Negrin 2014, Sureda et al. 2015*), with numbers of transplanted patients steadily increasing (Figure 3).

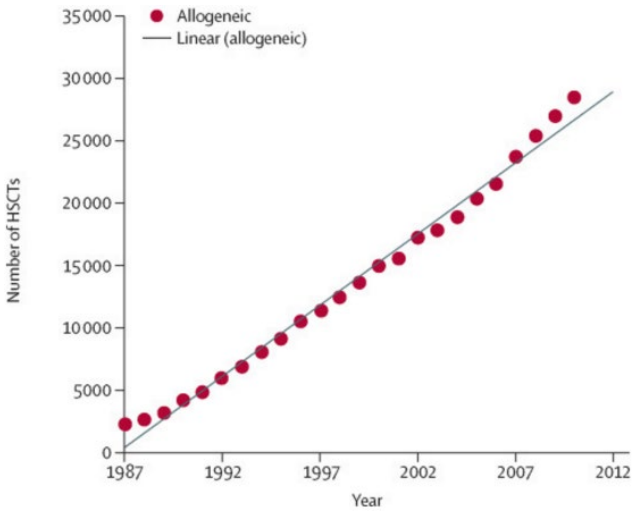


Figure 3: Global development of allogeneic HSCTs per year 1987-2010
Graph from Gratwohl (*Gratwohl et al. 2015*).

1.2.2 The allogeneic HSCT procedure

In summary, allogeneic HSCT can be described as a medical procedure with the purpose to replace a defective hematopoietic or immune system in a diseased patient with healthy HSCs from a donor. After transplantation, the donated HSCs have the potential to engraft, successively develop to a healthy hematopoietic system in the host and to reconstitute adequate immune function.

The allogeneic HSCT procedure can roughly be divided into the following arbitrary parts (Figure 4):

- A pre-transplantation assessment considering the indication for treatment and overall medical status of the patient to determine eligibility for allogeneic HSCT.
- A coordinated search for a suitably HLA-matched, healthy and available stem cell donor.
- A conditioning regimen, consisting of cytotoxic drugs with or without radiation therapy, given prior to stem cell infusion (n.b., patients diagnosed with severe combined immunodeficiency can be transplanted without prior conditioning).
- Infusion of the donated HSC containing graft into the patient.
- Prophylaxis against the development of graft-versus-host disease (GVHD).
- Supportive care including symptomatic treatment of toxic side effects of the conditioning as well as prophylaxis against bacterial, viral and fungal infections during both the neutropenic phase and throughout the initial phases of immune reconstitution in the host.
- Continuous follow-up of to monitor and evaluate the status of the evolving hematopoietic system and immune reconstitution and to treat detectable complications (such as signs of disease relapse, GVHD, infections, impaired quality of life, etc.).

It is commonly agreed that the success of allogeneic HSCT for treatment of hematologic malignancies is dependent on efficient eradication or control of the malignant clone, successful reconstitution of the host's hematopoiesis and immune system, and a low incidence of transplantation complications (infections, severe acute and/or chronic GVHD, relapse, etc.) (*Ringden et al. 2009, Remberger et al. 2011, Ziakas et al. 2014*).

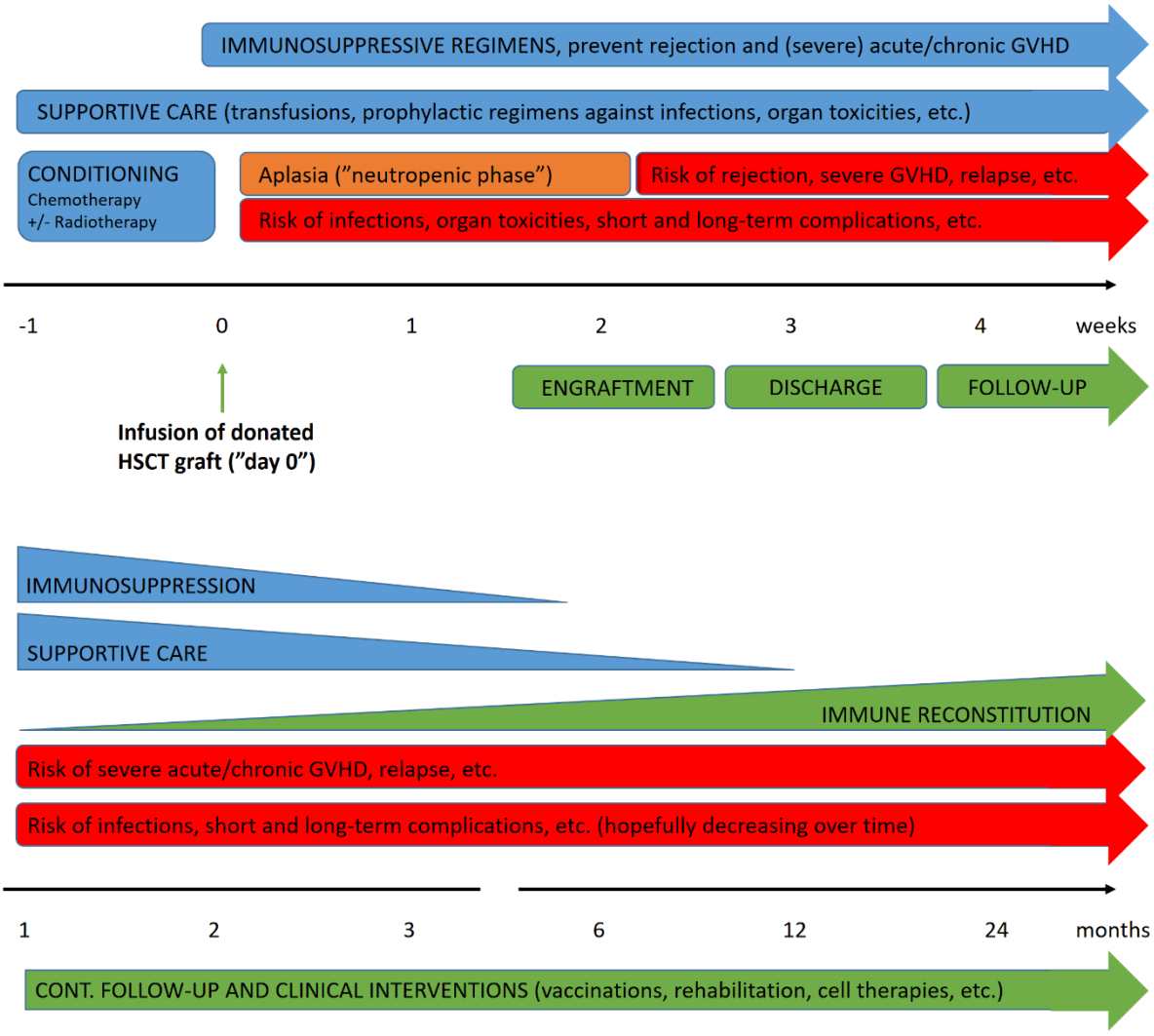


Figure 4: Summary of the clinical allogeneic HSCT procedure during the first month (top) and months/years (bottom).

1.2.3 Pre-transplantation assessments

Indications for allogeneic HSCT have varied over time. They depend on the development of transplantation procedures and on existing or novel treatment options available for the diseases for which transplantation can be considered (*Gratwohl et al. 2013, Gratwohl et al. 2015*). For every patient an individual assessment is performed prior to the final treatment decision. The purpose is to verify the disease as a valid indication for allogeneic HSCT and to evaluate the disease status and general medical condition of the patient, with special regard to previous identified risk factors for transplantation-related mortality (TRM), such as patient age, disease type/stage, existing comorbidity, the time interval from diagnosis to transplantation and available donors (*Gratwohl 2012*). Specific tools for risk assessment prior to allogeneic HSCT have been developed, showing that outcome depends on the disease and disease stage, as well as comorbidity burden at time of transplantation (*Diaconescu et al. 2004, Sorror et al. 2004, Sorror et al. 2005, Boehm et al. 2008, Pollack et al. 2009, Barba et al. 2010a, Kataoka et al. 2010, Armand et al. 2012a, Thanarajasingam et al. 2013*).

Pre-existing comorbidities have been of interest in the allogeneic HSCT field since the introduction of reduced intensity conditioning (RIC) regimens, which made transplantation accessible to broader patient groups (i.e. older patients and patients with comorbidities) whom were previously not eligible for allogeneic HSCT with myeloablative conditioning (MAC) because of treatment-related toxicities and high TRM (*Slavin et al. 1998, Martino et al. 2001, Luger et al. 2012*). To specifically assess the impact of patient comorbidities in allogeneic HSCT, the Hematopoietic Cell Transplantation-specific Comorbidity Index (HCT-CI) was developed in 2005 (*Sorror et al. 2005*). It was created from the Charlson Comorbidity Index, previously introduced to evaluate the impact of comorbidities in longitudinal studies (*Charlson et al. 1987*).

The HCT-CI is constructed to analyze 17 different comorbidities and their respective severity (Figure 5), which are subsequently summarized to assign the individual patient into one of three risk groups based on the total score (HCT-CI = 0 [low], HCT-CI = 1-2 [intermediate], and HCT-CI ≥ 3 [high]).

Comorbidity	Definition	Yes	Score
Arrhythmia	Atrial fibrillation*		1
	Atrial flutter*		
	Sick sinus syndrome*		
	Ventricular arrhythmia*		
Cardiovascular	Coronary artery disease*		1
	Congestive heart failure*		
	Myocardial infarction*		
	Ejection fraction ≤50% §		
Inflammatory Bowel Disease	Crohn's disease*		1
	Ulcerative colitis*		
Diabetes	Treated with insulin*		1
	Treated with oral hypoglycemic drugs*		
Cerebro-vascular	Transient ischemic attacks*		1
	Ischemic/hemorrhagic stroke*		
Psychiatric disturbances	Requiring consultation §		1
	Requiring specific treatment §		
Hepatic - Mild	Chronic hepatitis §		1
	Bilirubin >ULN- 1.5 x ULN §		
	AST/ALT >ULN- 2.5 x ULN §		
Obesity	Body mass index ≥35 §		1
Infection	Requiring anti-microbial treatment before, during and after the start of conditioning §		1
Rheumatologic	Required treatment*		2
Peptic ulcer	Confirmed (endoscopy) and required treatment*		2
Renal	Serum creatinine >177 µmol/L §		2
	On dialysis §		
	Prior renal transplantation*		
Pulmonary - Moderate	DLco 66-80% of predicted (corrected for Hb) §		2
	FEV1 66-80% of predicted §		
Pulmonary - Severe	DLco ≤65% of predicted (corrected for Hb) §		3
	FEV1 ≤65 of predicted §		
Heart valve disease	(Except asymptomatic mitral valve prolapse) §		3
Prior solid malignancy	Treated with surgery, chemotherapy, and/or radiotherapy (excl. non-melanoma skin cancer) §		3
Hepatic - Moderate/Severe	Liver cirrhosis §		3
	Bilirubin >1.5 x ULN §		
	AST/ALT >2.5 x ULN §		
		Total score:	

* Diagnosed at any time in the patient's past history.
§ Values detected at the closest time prior to start of conditioning treatment.

Abbreviations: ULN, upper limit of normal; DLco, diffusion capacity of carbon monoxide; FEV1, forced expiratory volume in one second; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Figure 5: The HCT-CI scoring chart for adult patients. Extrapolated from Sorror (*Sorror 2010*).

To further estimate allogeneic HSCT outcome from pre-existing patient data and build a tool to adjust for patient heterogeneity in allogeneic HSCT studies, the disease risk index (DRI) was developed in 2012 (*Armand et al. 2012a*) (Figure 6). The DRI was created from multivariate models for overall survival (OS) in allogeneic HSCT-patients. The seminal paper showed that DRI category (low, intermediate, high, and very high) was a significant factor associated with OS, progression-free survival and cumulative incidence of relapse (independently of comorbidity burden or intensity of conditioning). The HCT-CI and DRI have since then undergone multiple refinements (*Sorror et al. 2009, Armand et al. 2014, Sorror et al. 2014*) and both indices have been validated in numerous allogeneic settings (*Sorror et al. 2007a, Sorror et al. 2007b, Majhail et al. 2008, Xhaard et al. 2008, Barba et al. 2010a, El Kourashy et al. 2011, Raimondi et al. 2012*).

Summary of disease and stage risk groups

Disease	Risk
AML - favorable cytogenetics	Low
CLL	Low
CML	Low
Indolent B-cell NHL	Low
ALL	Intermediate
AML - intermediate cytogenetics	Intermediate
MDS - intermediate cytogenetics	Intermediate
Myeloproliferative neoplasms	Intermediate
Multiple myeloma	Intermediate
Hodgkin lymphoma	Intermediate
DLBCL/Transformed indolent B-NHL	Intermediate
Mantle cell lymphoma	Intermediate
T-cell lymphoma, nodal	Intermediate
AML - adverse cytogenetics	High
MDS - adverse cytogenetics	High
T-cell lymphoma, extranodal	High

Stage	Risk
1st CR	Low
2nd or subsequent CR	Low
1st PR	Low
Untreated	Low
Chronic phase (CML)	Low
2nd or subsequent CR (if RIC)	Low
2nd or subsequent CR (if MAC)	High
Induction failure	High
Active relapse	High
Accelerated or blast phase (CML)	High

Summary of overall risk groups

Disease risk		Stage risk	→	Overall risk
Low	+	Low	→	Low
Low	+	High	→	Intermediate
Intermediate	+	Low	→	
Intermediate	+	High	→	High
High	+	Low	→	
High	+	High	→	Very high

Abbreviations: AML, acute myeloid leukamia; CLL, chronic lymphocytic leukamia; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma; MDS, myelodysplastic syndrome; DLBCL, diffuse large B cell lymphoma; CR, complete remission; PR, partial remission; RIC, reduced intensity conditioning; MAC, myeloablative conditionng.

Figure 6: Schematic DRI risk group assessment (adults). Extrapolated from Armand (*Armand et al. 2012a*).

1.2.4 Donor selection

To perform an allogeneic HSCT, a suitable HSC donor must be identified, informed, and approved for graft donation. This is a crucial step in the pre-transplantation process since the donor type is known to impact the outcome of transplantation (*Gratwohl 2012*). The initial donor search is made primarily on compatibility of the human leukocyte antigen (HLA) match between the donor and the recipient. The HLA genes, the human equivalents of MHC molecules described above, were first described and characterized by Dausset in the 1950's (*Dausset 1958*). They are highly polymorphic with a wide variety between individuals (*Turner 2004, Latham et al. 2014*).

If available, the primary donor of choice is an HLA-identical sibling, preferably avoiding a female donor to a male recipient to avoid grafts containing antibodies formed against the Y-chromosome during pregnancy (resulting in H-Y antibodies) (*Atkinson et al. 1986, Popli et al. 2014*). The HLA genes are encoded for on chromosome 6 and are inherited according to the Mendelian pattern of genetics. This implies that the chance of a healthy sibling to carry the same two haplotypes as the diseased sibling is 1 out of 4 (25%). A donor with one identical HLA-haplotype (e.g. a father or mother donating to his/her biological child, or vice versa) is called haploidentical, i.e. 50% HLA-identical (Figure 7).

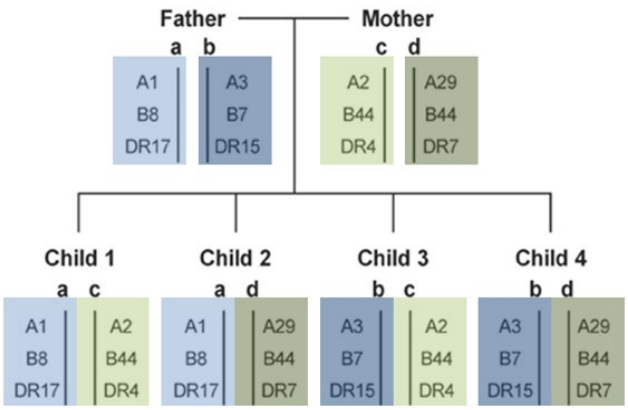


Figure 7: Mendelian inheritance of HLA-haplotypes.
Figure modified from Choo (*Choo 2007*).

If no HLA-identical sibling is available, matched unrelated donors (URD) can be used as graft donors in allogeneic HSCT (*Hansen et al. 1980*). At present, a matched URD is the second donor choice, as recommended in the European Society for Blood and Marrow Transplantation (EBMT) donor choice algorithm (*Apperley et al. 2012*). When searching for an URD in worldwide donor registries, HLA-matching of at least 8 HLA-antigens (2 antigens for each HLA-A, -B, -C, and -DR) is commonly sought for, i.e. an "8/8 match".

However, in recent years donor choice practices may be altered for specific patient groups. For example, there might be patients with MDS for whom a young and healthy URD can be considered a better donor choice than an older HLA-identical sibling to avoid a possibly shared genetic predisposition to disease (e.g. GATA2, RUNX1) (*Ljungman 2019*).

The number of HLAs analyzed as well as processes and techniques for analyses during donor selection varies among transplantation centers, which take factors such as number of samples, level of resolution, cost and turnaround time into consideration (*Latham et al. 2014*). At the Karolinska University Hospital HLA-A, -B, -C, -DRA, -DRB1, -DRB3, -DRB4, -DRB5, -DQA1, -DQB1, DPA1, and -DPB1 are analyzed. Previously, genomic molecular high-resolution typing was used for both HLA class I and class II antigens using sequence-specific primers (PCR-SSP) (*Olerup et al. 1992*). In 2017, the method of HLA-typing used at our center was replaced by next-generation sequencing (NGS) protocols, covering all loci in the same panel using long-range PCR (*Shiina et al. 2012*).

When searching for an URD a "12/12" HLA-match is desirable if possible, but certain HLA-mismatches may be accepted for individual patients (*Hauzenberger et al. 2008, Magalhaes et al. 2017*). In addition to the HLA-match, other factors should be considered when choosing the most suitable donor for each patient prior to an allogeneic HSCT. These include cytomegalovirus (CMV) serology status for donor and recipient, sex, age, weight and sometimes ABO-blood group match (*Ringden et al. 2004, Apperley et al. 2012*). All potential donors must be eligible and medically approved prior to graft donation. They must sign an informed consent to donate HSCs, and they must fulfill the requirements stated by applicable national legislations bodies.

If no available HLA-identical sibling or acceptable well-matched URD is available, umbilical cord blood (UCB) (*Gluckman et al. 1989, Ballen et al. 2013*) or partially HLA-matched relatives can be considered. In recent years it has been shown that allogeneic HSCT outcomes using related haploidentical donors can possibly be comparable to those with a matched related or unrelated donor (possibly with the limitation of relatively short follow-up of haploidentical HSCT recipients at present) (*Luznik et al. 2008, Raiola et al. 2014*). To prevent graft rejection and/or severe GVHD in these patients large doses of T cell depleted stem cells are used to enhance the chance of donor engraftment and post-transplantation cyclophosphamide can be given to achieve T cell-depletion in the haploidentical HSCT recipient (*Aversa et al. 2007, Symons et al. 2008*). Prospective randomized trials comparing URD HSCT and haploidentical HSCT to confirm previous retrospective results are currently lacking, though under development.

1.2.5 Pre-transplantation conditioning regimens

Prior to scheduled HSC graft infusion, the patient receives a cytotoxic conditioning treatment. The purpose of conditioning is to weaken the recipient's immune system to prevent graft rejection, to exterminate as many remaining malignant cells as possible to reduce relapse risk (valid for malignant diseases) and to create space for the graft to obtain access to appropriate BM niches (*Santos et al. 1972, Thomas et al. 1979*). The regimen of choice is derived from standard operating protocols determined by disease requirements and the overall health condition of the specific patient. An optimal conditioning regimen should deliver consistent engraftment, maximal malignancy elimination and minimal toxicity to the recipient to reduce the risk of relapse and TRM after allogeneic HSCT.

Conditioning regimen research started off as scientific experiments enforced by the reality of World War II and subsequent threats of nuclear warfare and aimed to find methods to cure soldiers and civilian by-standers from lethal radiation damage (i.e. high-dose total body irradiation [TBI] exposure). In one of the publications from that era, Jacobson et al demonstrated that a significantly higher proportion of mice survived lethal radiation doses if their spleen was shielded or if the mouse was injected with spleen cells after TBI exposure (*Jacobson et al. 1949*).

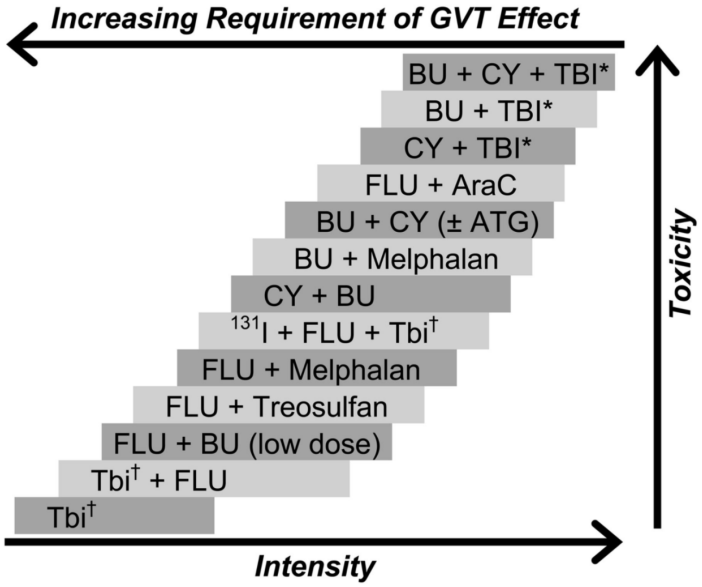
All conditioning regimens in early HSCT were highly toxic TBI protocols due to their effects against leukemia and lymphomas, immunosuppressive properties, and ability to penetrate to sanctuary sites. A decade later, experiments with cyclophosphamide allowed allogeneic engraftment (*Santos et al. 1972*), and over time it became evident that synergistic effects could be achieved by combining TBI and different alkylating agents. By combining multiple alkylating agents, TBI could even be omitted. The choice of alkylating agents was most likely because that was the cytotoxic drug class available at that time, and because BM toxicity is the dose-limiting toxicity for these cytostatic compounds (making rapidly dividing cell lines more susceptible to their DNA-alkylating effects) (*Puyo et al. 2014*).

Reducing toxicity without compromising the anti-malignant effect of allogeneic HSCT was important to improve patient outcome. Already in the late 1950s, it was shown that the treatment modality gave additional effects besides myeloablative disease treatment (*Barnes et al. 1957, Burchenal et al. 1960, Weiden et al. 1979a*). Today it is widely acknowledged that the curative effect of allogeneic HSCT is mediated both by the administration of cytotoxic conditioning and the graft-versus-leukemia (GVL) effect by immune competent cells in the graft (*Weiden et al. 1981, Barrett 1997, Champlin et al. 2000, Bacigalupo et al. 2009*).

Based on these findings, the concept of RIC regimens was developed, which depress the immune response of the recipient to a higher extent rather than fully eradicating remaining tumor cells (*Giralt et al. 1997, Slavin et al. 1998*). These protocols have been shown to reduce TRM and have, thereby,

made allogeneic HSCT a valid treatment option for older patients or patients with heavier comorbidity burden than was previously accepted. Over the last decade, the pendulum swung even more towards induction of GVL (or graft-versus-tumor, [GVT]) as the primary goal of transplantation such that non-MAC and RIC regimens are currently used more frequently in clinical practice (*Blaise et al. 2007, Gooley et al. 2010*).

Since the number of described conditioning protocols have expanded rapidly during the last decades, guidelines to define them as MAC, RIC or non-MAC have been developed. A report from the 'RIC regimen Workshop', held at the BMT-Tandem meeting in 2006, presented the "Champlin criteria" to define a RIC regimen. It states that exposure to a RIC regimen shall result in reversible myelosuppression when given without stem cell support and leads to mixed chimerism in a proportion of patients at the time of first assessment, with low rates of non-hematologic toxicity (*Giralt et al. 2009*). Full consensus is still not reached within the field of allogeneic HSCT regarding which regimen belongs to which of the intensity groups (applies primarily to "borderline" protocols). Regimens in between obvious MAC and non-MAC protocols are classified as RIC, which consequently results in a very diverse RIC group. The spectra of available regimens rather constitute a continuous scale of both intensity and toxicity, correlating to the declining need of GVL after transplantation (Figure 8).



* High-dose TBI (800-1320 cGy)
† Low-dose TBI (200-400 cGy)

Abbreviations: GVT, graft-versus-tumor; BU, busulphan; CY, cyclophosphamide; TBI, total body irradiation; FLU, fludarabine (various dosing schedules); AraC, cytarabine; ATG, anti-thymocyte globulin; ^{131}I, anti-CD45 antibody conjugated to ^{131}I; cGy, centigray.

Figure 8: Examples of conditioning regimens of different dose intensities in allogeneic HSCT
Reproduced from Gyurkocza (*Gyurkocza et al. 2014*).

1.2.6 Hematopoietic stem cells, grafts and cell doses

Another challenge in allogeneic HSCT is to harvest a sufficient number of viable HSCs from the graft donor. It is estimated that approximately 1 in 10,000 cells in the BM, and 1 in 100,000 cells in peripheral blood, is a HSC. Morphologically, HSCs are similar to white blood cells and defined as being CD34+, but a vast array of additional markers have been identified over time (*Berenson et al. 1988, Baum et al. 1992, Petzer et al. 1996a, Petzer et al. 1996b, Ng et al. 2017*). Consequently, techniques designed for identification and isolation of HSCs often depend on CD antigen detection.

The HSCs for graft infusion can be harvested from the donor's BM by repeated iliac aspirations, from apheresis of peripheral blood, or from previously collected and banked UCB. The recommended cytokine for mobilization of peripheral blood stem cell (PBSC) donors prior to apheresis is G-CSF, which induces myeloid hyperplasia and the release of CD34+ cells into the circulation (*Petit et al. 2002*).

The choice of graft source depends on transplantation indication, donor preference and availability. A sufficient number of CD34+ cells in the graft is important to achieve engraftment in the transplanted host and subsequent survival (*Storb et al. 1977*). Higher cell numbers in the graft also decreases the risk of rejection and shortens the neutropenic phase (*Niederwieser et al. 1988, Zaucha et al. 2001, Bittencourt et al. 2002, Mohty et al. 2003, Kamel et al. 2005*). These facts partly constitute the rational for using PBSC grafts in allogeneic HSCT, since these often contain higher CD34+ cell numbers and approximately 5-10 times more T cells (CD3+) compared with BM grafts (Table 1) (*Remberger et al. 2001, Apperley et al. 2012*). At the same time, studies have shown that the risk of chronic GVHD is increased after PBSCT compared to BM HSCT, probably because of the higher cell content (*Singhal et al. 2000, Ringden et al. 2002, Eapen et al. 2004*). In recent years, only a few studies have evaluated the effect of cell dose on outcome after allogeneic HSCT, and it is primarily MAC BM HSCT that have been studied (*Kamel et al. 2005, Singh et al. 2007, Pulsipher et al. 2009, Tsirigotis et al. 2010*). Hence, it is still important to establish optimal cell doses to improve outcome in other HSCT-settings, e.g. in RIC PBSCT.

	Volume collected	**Median CD34+ cell content**	**Median CD3+ cell content**	**Target cell dose**
Bone marrow	10–20 mL/kg	$2–3 \times 10^6$ /kg	25×10^6 /kg	2×10^8 TNC/kg
Peripheral blood	150–400 mL	8×10^6 /kg	250×10^6 /kg	$5–10 \times 10^6$ CD34+/kg
Umbilical cord blood	80–160 mL	0.2×10^6 /kg	2.5×10^6 /kg	$> 3 \times 10^7$ TNC/kg

Table 1: Number of cells per kg recipient body weight, according to stem cell source
Table from the EBMT Handbook (*Apperley et al. 2012*).

Given the described properties of PBSC grafts above, they are usually preferred in allogeneic HSCT for malignant diseases to decrease the risk of relapse (*Horowitz et al. 1990, Apperley et al. 2012, Holtick et al. 2014, Wu et al. 2015*). On the other hand, BM is usually preferred in patients with non-malignant disease to reduce the risk of GVHD (which for such indications is undesirable).

1.2.6.1 Graft cell dose and GVHD

To overcome, or limit, the correlation of higher cell doses and increased risk of GVHD development seen in the majority of cell dose studies in allogeneic HSCT, different protocols for graft engineering have been introduced. Since T cells play a major role in acute GVHD pathophysiology, current transplantation approaches use various forms of immunosuppressive techniques before (e.g. *in vivo* T cell depletion by anti-thymocyte globulin [ATG], or alemtuzumab) or after transplantation (calcineurin inhibitors, methotrexate, or post-transplantation cyclophosphamide), discussed in the next section.

Another approach is to manipulate the stem cell product. Previous publications include specific *in vitro* T cell depletion of stem cell grafts, possibly having the potential to reduce GVHD but negatively delay immune recovery (*Anandi et al. 2017*). To mitigate prolonged immune deficiency, a delayed add-back of T cells (after T cell depleted allogeneic HSCT) has been tested with mixed results and no evidence of clear outcome benefit (*Elmaagacli et al. 2003*).

Other protocols strive to retain beneficial immune donor cells and deplete harmful immune cells (i.e. enhance the GVL effect and reduce risks of GVHD) by selectively depleting specific T cell populations from the infused graft. Regarding T cell subsets, the γδT cells are of specific interest since they can be activated without prior binding to MHC molecules, enabling them to bridge innate and adaptive immune responses (*Bonneville et al. 2010*). The γδTCR recognize phosphoantigens and express receptors of NK cells and natural cytotoxicity families making them potential "GVL-enhancers" by recognition and killing of leukemia cells. Such MHC-independent antigen-recognition allows the preferential possibility to mediate GVL effects without GVHD complications (*Minculescu et al. 2015*). The approach is currently evaluated in selected patient settings and may prove an important clinical tool in the future (*Radestad et al. 2014, Perko et al. 2015*).

1.2.7 Immunosuppression

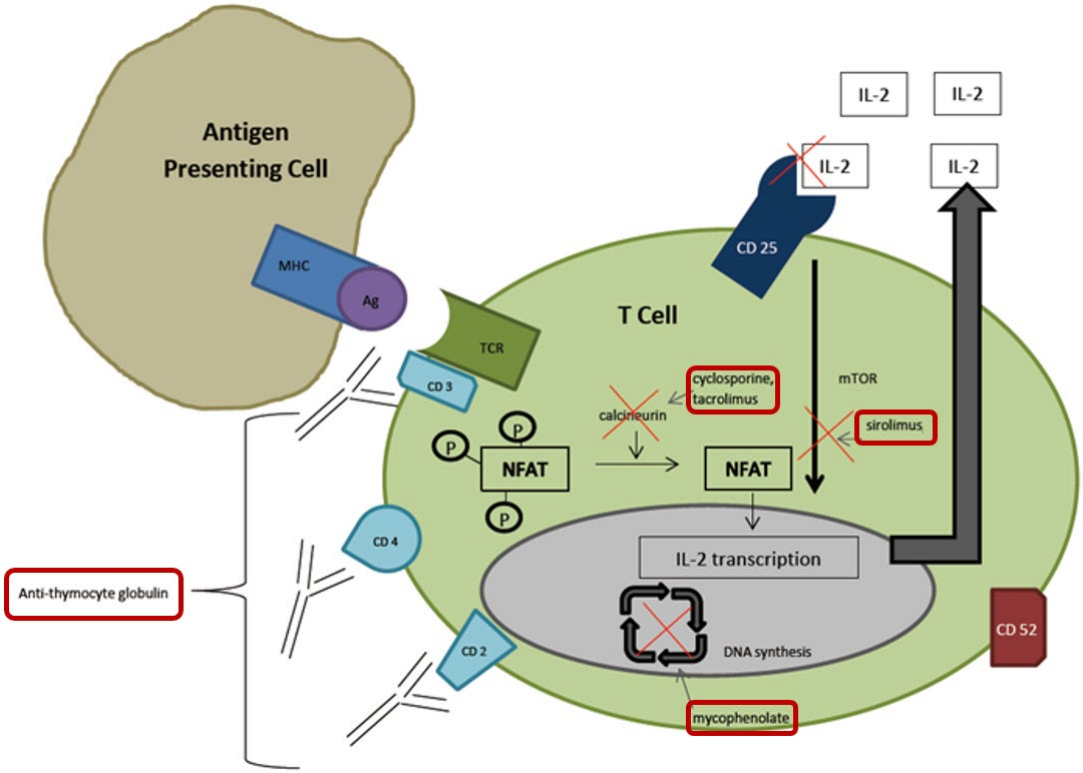
Immunosuppressive treatment is given to all allogeneic HSCT-patients to prevent graft rejection and severe forms of GVHD. The first doses are usually administered the day(s) before graft infusion to prepare the humoral milieu in the recipient prior to HSC infusion. The mode of action of the most commonly used immunosuppressive drugs is inhibition of T cell proliferation and effector function through blocking of different intracellular T cell pathways (Figure 9). By suppressing the activation and differentiation of naïve T cells, these drugs prevent transplantation recipients from mounting an adaptive immune response against the non-self antigens of the allogeneic graft (*Abo-Zena et al. 2002, Scheffert et al. 2014*).

One of the most common protocols in allogeneic HSCT today is a combination of a calcineurin inhibitor (cyclosporine A or tacrolimus) with 3-4 intermittent doses of methotrexate (*Storb et al. 1986, Storb et al. 1988, Ringden et al. 1993*). In UCB HSCT, where the marrow-depressing properties of methotrexate are to be avoided, a combination of steroids and cyclosporine is normally used (*Eapen et al. 2007*).

Both cyclosporine and tacrolimus, when complexed with their respective intracellular binding proteins, form a ternary complex with calcineurin (a Ca+ calmodulin-dependent serine/threonine phosphatase), causing its inactivation (*Liu et al. 1992*). This inhibits the ability of calcineurin to dephosphorylate the cytoplasmic subunit of the nuclear factor of activated T cells (NFAT). This process blocks the NFAT translocation into the nucleus, a step required for the transcription of cytokine genes, primarily IL-2 (*McCaffrey et al. 1993*).

In the last decade, new immunosuppressive strategies have been evaluated in solid organ transplantation, making way for their introduction in allogeneic HSCT (*Macdonald 2007, Knoll et al. 2014*). A regimen that has shown promising results is the combination of sirolimus and tacrolimus, which to some extent has different mechanisms of action compared to cyclosporine and methotrexate (*Cutler et al. 2004, Cutler et al. 2007*).

Sirolimus has been of interest due to its promising mode of action, which theoretically offers potential advantages over the immunosuppressive agents currently in use. Its actions include immunosuppression through inhibition of T cell and DC activity while promoting regulatory T cells (*Hackstein et al. 2003, Koenen et al. 2003*). Furthermore, sirolimus has antifibrotic, antineoplastic, antiviral and antifungal activities as well as synergistic action when combined with tacrolimus (*Sehgal 2003, Zaytseva et al. 2012, Li et al. 2014, Sheng et al. 2015*).



Abbreviations: MHC, major histocompatibility complex; Ag, antigen; TCR, T cell receptor; CD, cluster of differentiation; mTOR, mammalian target of rapamycin; P, phosphor; NFAT, nulear factor of activated T cells; IL, interleukin.

Figure 9: Schematic targets of common immunosuppressive agents in allogeneic HSCT.
Picture modified from Sheffert (*Scheffert et al. 2014*).

Since T cells are recognized as crucial mediators of the GVHD pathophysiology (see below) (*Prentice et al. 1984, Jaksch et al. 2005*), *in vivo* T cell depletion using anti-T cell antibodies such as ATG can be of interest. Conditioning regimens containing ATG is primarily given to recipients of grafts from unrelated donors and to patients with non-malignant disorders to enhance the GVHD prophylactic effect (*Remberger et al. 1999, Uzunel et al. 2006, Mohty 2007*). However, this approach may result in a higher risk of relapse after allogeneic HSCT, at least in groups of patients treated with high ATG-doses (*Baron et al. 2017*).

1.2.8 Graft-versus-host disease

When the hematopoietic system from a graft of a non-genetically identical donor evolves in the transplanted host, resulting immune-mediated alloreactivity can lead to GVHD. It was first described by Barnes and Loutit as a "secondary disease" in transplanted mice, different in character compared to the primary disease of radiation sickness. Mice transplanted with allogeneic spleen cells after irradiation developed fatal reactions (primarily skin abnormalities and diarrhea), as a result of the introduction of immunologically alloreactive cells into an immunocompromised host (*Barnes et al. 1962, Simonsen 1985*).

In humans, GVHD is commonly separated into an acute and a chronic form depending on the time of onset after allogeneic HSCT as well as observed clinical symptoms and organ manifestation (*Glucksberg et al. 1974, Przepiorka et al. 1995, Jagasia et al. 2015*). In 2005, the National Institutes of Health in United States (US) published a consensus document to address several aspects of chronic GVHD (*Filipovich et al. 2005*), making it possible to more clearly distinguish between different forms of GVHD. Historically, the cut off between acute and chronic GVHD was arbitrary set to 100 days (≈ 3 months) post-HSCT (*Filipovich et al. 2005, Apperley et al. 2012*), but the introduction of RIC, donor lymphocyte infusion (DLI) and late tapering of immunosuppressive drugs have made GVHD distinctions more diverse in recent years (Figure 10) (*Filipovich et al. 2005, Lee 2017*).

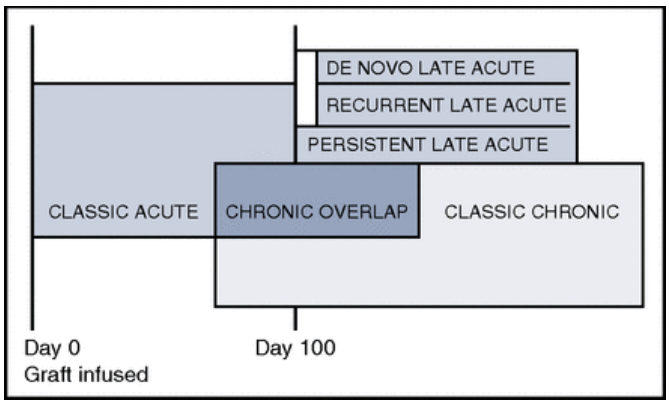


Figure 10: Different nomenclature of GVHD after allogeneic HSCT (box sizes do not reflect prevalence). Image from Lee (*Lee 2017*).

The incidence of acute GVHD is widely varying, between 10-90% in the literature (*Murphy et al. 1999*), depending predominantly on the type of donor and degree of matching (30–50% after matched sibling transplantations and 40–70% in recipients of matched URD grafts) (*Deeg et al. 1986, Hansen et al. 1990, Ringden et al. 1993, Aschan et al. 1994, Apperley et al. 2012*).

Despite continuous advances in allogeneic HSCT over time, GVHD remains a frequent and serious iatrogenic complication to given treatment. Both acute and chronic GVHD contribute significantly to morbidity and are associated with high mortality (*Deeg 2007, Salmasian et al. 2010, Ziakas et al. 2014*). Regarding the alloreactive processes, it is still a reality that GVL effects are sometimes strongly correlated to clinical GHVD manifestations (*Weiden et al. 1979b, Ringden et al. 1996, Passweg et al. 1998, Jenq et al. 2010*).

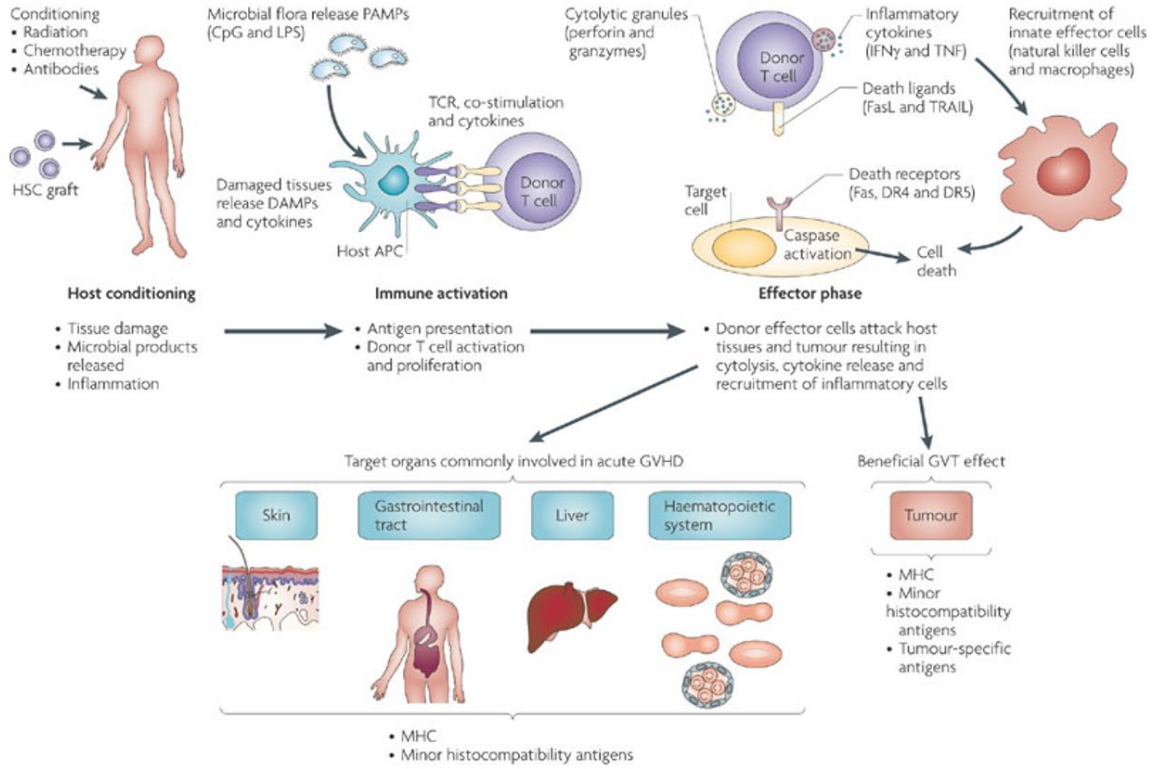
1.2.8.1 Acute graft-versus-host disease

Acute GVHD is the result of a complex cascade of immunocompetent cell interactions from the transplanted graft and host cells/tissues in an inflammatory milieu involving both innate and adaptive immune responses. According to the current paradigm, to a large extent based on murine models, the pathophysiology is commonly described in three sequential steps (*Krenger et al. 1996, Ferrara et al. 1999, Hill et al. 2000, Jaksch et al. 2005, Ferrara et al. 2009, Zeiser et al. 2017a*) (Figure 11).

1. Previous conditioning treatment and/or underlying disease prior to graft infusion inflicts damage and immunologic activation of the intestinal mucosa (loss of gastrointestinal homeostasis) and other host tissue barriers. Subsequent inflammatory triggers include sterile DAMP molecules (e.g. ATP, uric acid, IL-33) and PAMPs. Release of microbial products (e.g. translocation of LPS into the circulation) and inflammatory mediators (e.g. TNF-α and IL-1) activates innate immune cells by Toll- and/or NOD-like receptors on APCs. This increase the expression of MHC antigens and adhesion molecules on host tissues, enhancing the possibility of recognition of MHC and minor histocompatibility antigens by mature donor T cells.

2. The APCs that express host MHC or minor histocompatibility antigen-peptides interact with donor T cells from the transplanted graft leading to subsequent activation, proliferation, differentiation and migration of alloreactive donor T cells in the host. Proliferation of Th1 T cells occurs in the presence of IL-12 and the secretion of IL-2 and IFN-γ. IL-2 and IFN-γ induce further T cell expansion and cytotoxic T cell and NK cell responses. Both CD4+ and CD8+ T cells are involved, but CD4+ T seem to be of greatest importance at the initiation stage (*Blazar et al. 2012*). CD4+ T cells can cause GVHD through cognate interactions with MHC class II or with minor histocompatibility antigens, or by cytokine release (including TNF-α which induce apoptosis in epithelial cells (*Borsotti et al. 2007*)).

3. A complex cascade of multiple cellular (cytotoxic T cells and NK cells damage tissue by perforin/granzyme, Fas-ligand, and TNF-α) and inflammatory effectors are released in a "cytokine storm" (including IL-1β, INF-γ, IL-2) that further modulate the dysregulated immune responses, ultimately leading to aggravated target organ/tissue damage.



Abbreviations: PAMPs, pathogen-associated molecular patterns; CpG, oligodeoxynucleotides; LPS, lipopolysaccharide; DAMPs, damage-associated molecular patterns; TCR, T cell receptor; FasL, Fas ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; GVHD, graft-versus-tumor disease; GVT, graft-versus-tumor; MHC, major histocompatibility complex.

Figure 11: Summarized pathophysiology of acute GVHD and GVL/GVT effects.
Image from Jenq (*Jenq et al. 2010*).

The major organs affected by acute GVHD are the skin, the gastrointestinal tract and the liver; organs with continuous microbial pathogenic exposure (*Martin et al. 1990*). The diagnosis is mainly clinical, but histopathological evaluation by biopsies is sometimes needed for the distinction from other disorders, such as CMV colitis or treatment-related toxicities (*Einsele et al. 1994*). Consensus guidelines on grading of acute GVHD were published in 1995 (*Przepiorka et al. 1995*).

The stage of acute GVHD is at first determined separately in the three organ systems affected, and the respective grades for each organ are then combined to determine the overall acute GVHD grade using the Glucksberg criteria (*Glucksberg et al. 1974*) or the Center for International Blood and Marrow Transplant Research (CIBMTR) (*Rowlings et al. 1997*) criteria. Both systems have been prospectively validated and are predictive of mortality at 100 days and 1-year post-HSCT (*Cahn et al. 2005*). The overall grade correlates to survival, with approximately 25% long-term survivors in patients with grade III and < 5% survivors for grade IV.

First-line treatment of acute GVHD is methylprednisolone (*Ruutu et al. 1997, Salmasian et al. 2010*) in combination with the continuous use of the calcineurin-inhibitor based prophylaxis that the patient receives after transplantation. However, durable complete recovery is only achieved in about 35% of patients treated with steroids alone (*MacMillan et al. 2002, Salmasian et al. 2010*). If the patient does

not respond to prednisolone (defined as no response after 7 days or symptom progression after 5 days), there is no standard or consensus second-line treatment option (*Deeg 2007, Ruutu et al. 2014*) and the general recommendation is to include and treat such patients in clinical trials. Some emerging second-line therapies are discussed briefly in the section 'Future prospects and concluding remarks' later in this thesis.

1.2.8.2 Chronic graft-versus-host disease

Chronic GVHD is a multi-organ disorder characterized by immune dysregulation and constitutes one of the leading causes of late morbidity and mortality after allogeneic HSCT (*Sullivan et al. 1981, Lee et al. 2003, Baird et al. 2006, Arai et al. 2015*). It is diagnosed based on the presentation of clinical symptoms in the organs involved and overall stage is defined as mild, moderate or severe according to the degree of organ involvement and severity of graded symptoms (*Shulman et al. 1980, Filipovich et al. 2005, Jagasia et al. 2015*).

The pathophysiology of chronic GVHD is less understood compared to the processes in acute GVHD, but contributing immunologic factors have been identified (*Blazar et al. 2012, Socie et al. 2014, Zeiser et al. 2017b*). The first phase is largely the same as for acute GVHD. Briefly summarized, tissue damage results in translocation of pathogens and release of PAMPs and DAMPs and activation of TLRs and the NOD-like receptor inflammasome. As a consequence of the conditioning-related tissue damage, DCs upregulate costimulatory molecules. In the next phase, APCs prime alloreactive T and B cells, which leads to their expansion and polarization towards Th1, Th2 and Th17 (maintaining inflammation by cytokine release) and antibody generation. Thymic injury is caused by alloreactive T cells, causing impaired central tolerance and hampered thymopoiesis, including the loss of thymic epithelial cells required for the generation of regulatory T cells (regulatory cell cohorts of the B and NK cell populations are also reduced in this phase). In the last phase, infiltrating lymphocytes and myeloid elements can develop into cytotoxic effectors, causing local damage to overlying epithelia (in skin, mucosa and gut), or to secretory epithelia (salivary or lacrimal glands). Aberrant tissue repair promoted by macrophages leads to the activation of fibroblasts, which release factors that cross-link collagen and increase tissue-stiffness.

Standard treatment of chronic GVHD is corticosteroids, an approach unchanged over the last decades (*Ruutu et al. 2014*), which is usually combined with the use of calcineurin inhibitors to reduce steroid duration and its long-term side effects (including type 2 diabetes, osteoporosis, hypertension, physiological disturbances and impaired response against infections).

In terms of second-line therapy there is no evident standard of care. A published survey of EBMT centers in 2010 reported extracorporeal photopheresis, mycophenolate mofetil, rituximab, calcineurin

inhibitors, mTOR-inhibitors and tyrosine kinase inhibitors all being used in clinical practice (*Ruutu et al. 2012*). A number of small, non-randomized studies or case series have previously reported various experimental therapies (*Martin et al. 2011, Wolff et al. 2011*). Recently, the use of the tyrosine kinase ibrutinib (*Miklos et al. 2017*) was approved by the Food and Drug Administration (FDA) in the US to treat chronic GVHD in adults after documented failure of one or more previous therapies, and the selective Janus kinase (JAK1/2) inhibitor ruxolitinib has recently emerged as a second-line approach in patients with steroid-refractory chronic GVHD (*Modi et al. 2019*). Due to the lack of a robust second-line treatment, the prognosis for patients suffering from steroid-refractory chronic GVHD remains poor, valid for both overall survival and quality of life.

1.2.9 Immune reconstitution after allogeneic HSCT

A main concern after allogeneic HSCT is the profound immunodeficiency seen in treated patients. Following transplantation, patients need a quantitative and qualitative reconstitution of the various lymphocyte (B cells, T cells, NK cells) and myeloid (monocytes, macrophages and DCs) cell populations (Figure 12). Long lasting and/or persistent immune defects in the host may result in severe post-transplantation infections, disease relapse or secondary malignancies (*Mackall et al. 2009*). A vast array of factors affect the immune reconstitution in allogeneic HSCT recipients. These include age, sex, conditioning regimen, degree of genetic differences (incl. HLA minor histocompatibility antigens), stem cell source and graft manipulation, as well as post-HSCT events such as GVHD, relapse and infectious complications (*Paulin et al. 1987, Apperley et al. 2012, van den Brink et al. 2015*).

Parts of the innate immune system, including granulocytes, monocytes and NK cells normally reconstitute rapidly during the first month(s) after graft infusion (*Petersen et al. 2003, Storek et al. 2008, Bosch et al. 2012*). However, distinct properties of these innate cells such as chemotaxis and phagocytosis can be impaired over a longer time-period, especially in patients that develop GVHD (*Zimmerli et al. 1991*). Neutrophils are the first leukocytes to appear in peripheral blood post-transplantation, and their persistence is used clinically as a proxy marker for donor engraftment. By common definition in allogeneic HSCT, a neutrophil engraftment is considered when the absolute neutrophil count is $\geq 0.5 \times 10^9$/L for three consecutive days. Upon reaching this level of granulocytes in blood, the period of "isolation" for the patient can be revoked, since the most basic level of the rudimental immune competence is believed to be restored.

The adaptive, lymphoid part of the immune system reconstitutes more slowly in the host and with persistent (or at least prolonged) deficits in terms of global immune competence (*Maris et al. 2003*). Early expansion of mature T cells in the graft forms a limited repertoire during the first year after transplantation, followed by thymus-dependent development of naïve T cells, a process naturally

affected by older age and GVHD, and other factors that can impair thymopoiesis (*Clave et al. 2005, Gress et al. 2010, Sairafi et al. 2012*). Circulating B cells are at first undetectable in the periphery and may not reach normal numbers until 12 months (or longer). However, B cells can maintain an immune phenotype with limited immunoglobulin (IgG) production for up to 2 years post-HSCT, affecting immune competence and susceptibility to infectious pathogens (*Ringden et al. 1979, Small et al. 1990*).

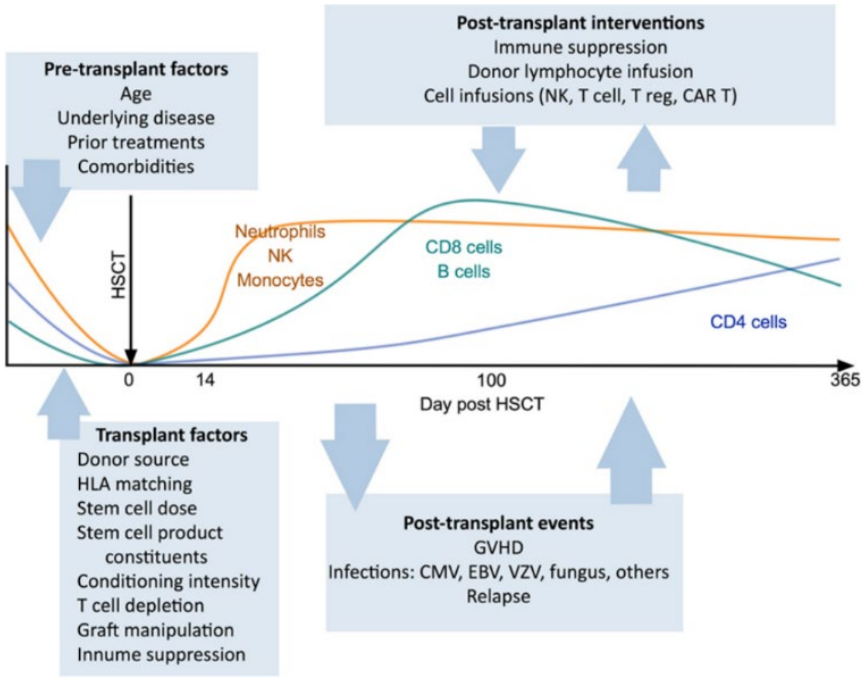


Figure 12: Schematic recovery of immune cell counts after allogeneic HSCT, and relations to different allogeneic HSCT factors and interventions.
Image from Stern (*Stern et al. 2018*).

As a consequence of impaired immune function, the immunocompromised host is at great risk for post-transplantation infections (*Rubin et al. 2002*). During the initial neutropenic phase, patients are at risk of bacterial and candida infections, not least since protective mucosal barriers are damaged after conditioning therapy (*Sparrelid et al. 1998, Blennow et al. 2014b*). Prolonged immunodeficiency in lymphoid cell subsets, augmented by continuous immunosuppressive therapy, further provides a risk for viral reactivations and/or infections (*Atkinson et al. 1979*). Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are major threats and are closely monitored and preemptively treated during the first months after HSCT (Figure 13) (*Ljungman et al. 2006, Blennow et al. 2014a, Uhlin et al. 2014*).

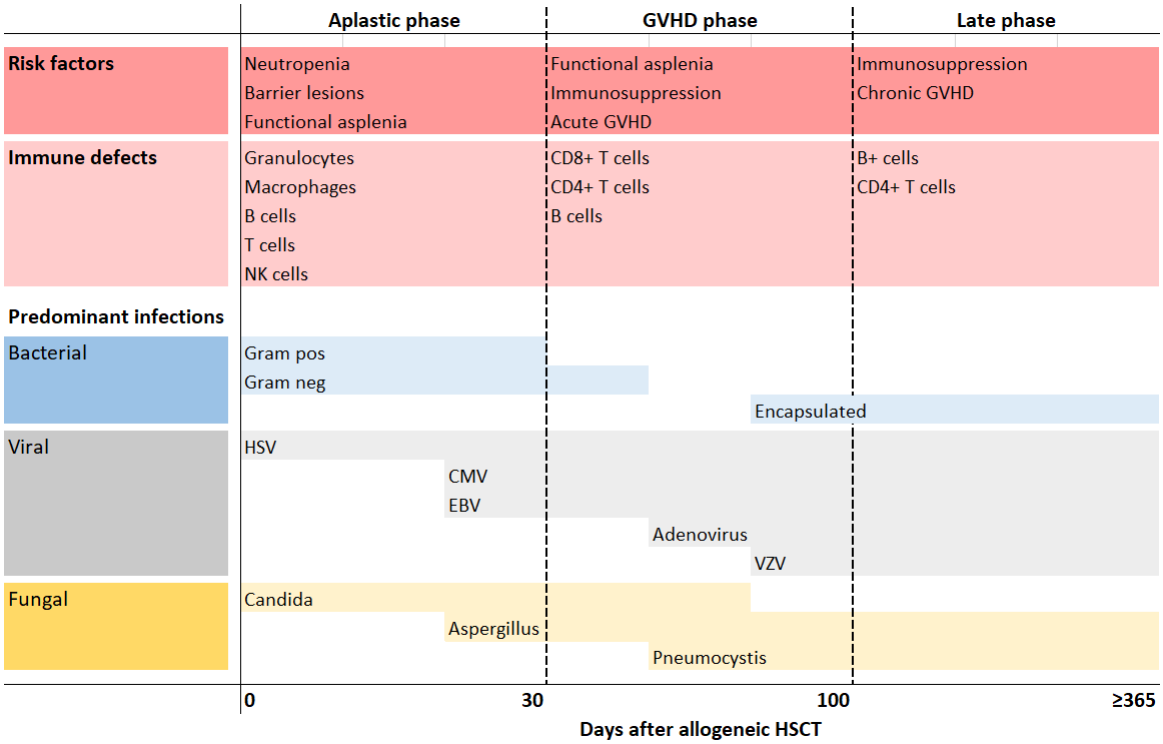


Figure 13: Major immune defects and selected spectra of predominant infections after allogeneic HSCT. Extrapolated from Tomblyn and Ljungman (*Tomblyn et al. 2009, Ljungman et al. 2016*).

To reduce infectious complications in the immunocompromised host, antibiotic and antifungal prophylaxis is routine during the neutropenic phase and the first months after transplantation. Valaciclovir prophylaxis, to prevent reactivation of Herpes Simplex virus and Varicella-Zoster virus, is usually administered during the first year. Invasive fungal infections are primarily a risk in patients developing GVHD and enhanced prophylaxis with an azole is then recommended (*Harrison et al. 2015*).

1.2.10 Chimerism

To monitor engraftment in patients after allogeneic HSCT, methods have been developed that takes advantage of the genetic disparity between the donor and the recipient (*McCann et al. 1993*). Chimerism analyses measure the percentage of donor-derived cells post-HSCT in whole blood, or in specific hematopoietic lineages in the host (usually CD3+ T cells, CD19+ B cells, CD33+ myeloid cells, and CD34+ hematopoietic stem cells) by using PCR to measure tandemly repeated DNA sequences in the genome (*van Dongen et al. 1992, Mattsson et al. 2001a*). Variation in chimerism results is analyzed over time, and deviations can be an early indicator of threatening graft rejection or disease relapse (*Bader et al. 2005*), in need of clinical action (e.g. reduced immunosuppression or administration of DLI (*Kolb et al. 1990*)). It has also been shown that early complete chimerism can be of importance for rapid GVL-induction to limit risks of early relapse (*Mattsson et al. 2001b, Shimoni et al. 2001*).

2 AIMS

The research presented in this thesis originated from the aim to increase the scientific knowledge in the field of allogeneic HSCT in general and to investigate relevant transplantation-related factors and their relation to clinical outcome in patients after treatment. To exhaustively cover such a vast research objective in one Ph.D. thesis is not possible, and aims that were more specific were necessary to construct relevant research hypotheses and to design the different projects included.

Based on this reasoning, the specific aims of the work presented in this thesis are:

- To independently validate two established pre-transplantation indices (HCT-CI and DRI) in a center-specific patient population to evaluate their clinical utility and ability to predict overall survival and TRM in patients after allogeneic HSCT.

- To determine the optimal CD34+ cell dose in allogeneic RIC and non-MAC PBSCT for patients with AML or MDS that may improve survival outcomes after transplantation.

- To prospectively compare clinical outcomes in allogeneic HSCT-patients randomized to one of two GVHD-prophylactic regimens (the established CsA/Mtx vs. the novel Tac/Sir) after allogeneic HSCT, with primary focus on acute GVHD and TRM.

- To study TREC and KREC kinetics and telomere length as proxy markers for immune reconstitution in allogeneic HSCT patients randomized to one of two GVHD-prophylactic regimens (the established CsA/Mtx vs. the novel Tac/Sir) in relation to long-term outcome after transplantation.

3 MATERIALS AND METHODS

3.1 ETHICS

3.1.1 Paper I

The Regional Review Board of Ethics in Stockholm (DNR 2014/1376-31/3) approved the retrospective study. Informed consent to data collection from the allogeneic HSCT procedure was obtained from each patient prior to start of conditioning treatment.

3.1.2 Paper II

This project was accomplished in close collaboration with the CIBMTR, situated in the US. The CIBMTR is a voluntary working group of more than 450 transplantation centers worldwide that continuously report data on performed allogeneic HSCTs to the organization. Participating centers are required to report all transplantations consecutively to avoid any selection bias and compliance is continuously monitored. Patients are followed longitudinally to time of death or lost to follow-up. All patients must provide written informed consent for data submission/transfer and research participation, and the research organization is governed by the "The US Health Insurance Portability and Accountability Act".
The project reported in paper II was approved as a specific research project (CIBMTR, GS12-01) by "The Institutional Review Boards of the Medical College of Wisconsin" (MCW/FH IRB, Milwaukee, Wisconsin, US) and "The National Marrow Donor Program" (NMDP, Minneapolis, Minnesota, US), where the CIBMTR is based (IRB-2002-0063).

3.1.3 Paper III

The Regional Review Board of Ethics in Stockholm (DNR 2006/1430-31/3) and Helsinki (541/2007, DNR 360/E5/07) and the Swedish and Finnish Medical Products Agencies (DNR 151:2007/38987 and KLNR 57/2008, respectively) approved the study. The trial was registered at ClinicalTrials.gov (identifier NCT00993343) and the European Clinical Trials Database (identifier 2006-006577-25). Written informed consent was obtained from each patient, or from parents/guardians of patients who were < 18 years of age, before the start of allogeneic HSCT conditioning treatment.

3.1.4 Paper IV

The study was approved by the Regional Review Board of Ethics in Stockholm (DNR 2016/317-31/1), and an additional approval regarding retrospective access to patient samples from the applicable biological repository (the biobank "Chimerism-DNA") at the Clinical Immunology department at the Karolinska University Hospital was obtained from the Stockholm Medical Biobank (Bbk-01501).

3.2 PATIENT AND ALLOGENEIC HSCT CHARACTERISTICS

Patient, donor and transplantation characteristics for subjects included in this thesis are summarized in Table 2. The choice of conditioning regimen depended on disease indication, patient age and standard operating procedures at participating centers. Applicable characteristics are described in additional detail in the Method-sections of each enclosed paper (I-IV). Supportive care and GVHD prophylaxis for all patients followed institutional standards at participating centers, or applicable study protocols.

Factor	Paper I	Paper II	Paper III	Paper IV
Number of patients	521	1057	209	200
Age at allogeneic HSCT, range, years	18–69	45–75	0.6–71	0.6–71
Sex, male/female	304/217	657/400	127/82	120/80
Year of allogeneic HSCT, interval	2000–2012	2002–2011	2007–2014	2007–2014
Diagnoses				
AML/ALL	190/61	858/0	57/42	49/41
CLL	30	0	22	22
MDS	84	199	34	33
Lymphoma	59	0	27	27
Other malignancies	83	0	14	15
Non-malignant	14	0	13	13
Disease Stage (CR1 / CR≥2 / Relapse, RAEB)	233/288/0	552/165/34	96/113/0	89/111/0
Donor				
Matched, related	186	370	62	59
URD (matched* or mismatched♮)	335	687	103	141
Conditioning regimen				
MAC/RIC	240/281	0/1057	72/137	56/144
Including ATG	371	360	154	147
GVHD prophylaxis regimen				
CsA/Mtx	417		106	103
Tac/Sir	64		103	97
Other	40		0	0
Graft source				
BM/PBSC/UCB	57/443/21	0/1057/0	39/170/0	39/161

* HLA-A, -B, and -DR matched, ♮ HLA-A, -B, or -DR allele/antigen-mismatched.

Abbreviations: HSCT, hematopoietic stem cell transplantation; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; CR, complete remission; RAEB, refractory anemia with excess blasts; URD, unrelated donor; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; ATG, anti-thymocyte globulin; GVHD, graft-versus-host disease; CsA, cyclosporine A; Mtx, methotrexate; Tac, tacrolimus; Sir, sirolimus; BM, bone marrow; PBSC, peripheral blood stem cells; UCB, umbilical cord blood; HLA, human leukocyte antigen.

Table 2: Patient, donor and transplantation characteristics in paper I-IV (not exhaustive).

3.3 STUDY DESIGN

3.3.1 Paper I

The study was designed to evaluate and compare the predictive capacity of the pre-transplantation indices HCT-CI and DRI for overall survival and TRM in our adult allogeneic HSCT-patient cohort at the Karolinska University Hospital in Stockholm. Patients ≥ 18 years transplanted for a hematological malignancy during the interval between January 2000 to December 2012 were assessed for inclusion (n = 644). Patients transplanted for solid tumors (n = 57), non-malignant diseases (n = 31) and patients with a previous allogeneic HSCT (n = 35) were excluded, resulting in a study cohort of 521 patients.

Pre-HSCT patient data regarding any previous or present HCT-CI listed comorbidity at time of pre-transplantation assessment was extracted from applicable medical records. If no information was available or indicated for a specific HCT-CI parameter, no score was assigned to that comorbidity as recommended by Sorror, the developer of the HCT-CI (*Sorror 2013*). The same data collection process was used to obtain enough diagnostic information for DRI classification, accurate at time of pre-transplantation assessment and/or admittance for allogeneic HSCT. Cytogenetic data for AML or MDS were classified according to previously proposed risk schemes (*Armand et al. 2010, Armand et al. 2012b*).

Included patients were classified to a risk group for each index according to HCT-CI and DRI procedures described previously by Sorror and Armand, respectively (Figure 5, Figure 6) (*Sorror et al. 2005, Armand et al. 2012a, Sorror 2013*) and set in relation to registered allogeneic HSCT outcomes from the transplantation registry.

3.3.2 Paper II

The study was designed to determine the optimal CD34+ cell dose for grafts used in allogeneic RIC HSCT by using non-relapse mortality (NRM) as primary outcome. Exploratory analyses of the effect of CD34+ cell dose were performed separately for HLA-identical sibling and unrelated donor transplantations.

The CIBMTR database was used to obtain applicable study data for registered patients eligible for inclusion according to the study protocol; consecutive patients from 45 to 75-years old with AML or MDS who received their first allogeneic HSCT with RIC or non-MAC regimens during the period between 2002 and 2011. All patients received PBSC grafts, and the CD34+ cell dose was determined by the cell processing laboratories at each reporting center.

3.3.3 Paper III

The study was designed to compare two different GVHD prophylaxis regimens used after allogeneic HSCT: cyclosporine/methotrexate (CsA/Mtx) versus tacrolimus/sirolimus (Tac/Sir) (Figure 14). The primary endpoint was acute GVHD of grades II-IV in the treatment groups. Secondary endpoints included engraftment outcomes, treatment-related toxicities, infections, chronic GVHD, disease relapse, TRM and OS.

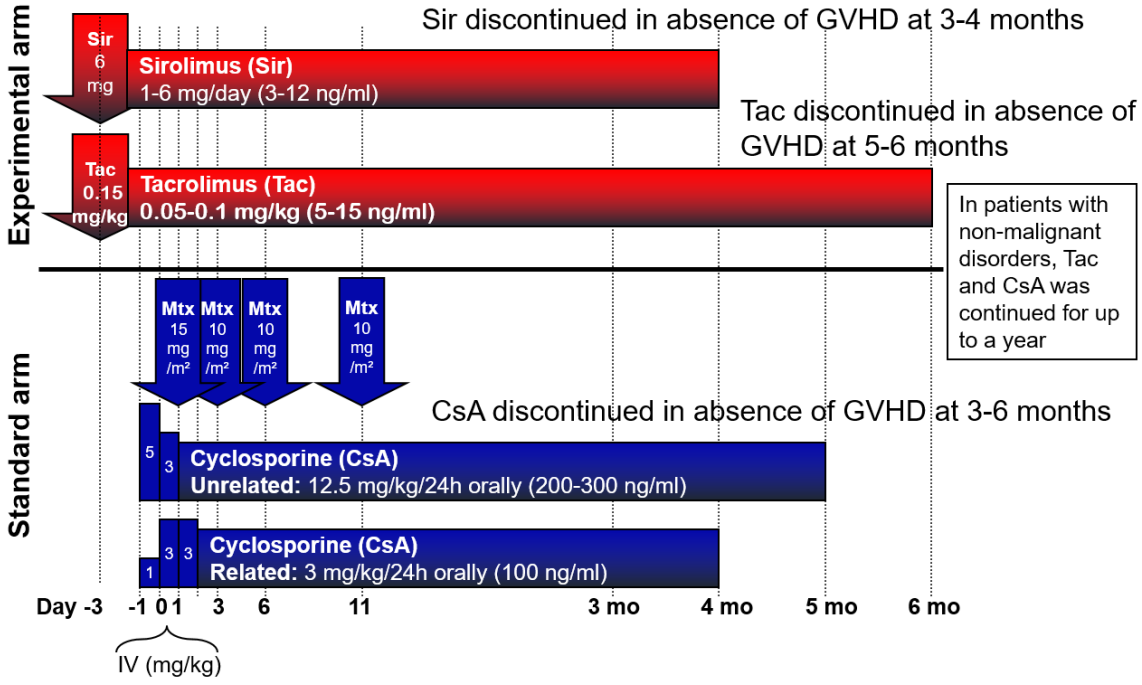
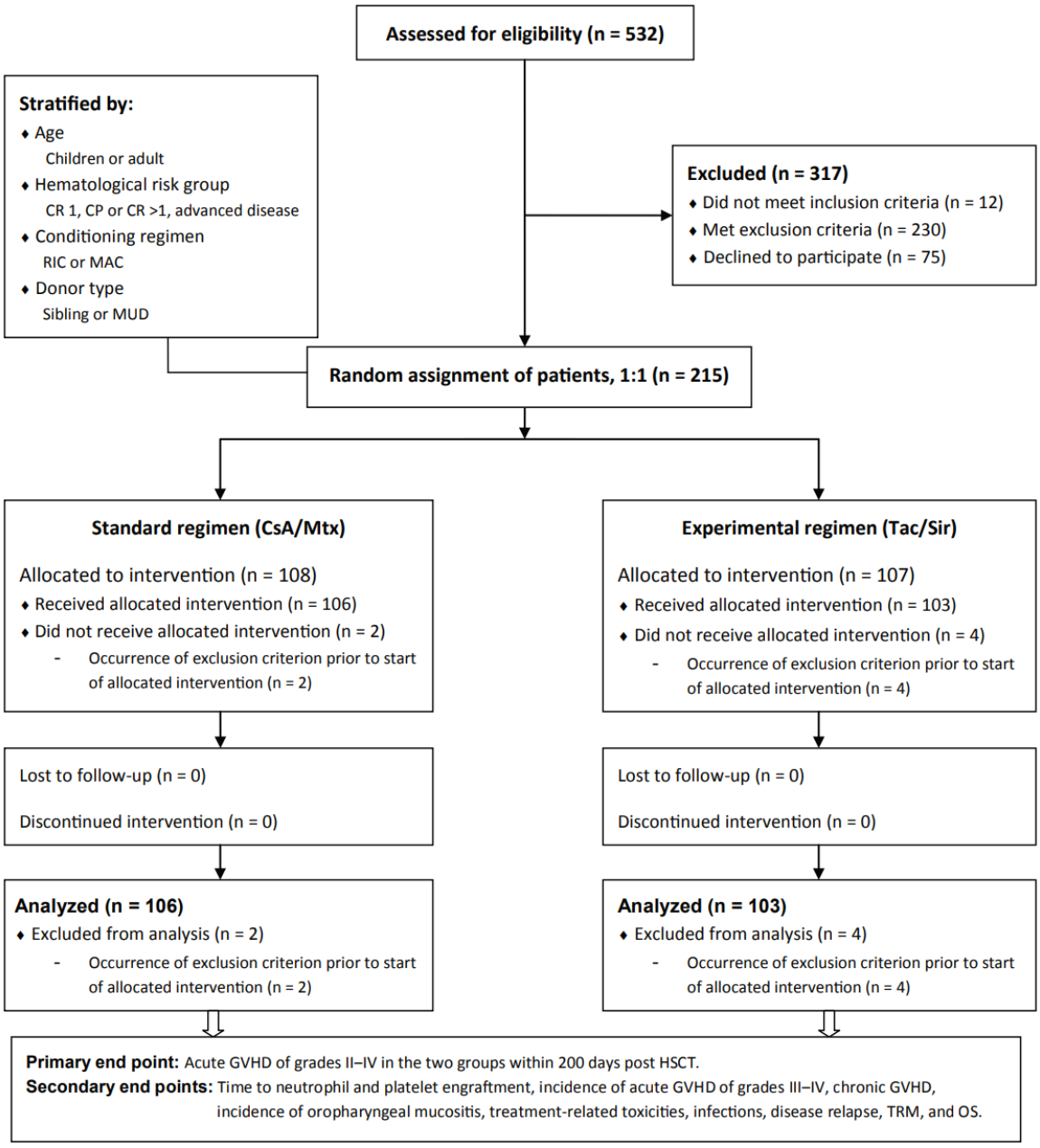


Figure 14: Treatment arms in the randomized clinical trial of GVHD prophylaxis (paper III).

The study was performed as a prospective, randomized, open, phase III trial. The hypothesis was that the novel combination of Tac/Sir would lead to less acute GVHD compared to the standard CsA/Mtx regimen. This assumption was based on previous publications which reported low incidence of grades II-IV acute GVHD using the Tac/Sir regimen (including a phase II study by Cutler et al (*Cutler et al. 2004*), and a safety pilot study by Ringdén et al (*Ringden et al. 2011*)).

Inclusion criteria included all patients 0.5-75 years of age with hematological diagnoses, immunodeficiencies or metabolic disorders with indication for a first allogeneic HSCT using BM or PBSC grafts. Exclusion criteria were relapse of malignant disease, HLA-A, -B, or -DR mismatched grafts on the allele level, addiction to drugs or alcohol, uncontrolled infection, pregnancy or breastfeeding within 4 weeks of study entry, impaired organ function (defined in paper III), Karnofsky performance status < 70% or requirement of voriconazole at time of study entry. Due to the risk of toxicity, the intention to use a MAC regimen with busulphan and cyclophosphamide (BuCy) was added as an exclusion criterion during the trial period (see below).

The aim was to include 200 patients (100 in each study arm), which should be sufficient to detect a statistical difference in incidence of grades II-IV acute GVHD of at least 24 percentage points between the treatment groups with a two-sided probability of $p < .05$. Patients were assessed for eligibility and included between September 2007 and January 2014 at Karolinska University Hospital in Stockholm and Turku University Hospital. After assessment for eligibility, 215 patients were included in the trial (Figure 15).



Abbreviations: CR, complete remission; CP, chronic phase; RIC, reduced-intensity conditioning; MAC, myeloablative conditioning; MUD, matched unrelated donor; CsA, cyclosporine A; Mtx, methotrexate; Tac, tacrolimus; Sir, sirolimus; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantaion; TRM, transplantation-related mortality; OS, overall survival.

Figure 15: CONSORT diagram of the clinical trial of GVHD prophylaxis (paper III).

3.3.4 Paper IV

The study was designed as a retrospective laboratory study based on the patient population included in the previous randomized trial of GVHD prophylaxis (paper III). The purpose of the subsequent study was to investigate the effect of the two different immunosuppressive protocols (CsA/Mtx vs. Tac/Sir) on certain parts of the immune reconstitution after allogeneic HSCT.

All patients included in the clinical trial (n = 209) were assessed for inclusion. Study samples were collected from biobanks created from standardized follow-up after allogeneic HSCT, and patients with ≥ 1 retrievable sample fit for analyses of TREC (2, 3, 6, 12, 24 months), KREC (2, 3, 6, 12 months) and/or telomere length (3, 12, 24 months) were included (n = 200). The laboratory methods used are described in the following section, and the results from laboratory analyses were set in relation to registered allogeneic HSCT outcomes from the transplantation registry.

3.4 LABORATORY METHODS

3.4.1 Introduction to polymerase chain reactions

The PCR is a well-established laboratory technique used in a vast array of medical research to amplify pre-specified segments of DNA to facilitate further analyses. The reaction is performed in a stable buffer solution to which DNA from a study sample is added together with DNA primers (designed to bind to the specific DNA sequences of interest), raw nucleotides to form the DNA copies and a thermal stable polymerase enzyme to synthesize new strands.

The reaction is performed in repetitive temperature cycles, each with a step-wise change of solution temperature (*Lorenz 2012*). It starts with a holding stage in which the temperature is raised (94-95◦C for 5-10 minutes) to activate the polymerase and separate the double-stranded DNA helix into two single strands due to breakage of the hydrogen bonds (denaturation). After subsequent cooling, the added primers bind to the specific DNA regions of interest (annealing). In the third step, the temperature is raised again to > 70◦C. This allows the DNA polymerase to bind to the single-strands at location of the primers and synthesize new DNA products using the single strand as template (elongation). The temperature cycles are repeated until a sufficient number of DNA copies have been formed.

In TaqMan real-time PCR, the amplified PCR products are quantified by measuring fluorescence emitted by reporter molecules. The result is presented as a cycle threshold (Ct), representing the level of detection at which the reaction reaches a fluorescence intensity above background levels. The Ct can then be used to calculate the quantity of copies produced, often in relation to a reference gene, which acts as the baseline of gene expression in the analyzed sample.

3.4.2 Chimerism analyses

In paper III, samples from peripheral blood and/or BM were used for chimerism analyses, primarily for whole blood chimerism and split chimerism analyses, respectively. Genomic DNA from applicable samples was extracted using standardized kits, and analyses were performed with real-time PCR based on single nucleotide polymorphisms (*Alizadeh et al. 2002*). Hematopoietic cell lineages analyzed by split chimerism were T cells (CD3+), B cells (CD19+), myeloid cells (CD33+), and hematopoietic precursors (CD34+; applicable to BM samples), by methods described previously (*Mattsson et al. 2001b*).

3.4.3 TREC and KREC quantification

In paper IV, a TaqMan real-time PCR method was used to quantify lymphocyte excision circles from previously collected blood samples. Using DNA extraction kits, genomic DNA was obtained from thawed CD3+ and CD19+ sorted samples (for TREC and KREC analyses, respectively).

Using specific primers directed towards the δRec-ψJα signal joint TREC and the joint recombination signal sequence intron κ-deleting element (i.e. KREC), PCR products were quantified separately by real-time PCR. The PCR was run as a multiplex in which both TREC (or KREC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified in the same reaction. Primer and probe sequences are listed below (Table 3).

The PCR amplification was performed on a real-time PCR system with a cycling program of 1 × (95°C for 10 min), 40 × (95°C for 15 sec, 60°C for 1 min). Levels of excision circles were calculated by the ΔCt method using the ratio between amplified TREC or KREC copies and the housekeeping gene GAPDH (TREC/GAPDH and KREC/GAPDH, respectively) by reactions and methods previously published (*Sottini et al. 2010, Sairafi et al. 2012*). The quantity of lymphocyte excision circles was then calculated using the formula $2^{(\Delta Ct)}$, where ΔCt = Ct GAPDH - Ct TREC (or ΔCt = Ct GAPDH - Ct KREC).

3.4.4 Analyses of telomere length

In paper IV, relative average telomere length was measured by a SYBR green PCR-based method using a specific telomere assay (primers and probes are listed in Table 3). Telomere DNA repeats were amplified by PCR, and the telomere amplification product (T) was set in relation to the single-copy gene β-globulin (S) according to methods described previously (T/S ratio; telomere repeat copies to single gene copies) (*McGrath et al. 2007*).

Primers/Probes		Sequence
sjTREC	Forward	5'-CAC ATC CCT TTC AAC CAT GCT-3'
	Reverse	5'-GCC AGC TGC AGG GTT TAG G-3'
	Probe	5'-FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT-TAMRA-3'
sjKREC	Forward	5'-TCC CTT AGT GGC ATT ATT TGT ATC ACT-3'
	Reverse	5'-AGG AGC CAG CTC TTA CCC TAG AGT-3'
	Probe	5'-FAM-TCT GCA CGG GCA GCA GGT TGG-TAMRA-3'
GAPDH	Forward	5'-GGA CTG AGG CTC CCA CCT TT-3'
	Reverse	5'-GCA TGG ACT GTG GTC TGC AA-3'
	Probe	5'-VIC-CAT CCA AGA CTG GCT CCT CCC TGC-3'
Telomere (Tel-1b)	Forward	5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3'
Telomere (Tel-2b)	Reverse	5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3'
Hbg 1	Forward	5'-GCT TCT GAC ACA ACT GTG TTC ACT AGC-3'
Hbg 2	Reverse	5'-CAC CAA CTT CAT CCA CGT TCA CC-3'

Abbreviations: sjTREC, signal joint T cell receptor excision circle; sjKREC, signal joint kappa-deleting recombination excision circle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hbg, human β-globulin.

Table 3: Primers and probes used for TREC, KREC and telomere quantification (paper IV).

3.5 STATISTICS

Models of biostatistics applied in allogeneic HSCT research are often used to mathematically describe and/or compare relations between different pre-defined events of interest in the study (e.g. engraftment, development of acute or chronic GVHD, relapse or death) and the time from the beginning of an observation period (e.g. start of conditioning or graft infusion) to either (i) the event studied, (ii) the end of the study period or (iii) censoring of the studied patient. Censoring is said to present in a study/trial when there is incomplete information about a study participant, observation or value of a studied measurement. In clinical research it is applicable when the studied event of interest does not happen while the patient is being monitored, or because the subject drop out of the trial. In practice, this means that nothing is known about that subject after the time-point of censoring.

Different events can compete with each other (e.g. TRM can compete with relapse), which is adjusted for by using different competing risk models (*Campbell et al. 2007*). The probabilities of the events were calculated using the cumulative incidence method (*Gooley et al. 1999*). Categorical parameters were compared using the chi-square test and continuous variables were compared using the Mann-Whitney U test (*Campbell et al. 2007*).

To describe the survival times of members in assigned study groups, Kaplan-Meier curves were used, and the probability of OS was calculated using the Kaplan-Meier estimator (*Klein et al. 2003*). The Log-rank test was used to compare survival times between different groups, whereas the Cox proportional hazards regression model was used to describe the effect of categorical or quantitative variables on OS (*Klein et al. 2001b, Klein et al. 2001a*).

Multivariate analyses were performed to study if possible prognostic variables were relevant for outcome in a specific patient cohort, analyzed with proportional hazard regression models (e.g. the Cox regression model, (*Klein et al. 2001a*)). Analyses of predictive factors for relapse, TRM and GVHD were performed using a proportional sub-distribution hazard regression model (*Fine et al. 1999*). Results generated are expressed in the enclosed papers as hazard ratios (HR) or odds ratios (OR) together with the 95% confidence interval.

For applicable statistical analyses, p-values were calculated to represent a measurement of how well the defined null hypotheses matched the specific tests and data. All calculated p-values were 2-tailed.

Statistical analyses were performed using the cmprsk package developed by Gray (*Gray 2001*), the Splus 6.2 software (Insightful, Seattle, WA, US), the Statistica 12 software (StatSoft, Tulsa, OK, US), the SAS 9.3 (Cary, NC, US) and SPSS Statistics (IBM, Armonk, NY, US).

The specific statistical methods and definitions used in each study in this thesis are described in additional detail in the Statistics-sections of each enclosed paper (I-IV).

4 RESULTS AND DISCUSSION

4.1 EVALUATION OF PRE-TRANSPLANTATION INDICES IN AN ALLOGENEIC HSCT SINGLE-CENTER COHORT (PAPER I)

4.1.1 Rational for studying the HCT-CI and DRI on the single-center level

As briefly reviewed in the introduction to this thesis, it has been shown that outcome after allogeneic HSCT is influenced by the patient's comorbidity burden and the specific hematologic disease and stage at time of pre-transplantation assessment.

The impact of comorbidities in patients with hematologic malignancies is related to the diverse physiologic burdens of the cancer itself, but also includes combinatory effects of previous chronic disease as well as damage inflicted by cancer treatment(s) (*Yancik et al. 2001*). The interactions between the disease and different comorbidities can vary, depending on the type and grade of organ involvement. Accordingly, the purpose of comorbidity indices is to function as applicable tools to rate the impact of different comorbidities in relation to a specific disease or treatment. As described in the introduction, Sorror et al used patient data to develop the HCT-CI in 2005 to specifically assess the impact of patient comorbidities in allogeneic HSCT (*Sorror et al. 2005*). The same research group published a subsequent prospective study in 2015, involving 8,115 allogeneic HSCT recipients in the CIBMTR registry, confirming the HCT-CI's usability and predictability of TRM (*Sorror et al. 2015*).

In 2012, Armand and colleagues developed the DRI to estimate transplantation outcome from pre-existing data regarding the patients' hematological diseases (*Armand et al. 2012a*). The DRI category was significantly associated with OS, progression-free survival, and cumulative incidence of relapse (independently of comorbidity burden or intensity of conditioning). In 2015, Lim et al confirmed the index to be a robust way to stratify survival and relapse after transplantation, but concluded that it should be evaluated and calibrated with local data before implementation due to possible differences driven by transplantation-center effects (e.g. patient selection criteria for allogeneic HSCT) (*Lim et al. 2015*).

To learn how to interpret, and to validate the predictive capacity of these increasingly used pre-transplantation indices in our center-specific patient cohort, we performed a retrospective study of 521 consecutive adult allogeneic HSCT-patients who underwent transplantation for hematological malignancy in the years 2000-2012 (*Torlen et al. 2017*). The purpose was primarily to investigate the indices capacity to predict OS and TRM.

This type of retrospective single-center analyses can be valuable in several aspects. Aside from serving as a quality control, they can identify improvable factors in local medical practice when results are compared to international data. Results from other studies cannot always be extrapolated to the

single-center level, not the least since differences in allogeneic HSCT practices and outcome exist between centers, despite having superficially similar transplantation protocols (*Ruutu et al. 1997, Frassoni et al. 2000*).

Individual patient data included in pre-transplantation indices is not the only factor that determines treatment outcome. It undoubtedly interacts with other patient and transplantation variables such as treatment protocols, common practices at different centers, and individual patient and donor characteristics. Consequently, it is difficult to apply these findings at the individual level without proper validation. Nevertheless, the patient has the right to receive proper counseling prior to an allogeneic HSCT decision, including risk-assessments of disease and patient's specific characteristics.

4.1.2 Predictive value of the HCT-CI

In paper I, included patients were categorized into the low (38%), intermediate (28%) or high (34%) risk group of the HCT-CI as described previously. The distribution of patients to each risk group was essentially the same as reported in other studies. Based on this partitioning, a significant difference in 5-year OS could only be demonstrated between the low and high HCT-CI risk groups ($p < .01$) and no significant difference in 5-year TRM between the risk groups could be detected ($p = .17$, Figure 16).

The only HCT-CI factor with a specific effect on 5-year OS in our study was "severe pulmonary comorbidity" (HR 1.81, $p < .001$). This implies that extensive pulmonary comorbidities shall be carefully considered during the pre-transplantation assessment of possible allogeneic HSCT candidates. The lungs represent one of the organs most frequently targeted by pathogens, and their role as the organ of gas exchange (and target of chronic GVHD (*Hildebrandt et al. 2011*)) makes their function critical under and after transplantation.

4.1.3 Predictive value of the DRI

In the patient cohort presented in paper I, the 5-year OS in patients classified to the very high DRI risk group ($n = 13$) was significantly poorer ($p < .001$). Even though the high and very high-risk groups were merged in statistical analyses (due to a small number allocated to the very high-risk group), DRI risk category significantly affected 5-year OS ($p = .004$, Figure 17) and RFS ($p = .003$). As expected, based on the construction of the DRI, the majority of deaths were due to relapse. No significant difference in TRM could be detected ($p = .54$, Figure 17), probably because the DRI primarily is a determinant of relapse and should not primarily predict TRM.

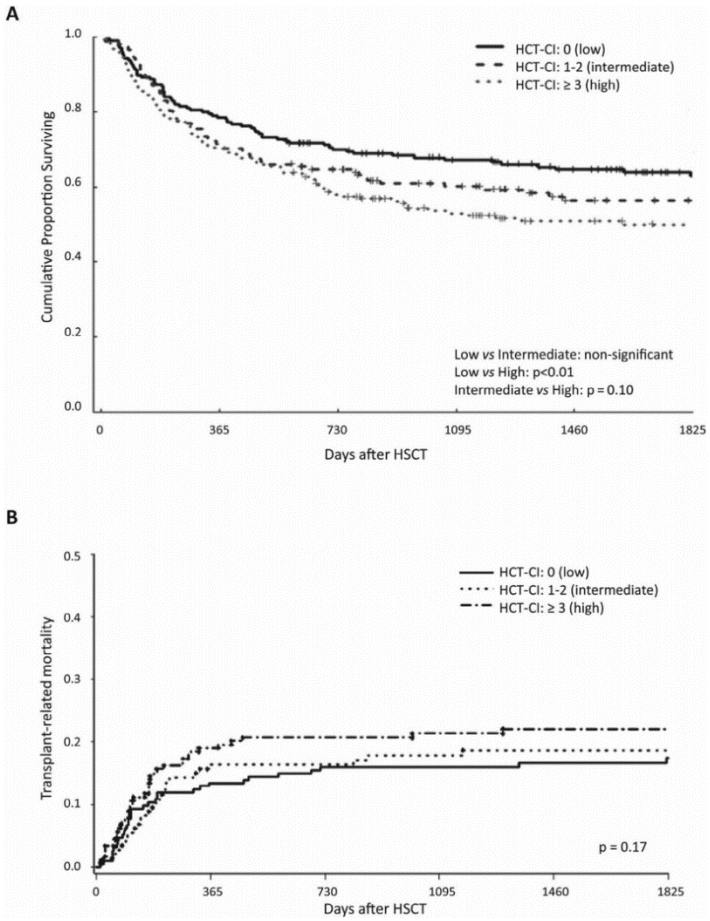


Figure 16: Allogeneic HSCT outcome in HCT-CI risk groups (paper I). (A) Shows OS, (B) shows TRM.

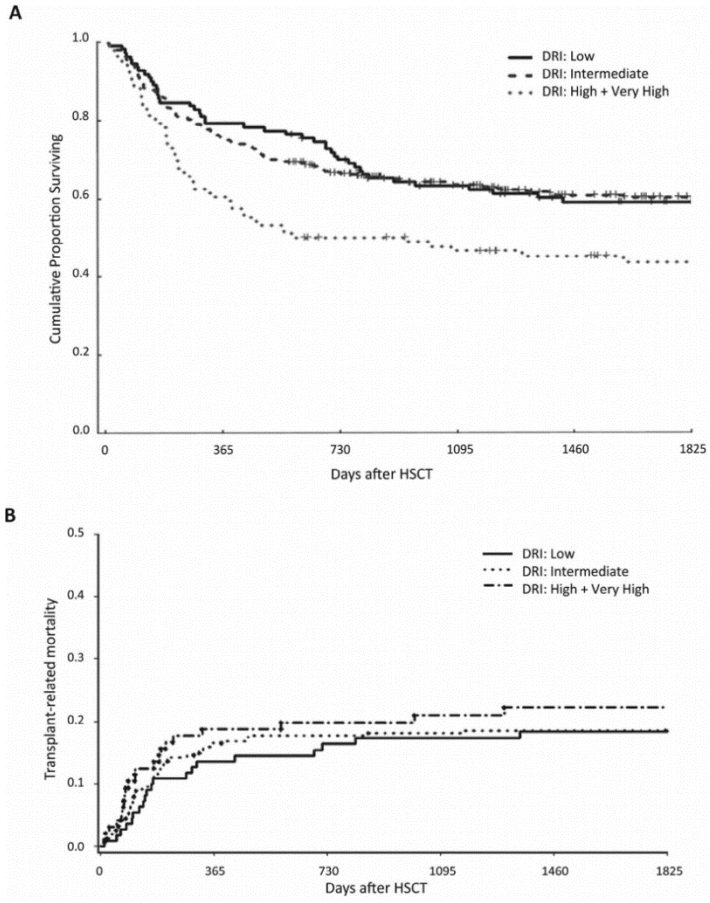


Figure 17: Allogeneic HSCT outcome in DRI risk groups (paper I). (A) Shows OS, (B) shows TRM.

4.1.4 Factors affecting OS in paper I

Aside from patient categorization to the merged high- and very-high risk group of DRI ($p = .01$), the multivariate analyses for mortality in paper I identified older age ($p < .001$), TBI conditioning ≥ 6 Gy ($p < .01$), administration of ATG ($p = .015$) and mismatched donor grafts ($p = .02$) as predictors of worse 5-year OS.

4.1.5 Comments on study results for HCT-CI and DRI

Since the first reports of successful results after allogeneic HSCT, the field has been under continuous development to improve patient outcome, driven by intensive research to improve clinical procedures. As a natural consequence, year of transplantation is significantly associated with patient outcome after treatment, with improved patient outcomes in more recent time-periods (*Gooley et al. 2010, Remberger et al. 2011*). In paper I, we analyzed patients who underwent allogeneic HSCT during the period of 2000-2012. The seminal works of HCT-CI and DRI were performed on patient cohorts transplanted during earlier periods (1997-2003 and 2000-2009, respectively). This fact may to some extent explain why HCT-CI had lower impact on TRM in our study compared with data from the original work.

Selection bias may be another explanation. Patients assessed for allogeneic HSCT at our center after publication of the original HCT-CI and DRI reports and classified to the highest HCT-CI and DRI risk groups may have been considered unsuitable for transplantation due to an increased risk of TRM or relapse (on reasonable terms). At the same time, knowledge about comorbidity impact has also made it possible to adjust and individualize allogeneic HSCT treatment protocols (and subsequent follow-up) to reduce, or at least limit, the effect of known comorbidities or relapse risks by proper intervention. Such action can include increased frequencies of tests during follow-up for selected patients to monitor lung function closely, or to individualize the dose of busulphan in applicable conditioning protocols using therapeutic drug monitoring to avoid excess liver damage (currently done for all allogeneic HSCT-patients at the Karolinska University Hospital (*Sandstrom et al. 2001*)).

Increased possibilities to follow patient-specific minimal residual disease (MRD) markers (e.g. based on high-throughput sequencing) can also speed up decisions for faster tapering of immune suppression and/or DLI-treatment with the opportunity to reduce impending relapse risks. Individualized treatment after allogeneic HSCT to prevent relapse in high-risk patients has been evaluated in different transplantation settings (e.g. FLT3 inhibitors in AML (*Larrosa-Garcia et al. 2017*), TKI treatment in Philadelphia-positive ALL (*Giebel et al. 2016*) or preemptive DLI (*Tan et al. 2014*)).

Another possibility is that patients with high HCT-CI scores received RIC regimens more frequently during the later years covered in the study, to reduce the risk of toxicity and subsequent TRM (*Ringden et al. 2013*). Such individualization of allogeneic HSCT therapy may have resulted in a TRM similar to that of lower-scoring patients receiving MAC. Less predictive power of the HCT-CI (and the EBMT-score) in such subgroups of patients have been reported previously, since some comorbidities are less strongly associated with mortality after RIC than after MAC (*Gratwohl et al. 2009, Barba et al. 2010b, Barba et al. 2014*). Indications of an attenuated predictive power of the established pre-transplantation risk indices emphasize that proper prediction of TRM requires a continued reassessment of risk scores in specific patient cohorts. One example, based on this knowledge, is the integrated index developed by the Acute Leukemia Working Party (ALWP) of the EBMT, created from the HCT-CI and EBMT risk scores, with increased predictive power in RIC HSCT (*Versluis et al. 2015*).

An additional explanation for the lower impact of HCT-CI on TRM in paper I can be limitations in study design. Study-patients were scored retrospectively based on data recorded in available medical charts prior to their admission for transplantation. These records were not designed to state a specific score for each HCT-CI comorbidity (the seminal HCT-CI paper was published in 2005 (*Sorror et al. 2005*)). Hence, potentially important comorbidity data might not have been available, underestimating the HCT-CI score for some patients (i.e. no score assessed for a certain HCT-CI category despite an existing, but not documented/retrievable, comorbidity). These patients could have died in TRM events after transplantation, consequently reducing the possibility to find a difference in TRM between the HCT-CI risk groups in the study.

It is also important to emphasize that the pre-transplantation indices discussed in paper I are not designed to be compared head-to-head. They rather function as complement to each other since they are designed to predict different outcomes (primarily TRM vs. relapse, even though both eventually predict OS). By evaluating HCT-CI and DRI in local transplantation data and adjusting allogeneic HSCT procedures accordingly, it might be possible to rule out differences in outcome between patient risk groups (at least regarding TRM). Additionally, these indices can be used to compare groups in clinical trials and to adjust outcome analyses.

4.1.6 Other risk scores used in allogeneic HSCT

Paper I was designed to specifically evaluate the HCT-CI and DRI. For the discussion of this thesis, it can be important to clarify that these are not the only indices used to predict outcome after allogeneic HSCT. The EBMT and the European Leukemia Net risk score combine five factors to predict OS and TRM (age, disease stage, time between diagnosis and transplantation, donor type and donor-recipient sex combination) (*Gratwohl et al. 2009, Gratwohl 2012*). In summary, outcome is worse for older

patients with advanced disease stage transplanted > 12 months after diagnosis with mismatched donors.

The updated Pre-transplantation Assessment of Mortality (PAM) score estimates the probability of survival at 2 years after allogeneic MAC HSCT. The score is valid for hematologic malignancies and is composed of parameters of age, donor relationship, disease/stage, FEV1 and CMV serology match between donor and recipient (*Au et al. 2015*).

Since the EBMT risk score and PAM score add some additional parameters of importance for outcome (not considered in the HCT-CI or DRI), they may be a relevant complement to the HCT-CI and DRI in clinical practice. Another supplement is the assessment tools developed for specific hematological malignancies (e.g. the WHO classification-based prognostic scoring system for survival in MDS (*Malcovati et al. 2007*) or the AML-Composite Model (*Sorror et al. 2017*) to estimate risk of mortality). Nevertheless, regardless of the indices used at pre-transplantation assessment, it is of importance to be familiar with their composition and impact in order to apply and interpret them correctly in clinical practice, not the least when used on the individual patient level.

4.2 INVESTIGATIONS OF OPTIMAL CD34+ CELL DOSE IN RIC HSCT TO IMPROVE OUTCOME (PAPER II)

4.2.1 Background and outcomes related to CD34+ cell dose

In previous parts of this thesis it has been shown that a high comorbidity burden and/or older age at time of allogeneic HSCT can result in worse patient outcome after treatment. Accordingly, RIC and non-MAC regimens are increasingly used for these patients today to alleviate some of the excess mortality risks. Since these protocols rely more on GVL effects after allogeneic HSCT than anti-leukemic effects of chemotherapy or radiation prior to transplantation, they may limit the toxicity to which the patient is exposed.

In clinical allogeneic HSCT protocols, HSCs remain identified as CD34+ cells in transplanted grafts (even if a vast array of additional markers have been identified). An adequate CD34+ count is important to achieve sustained engraftment after transplantation, and several reports have shown that CD34+ cell dose is of importance for transplantation outcome using MAC HSCT protocols. However, the impact of CD34+ cell dose in RIC HSCT has not been investigated to the same extent.

The predominant graft source for RIC HSCT is PBSCs. As mentioned previously, higher cell numbers shorten the phase of aplasia and decrease the risk of rejection or graft failure, and PBSC grafts contain approximately 5 to 10 times more CD34+ cells than BM grafts (*Remberger et al. 2001*).

According to the different prerequisites above, we sought to investigate the optimal CD34+ cell dose for RIC regimens in paper II (*Torlen et al. 2014*). To achieve a higher number of patients in the study, the project was proposed to, and completed in close co-operation with, the CIBMTR in the US. The retrospective study included 1,054 adults (45 to 75 years old) who underwent RIC HSCT during 2002-2011. To obtain a more homogenous patient group, the study population was limited to patients diagnosed with AML or MDS, the most common indications for allogeneic HSCT for older patients.

The results from multivariate analyses showed PBSC grafts from HLA-matched siblings containing $< 4 \times 10^6$ CD34+ cells/kg recipient were associated with higher NRM (HR 2.03, p = .004, Figure 18), overall mortality (HR 1.48, p = .008), and lower neutrophil and platelet recovery (p = .03). Similarly, grafts from unrelated donors containing $< 6 \times 10^6$ CD34+ cells/kg recipient were associated with higher NRM (HR 1.38, p = .02, Figure 18) and overall mortality (HR 1.20, p = .05).

Contrary to cell dose publications from MAC HSCT, the CD34+ cell dose was not significantly related to relapse rate or GVHD grades II-IV, and no upper cell dose limits associated with adverse outcomes was identified in the material (with either donor type).

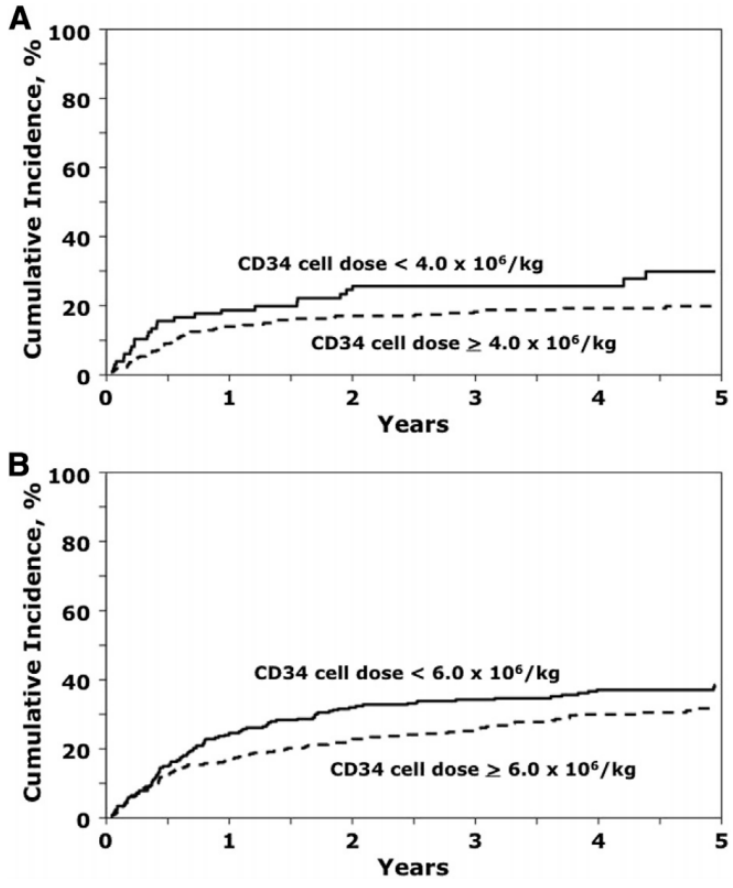


Figure 18: The incidence of non-relapse mortality after allogeneic PBSCT (paper II).
(A) HLA-identical siblings, (B) Unrelated donors.

4.2.2 CD34+ cell dose in RIC HSCT for adult patients

An important finding in paper II was that higher CD34+ cell doses predicted better survival in RIC PBSCT. This was valid for both HLA-matched and unrelated donor HSCT, but the threshold was lower for HLA-identical siblings. To some extent, these differences may be attributed to the level of HLA-disparity between the donor and the recipient. A single HLA locus mismatch (at HLA-A, -B, -C, or -DRB1 allele level) was associated with higher mortality, but this effect was independent of CD34+ cell dose.

At about the same time, I participated in a local project evaluating the effect of CD34+ cell dose and total nucleated cell (TNC) dose on outcome after allogeneic HSCT at our transplant center at Karolinska University Hospital in Stockholm (*Remberger et al. 2015*). In that paper, we studied 544 consecutive patients with hematological malignancies transplanted between 2000 and 2011. Patients with either BM or PBSC grafts from an HLA-identical sibling or an HLA-A, -B, and -DR matched URD were included. In this more heterogenic patient cohort, patients receiving very high CD34+ cell doses in PBSC grafts ($\geq 11 \times 10^6$ /kg recipient) had decreased survival rates and increased relapse. In summary, the study concluded that in PBSCT the CD34+ cell dose should be kept in the interval $2.5 – 11 \times 10^6$ /kg recipient. Lower ($< 2.5 \times 10^6$ /kg) or higher ($> 11 \times 10^6$ /kg) CD34+ cell doses were associated with worse OS ($p = .001$). Higher doses increased the incidence of relapse ($p = .02$), maybe because of a simultaneously high regulatory T cell content in these grafts, possibly down-regulating GVL effects. This theory could not be verified, but was to some extent supported by the finding that CD34+ cell dose in the study was not significantly associated to the incidence of chronic GVHD.

Taking these studies together and set in relation to the numerous cell dose studies present in allogeneic HSCT literature, it seems reasonable that CD34+ cell doses can be used in a broad but limited interval as long as doses exceed 4×10^6 CD34+ cells/kg recipient or 6×10^6 CD34+ cells/kg recipient for HLA-identical sibling transplants and unrelated donor transplants, respectively (at least for RIC HSCT). These doses should be enough for proper engraftment and safe transplants. Accordingly, an additional day of donor PBSC apheresis can be recommended when the CD34+ cell yield is below these levels after the first day of harvest, even if some studies have shown acceptable allogeneic HSCT results with PBSC graft cell doses of 1-2 $\times 10^6$ CD34+ cells/kg recipient (*Yamamoto et al. 2018*).

A retrospective ALWP-EBMT study recently indicated that the highest quartile in CD34+ cells (and CD3+ cells) associated with poorer outcomes after RIC PBSCT (*Czerw et al. 2016*). Hence, avoiding higher numbers of CD34+ cells might be reasonable. It is probably more beneficial to the patient if parts of the grafts are removed and frozen for later use. In such scenarios, it can be used as future CD34+ cell boost treatment in allogeneic HSCT-patients with poor graft function or as DLI in threatening relapse.

Discussing RIC HSCT in older patients with AML, it is also of interest to mention the prospective multi-center study by Brune et al comparing RIC HSCT with no transplantation in adults 50 to 70 years old with intermediate or high risk AML (*Brune et al. 2018*). At present, only preliminary data have been communicated, but future results may add valuable input to the discussion of allogeneic HSCT in older AML patients.

4.3 A PROSPECTIVE EVALUATION OF TWO IMMUNOSUPPRESSIVE REGIMENS AFTER ALLOGENEIC HSCT (PAPER III)

4.3.1 Rational for the clinical trial of GVHD prophylaxis regimens

As briefly reviewed in the introduction in this thesis, GVHD remains a most challenging complication to allogeneic HSCT. Data varies in different studies, but approximately 30–70% of allogeneic transplantation recipients will develop acute GVHD, contributing significantly to morbidity and high mortality rates after treatment. Approximately 15% of patients develop grades III-IV acute GVHD, and according to the latest publication of the CIBMTR summary slides, acute GVHD currently accounts for 8-11% of deaths within 100 days after transplantation (*D'Souza et al. 2019*). To further complicate the issue of acute GVHD, highly effective treatments to combat disease are lacking; high-dose steroid administration remains unsatisfactory, with 30–50% of the patients being steroid-resistant or dependent.

Since the randomized trials conducted by Storb et al in the 1980s (*Storb et al. 1986*), the regimens based on a calcineurin inhibitor (cyclosporine A or tacrolimus) in combination with intermittent doses of the folate antagonist methotrexate has been the standard GVHD prophylaxis used after allogeneic HSCT. European centers tend to use CsA/Mtx, while American centers primarily use Tac/Mtx (*Apperley et al. 2012*).

Due to the unmet need of improved GVHD prophylaxis, the promising properties of the mTOR inhibitor sirolimus (partly reviewed in the introduction of this thesis) was of interest to the allogeneic HSCT field after its discovery and isolation in 1972. It was first isolated as an antifungal agent, but additional investigations showed advantageous immunosuppressive capacities (*Sehgal 2003*). In contrast to the calcineurin inhibitors, sirolimus more potently suppressed expansion of conventional T cells than regulatory T cells. Based on these findings, Cutler et al introduced the novel combination of Tac/Sir (without methotrexate) as GVHD prophylaxis after allogeneic HSCT (*Cutler et al. 2004*). To determine whether the Tac/Sir combination would result in more favorable outcomes compared to the established GVHD prophylaxis with CsA/Mtx, a prospective randomized trial was designed to compare the regimens head-to-head (paper III) (*Torlen et al. 2016*).

4.3.2 Discussion of the results from the CsA/Mtx vs. Tac/Sir clinical trial

4.3.2.1 GVHD outcomes

Intention-to-treat analyses of the primary endpoint did not detect any significant difference in the cumulative incidence of grades II-IV acute GVHD between the two prophylaxis groups (51% in Tac/Sir-patients vs. 41% in CsA/Mtx-patients, p = .19, Figure 19). Multivariate analysis identified malignant diagnoses (RR 9.39, p = .03) and female donor to male recipient transplants (RR 2.44, p = .02) as risk factors for grades II-IV acute GVHD. The incidence in Tac/Sir-patients was almost twice as high compared to data from a clinical trial by Cutler et al in 2014, which reported an incidence of grades II-IV GVHD in Tac/Sir-patients of 26%). One reason for this discrepancy was probably the clinical objective at our center to achieve a grade I (-II) acute GVHD in patients with malignant diagnoses to reduce the risk of relapse. Consequently, patients were kept in the lower range of the relatively wide drug concentration intervals for tacrolimus and sirolimus stated in the study protocol (Figure 14). In clinical practice, the approach was rather to reduce the incidence of more severe acute GVHD in the study-patients, which also can explain the low incidence of grades III-IV acute GVHD in the trial (7% in Tac/Sir-patients vs. 13% in CsA/Mtx-patients, p = .09).

Sirolimus was also tapered and discontinued earlier (median treatment duration was 68 days) both in comparison to the referenced trial by Cutler et al (tapering started > 100 days post-HSCT) and to a trial by Pidala et al, which reported prolonged sirolimus administration using Tac/Sir (≥ 1 year) to be associated with reduced risks of chronic GVHD (*Pidala et al. 2015*). In addition, a prospective trial in adults transplanted for sickle cell disease using non-MAC allogeneic HSCT and GVHD prophylaxis with sirolimus alone (≥ 1 year), showed a high cure rate and absence of GVHD or mortality during a median follow-up of 22 months (*Saraf et al. 2016*).

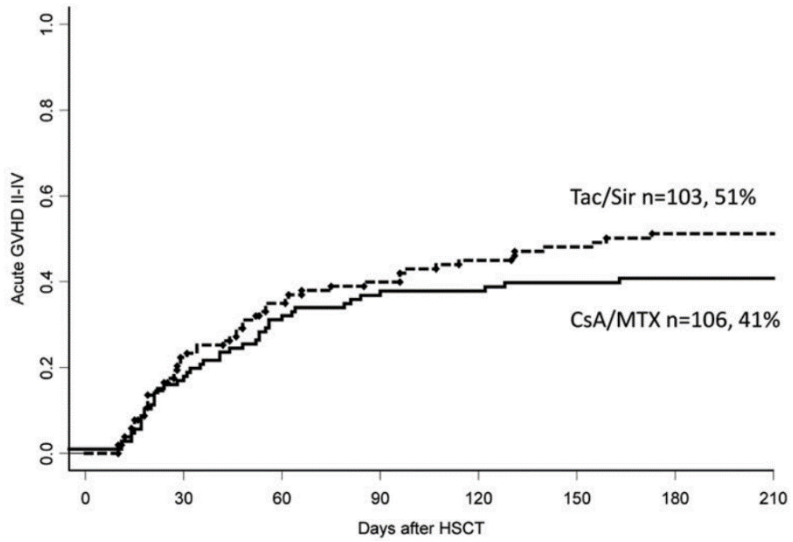


Figure 19: Cumulative incidence of grades II-IV acute GVHD in paper III (primary endpoint).

We could not identify a significant difference in any analyzed GVHD outcome between the treatment arms in the trial. If any minor difference did exist between the prophylactic regimens, it may have been equalized by the relatively frequent use of ATG administration prior to allogeneic HSCT in the trial. More than 70% of patients in both groups received ATG (4-8 mg per kg bodyweight) to reduce GVHD risk, with higher doses in patients with non-malignant diseases, in malignant diagnoses receiving MAC, and to recipients of HLA-mismatched grafts. This strategy possibly limited the detection of any beneficial effects between the groups (*Baron et al. 2017*).

4.3.2.2 Factors related to TRM and OS

No significant difference in TRM or OS between Tac/Sir-patients and CsA/Mtx-patients was detected in the trial (TRM: 12% vs. 18%, $p = .4$ and OS 71% vs. 72%, $p = .71$). Multivariate analysis identified older age ($p < .01$) and allele-mismatched grafts ($p < .01$) as significant risk factors for mortality, while RIC was detected as a protective factor against mortality ($p = .01$). The most frequent cause of death in both groups was relapse of malignant disease.

4.3.2.3 Comments on the VOD/SOS and TMA incidence

As reported in paper III, a suspected increase in incidence of hepatic veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS) and thrombotic microangiopathy (TMA) was noticed in recipients of Tac/Sir GVHD prophylaxis in the initial stage of the trial, primarily after previous conditioning with busulphan/cyclophosphamide. These cases resulted in a decision by the PI to stop further inclusion of patients scheduled for busulphan/cyclophosphamide conditioning into the trial. After that decision, no additional patient fulfilled strict VOD/SOS or TMA criteria.

Transplantation-related VOD/SOS has been a complication after allogeneic HSCT for decades, but the pathogenesis is not fully understood. The clinical symptoms of hepatic VOD/SOS (conjugated hyperbilirubinemia, hepatomegaly and weight gain due to fluid accumulation and ascites) are thought to be caused by sinusoidal obstruction in the liver, resulting from dysfunction of hepatic sinusoidal endothelial cells (*DeLeve et al. 2002, Mohty et al. 2016*). Cellular damage is probably caused by different interacting factors, initiated by toxicity from administered chemotherapy. This results in depletion of glutathione and nitric oxide, increased levels of vascular endothelial growth factor and inflammatory cytokines affecting the coagulation/fibrinolytic system (*Cheuk 2012*).

Onset of VOD/SOS have been associated with conditioning regimens based on cyclophosphamide combined with either TBI or busulfan (*Kalayoglu-Besisik et al. 2005*). In a prospective randomized study by Ringdén et al comparing busulphan with TBI conditioning, it was found that patients treated

with busulphan had a cumulative incidence of VOD/SOS of 12% compared to 1% in patients receiving TBI (p = .009) (*Ringden et al. 1994*). Hence, busulphan may be more potent in inflicting sinusoidal damage than TBI. Since busulphan has been identified as a risk factor for both VOD/SOS and TMA, therapeutic monitoring of busulphan concentrations was done for all study-patients, followed by continuous dose adjustments of busulphan (done for all allogeneic HSCT-patients receiving busulphan at the Karolinska University Hospital). This could have reduced the risk of excess busulphan toxicity (*Hassan et al. 2002, Malar et al. 2011*).

Nevertheless, a possible increase in VOD/SOS was noticed in Tac/Sir-patients receiving BuCy in the initial stage of the trial. Sirolimus may contribute to the described VOD/SOS processes through blocking of proliferative responses of sinusoidal endothelial cells to various important growth factors (*Khimani et al. 2018*). Endothelial toxicity has also been reported from *in vitro* studies of sirolimus in combination with tacrolimus (*Carmona et al. 2013*).

As a result of the increased use of RIC in allogeneic HSCT, in combination with prophylactic treatments to restore endothelial cell function and prevent thrombotic obstruction of sinusoids (e.g. administration of ursodiol), the cumulative incidence of VOD/SOS has dropped during the 2000s (*Carreras et al. 2011*).

According to the literature referenced above, the increased VOD/SOS rate in paper III may have been a result of additive effects from busulphan and cyclophosphamide toxicity, enhanced by tacrolimus and sirolimus administration. This reasoning may also explain the increased incidence of TMA in the same patient group, since endothelial damage by the same extrinsic factors can induce a cascade of aberrant complement activation and development of TMA (*Seaby et al. 2018*). However, Labrador et al. retrospectively analyzed TMA incidence in allogeneic HSCT recipients receiving GVHD prophylaxis with Tac/Sir (n = 68) or Tac/Mtx ± ATG (n = 34) and could not find any difference between the groups (7.4% vs. 8.8%; p = .8) (*Labrador et al. 2014*). Accordingly, TMA might primarily be an effect of high-dose busulphan-treatment, especially in combination with tacrolimus.

4.3.2.4 Other transplantation outcomes in paper III

Engraftment outcomes were similar between the Tac/Sir and CsA/Mtx group, except for a slightly delayed platelet engraftment in CsA/Mtx-patients (14 vs. 12 days; p < .01). No significant differences in incidence of oropharyngeal mucositis, number of CMV infections or time to full donor chimerism were detected between the studied groups in the trial. In conclusion, GVHD prophylaxis with Tac/Sir and CsA/Mtx provided comparable transplantation outcomes in patients after both matched related or unrelated allogeneic HSCT, but differences in toxicity may exist in certain transplantation settings.

4.4 STUDIES OF T AND B CELL RECONSTITUTION AFTER ALLOGENEIC HSCT (PAPER IV)

4.4.1 Rational for studies of TREC, KREC and telomere length after allogeneic HSCT

Previous sections in this thesis have to some extent discussed principles of conditioning and immunosuppressive protocols in allogeneic HSCT. Both are essential parts of the transplantation procedure, but their mechanisms of action constitute risk factors for long-term cellular and humoral immunodeficiency post-HSCT treatment. This is of importance since prolonged adaptive immunodeficiency after transplantation increases the risk of infections, disease relapse and secondary malignancies (*Mackall et al. 2009, van den Brink et al. 2015*).

To reconstitute a competent adaptive immune system after allogeneic HSCT, the lymphocyte pool needs to be restored with T and B cells capable of diverse antigen recognition. In general, lymphocyte reconstitution starts approximately 3–6 months after allogeneic HSCT. Two processes contribute to the reconstitution: peripheral expansion of naïve and memory cells transferred from the donor and *de novo* production from primary lymphoid organs. While expansion of mature T and B cells primarily provide a transient immune protection (as well as potential alloreactivity), the *de novo* production from HSCs is important for long-term protection and tolerance (*Lausen et al. 2004, Storek et al. 2008*). Accordingly, TREC and KREC levels have been utilized as proxy markers for lymphocyte reconstitution, with impact on outcome after allogeneic HSCT (*Corre et al. 2010, Mensen et al. 2013*).

Effective immune reconstitution in the transplantation recipient further requires a renewal capacity of hematopoietic cell lineages. As an effect, short lymphocyte telomeres (compared to their donors) have been observed in patients after allogeneic HSCT, especially in the first year after treatment (*Rufer et al. 2001*). In theory, this can lead to premature senescence of immune cells and related complications (*Baerlocher et al. 2009*). Notably, in a study of PBMCs from young and healthy donors by Welzl et al, shortening of telomeres *in vivo* was less prominent after incubation with sirolimus, compared to CsA and tacrolimus (*Welzl et al. 2014*).

According to this background and previously described laboratory methods, the aim of the project in paper IV (*Torlen et al. 2019*) was to assess TREC, KREC and telomere length in the two treatment arms from the randomized trial of GVHD prophylaxis (paper III) and to set data in relation to long-term outcomes after allogeneic HSCT.

4.4.2 Discussion of TREC and KREC levels and telomere length post-HSCT

At all assessed time-points after allogeneic HSCT, the median TREC and KREC levels and telomere length were not significantly different between patients in the two GVHD prophylaxis arms. Consequently, CsA/Mtx and Tac/Sir are probably interchangeable regimens in this respect, at least during the first months post-HSCT. However, as pointed out in the discussion of paper III, the median time of treatment with sirolimus was relatively short (68 days). Accordingly, any impact of the Tac/Sir regimen on lymphocyte excision circle at later time-points after allogeneic HSCT cannot be ruled out, but a Tac/Sir effect that would exceed or alleviate the significant impact of the other transplantation factors described are deemed unlikely.

From the initial patient samples taken at 2 months after HSC-infusion, there was a gradual increase in median levels of lymphocyte excision circles in the study population during follow-up. For KREC, higher frequencies were detected in samples taken at 12 months after transplantation compared with samples at 2 months ($p = .035$). For TREC, the increase was most evident when comparing levels in patient samples taken at 6 and at 12 months after transplantation ($p < .001$). This confirms results from other studies (*Sairafi et al. 2012, Mensen et al. 2013*) and coincides with the expected time-point for an increased thymus-dependent T cell development from naïve T cells in the host, as well as discontinuation of immunosuppression in the absence of GVHD. T cell neogenesis and output is also affected by factors impairing thymopoiesis, such as older age, and multivariate analyses identified patient age ≤ 40 years at time of transplantation to be associated with higher TREC levels at both 12 months (OR .96, $p < .001$) and 24 months (OR .92, $p < .0001$) post-HSCT.

In paper IV, lower TREC levels < 12 months after allogeneic HSCT were significantly associated to ATG-containing conditioning regimens ($p < .001$). This is most likely a result of T cell depleting effects through ATG-induced complement-dependent lysis and apoptosis, resulting in delayed reconstitution. This effect, together with the multifactorial properties of ATG on other parts of the immune system (including modulation of leukocyte/endothelium interactions, interference with DCs and induction of regulatory T cells (*Mohty 2007*)), calls for careful dose considerations when using ATG in allogeneic HSCT (*Remberger et al. 2004, Admiraal et al. 2017*). Its desirable potential to reduce risks for acute and chronic GVHD are close to unambiguous (*Arai et al. 2017*), but using high doses mechanistically may result in a slower or impaired reconstitution of adaptive immunity for a considerable period of time after allogeneic HSCT, which may increase the risk of severe infectious complications.

Compared with recipients of PBSC grafts, recipients of BM grafts had higher TREC-levels at 12 months ($p < .05$) and 24 months ($p < .01$) after transplantation. Since BM grafts have a more diverse hematopoietic cell composition and in general are associated to lower incidence of chronic GVHD post-HSCT, these grafts may aid restoration of an advantageous thymic microenvironment (i.e. a

thymus less affected by GVHD reactions) and enhance T cell reconstitution, especially if immuno-suppression can be reduced earlier after allogeneic HSCT. On the other hand, a confounding factor for the impact of graft source on immune reconstitution is recipient age. Of the patients in paper IV, only 3 patients < 20 years of age received PBSC grafts, meaning higher TREC levels in the BM HSCT cohort may be a surrogate marker for a preserved thymopoiesis in these patients.

TREC levels were not affected by development of acute GVHD during follow-up, but patients diagnosed with moderate/severe chronic GVHD had lower TREC levels at 12 months post-HSCT ($p < .05$). At the same time, grades II-IV acute GVHD was the only significant factor for lower KREC levels ≤ 6 months after transplantation ($p < .05$). It is known that hematopoietic dysfunction can be a manifestation of GVHD and that alloreactive effector cells can target class I and class II HLA proteins on hematopoietic cells in the host (*von Bonin et al. 2014*). Mensen et al have reported that delayed B cell recovery after allogeneic HSCT can be an effect of BM-infiltrating T cells during acute GVHD, and that cytokine release can impair donor-derived hematopoiesis due to a more toxic BM micro-environment (*Mensen et al. 2014*).

Telomere length decreased in study samples during follow-up, with significantly lower levels between 3 months and 24 months after transplantation ($p = .002$). This is confirmed in previous reports and is probably a result of the immense differentiation pressure on lymphocytes during repopulation of the host (*Akiyama et al. 2000*). Another finding was that female sex was correlated to reduced telomere length in multivariate analysis at 12 months (OR .43, $p < .05$). This can be a result of reduced telomerase activity, connected to insufficient estrogen-upregulation due to reduced hormone production in damaged granulosa cells after allogeneic HSCT (*Calado et al. 2009*). However, telomerase activity (or levels of sex hormones) was not measured in the study, so such an explanation cannot be verified in current study data. Nevertheless, it has been reported that onset of puberty can initiate thymic evolution and androgenic blockade to improve immune reconstitution have been discussed (*Leposavic et al. 2008*).

4.4.3 Lymphocyte excision circles in relation to survival outcomes

Analyses of data in study IV identified cut off levels of lymphocyte excision circles with impact on 5-year OS and TRM after allogeneic HSCT. Patients with TREC levels above median in study samples taken at 6 months after transplantation had lower TRM (3.1% vs. 15.4%, $p = .04$) and superior 5-year OS (84% vs. 68%, $p = .04$) compared to patients with lower levels (Figure 20). The same was observed in KREC data from samples taken 3 months after graft infusion; patients with KREC levels above median had lower TRM (8.5% vs. 21.7%, $p = .035$) and superior 5-year OS (81% vs. 60%, $p = .01$) compared to patients with lower levels (Figure 20).

Impaired lymphocyte reconstitution is a known risk factor for various (and more severe) infections after allogeneic HSCT. This was confirmed by a higher number of deaths due to infectious complications in the analyses of the dichotomized patient groups with lymphocyte excision circles below median during follow-up. The data must be confirmed in additional studies, and the findings must be considered in relation to other and more established outcome-related factors after transplantation. Nevertheless, it indicates that TREC and KREC assessment can be of value during follow-up after allogeneic HSCT. Interventions in selected patient groups may include a faster taper of immunosuppression in the absence of GVHD, or prolonged/enhanced infection prophylaxis during follow-up.

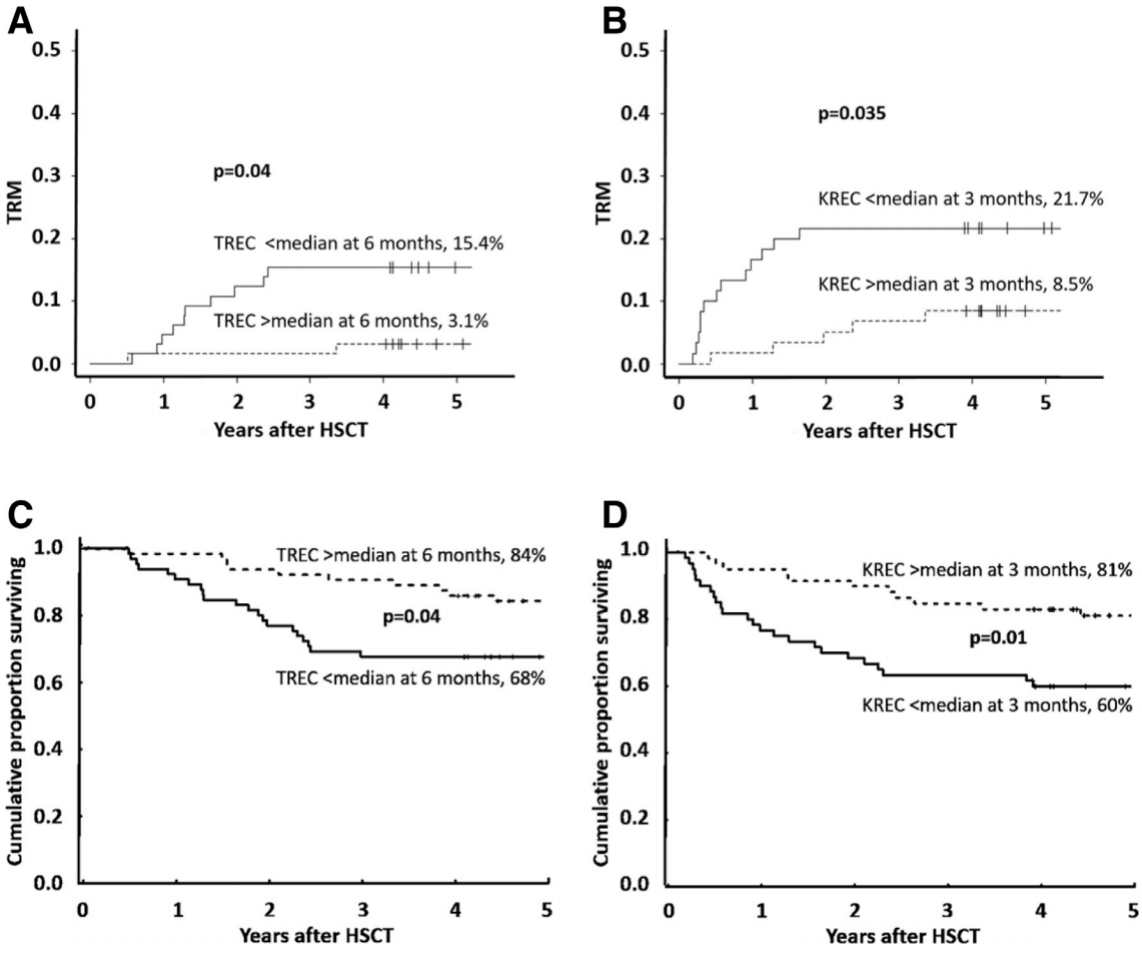


Figure 20: Impact of lymphocyte excision circle levels on allogeneic HSCT outcomes (paper IV).
(A) TREC levels above/below median, TRM. (B) KREC levels above/below median, TRM.
(C) TREC levels above/below median, OS. (D) KREC levels above/below median, OS.

4.4.4 Additional comments on KREC and TREC analyses in this thesis

In previous allogeneic HSCT research, TREC and KREC have been measured by different techniques, and expressed in different ways (e.g. TRECs per μl of blood [TREC number] or per cell [TREC content]). To compare data between studies, the same techniques for quantification must be used. In paper IV, we calculated TREC and KREC copies in relation to the house-keeping gene GAPDH analyzed in purified CD3+ and CD19+ cell samples, respectively. This method should improve accuracy, primarily since the calculated result is less affected by variations in cell concentrations or frequencies in the sample at time of collection/analysis.

It is important to remember that TREC levels are determined both by thymic output and by peripheral events (such as dilution) and that TREC-containing naive T cells are not recent thymic emigrants by definition (*Hazenberg et al. 2003*).

Despite some interesting findings in paper IV, it is probably premature to argue for the implementation of TREC or KREC analyzes as a routine follow-up after allogeneic HSCT. The results presented might rather serve as hypothesis generating and need to be confirmed in larger studies, preferably in prospective multi-center settings to compensate for center-driven effects.

It can also be stressed that lymphocyte reconstitution is influenced by multiple patient- and transplantation-related factors (some of them discussed in paper IV), and it is not possible to rely on single analysis models for prediction of outcome.

In relation to the other papers discussed in this thesis, it would also be of interest to study pre-transplantation levels of TREC and KREC in donors and recipients to investigate if levels prior to transplantation are important for immune reconstitution or allogeneic HSCT outcomes. If so, these analyses can be additional factors to consider as complement to current pre-transplantation indices used during patient assessment. Based on the results, preemptive actions may be taken to limit disadvantageous effects of impaired immune reconstitution.

5 CONCLUSIONS

5.1 GENERAL CONCLUSIONS FROM THIS THESIS (PAPER I-IV)

- Local validation of the pre-transplantation indices HCT-CI and DRI is recommended before making index-based decisions of allogeneic HSCT at the individual patient level.

- A higher HCT-CI score may be accepted in allogeneic HSCT patients receiving HLA-identical sibling grafts compared to patients receiving matched unrelated donor grafts.

- The CD34+ cell dose in grafts used for RIC PBSCT should exceed 4×10^6 /kg patient weight in HLA-identical sibling transplants and 6×10^6 /kg patient weight in unrelated donor transplants for older patients with AML or MDS.

- The CD34+ cell dose did not affect relapse or GVHD outcomes in HLA-identical sibling or unrelated donor RIC PBSCT for older patients with AML or MDS, and no upper cell dose limit was associated with adverse outcome.

- Tac/Sir is a valid GVHD prophylaxis regimen in allogeneic HSCT with comparable transplantation outcomes to CsA/Mtx.

- Type of GVHD prophylaxis (Tac/Sir or CsA/Mtx) did not affect levels of lymphocyte excision circles (TREC or KREC) after allogeneic HSCT, but treatment with ATG resulted in significantly lower TREC levels.

- Patients with TREC or KREC levels above median during follow-up had superior 5-year overall survival and lower transplantation-related mortality.

6 FUTURE PROSPECTS AND CONCLUDING REMARKS

6.1 NEW APPROACHES TO IMMUNOSUPPRESSION IN ALLOGENEIC HSCT

6.1.1 Preventive and therapeutic strategies in acute GVHD

Parts of this thesis have briefly described and, in some aspects, compared the most frequently used GVHD prophylactic agents in allogeneic HSCT today; primarily calcineurin inhibitors (cyclosporine and tacrolimus), the folate antagonist methotrexate, the mTOR inhibitor sirolimus and the pan T cell depleting reagent ATG (paper III and paper IV).

Despite combining these agents in various protocols, moderate to severe acute GVHD still affects approximately 20–50% of transplanted patients. In addition, modern treatment protocols for acute GVHD using glucocorticoids (*Mielcarek et al. 2015*) often remain unsatisfactory with around 30% of the treated patients being steroid-resistant or steroid-dependent over time. Hence, there is still an unmet need for improved GVHD prophylaxis and treatment in allogeneic HSCT.

Novel approaches with potential to prevent (or limit) acute GVHD in modern allogeneic HSCT include prevention of T cell migration to affected organs (e.g. by CCR5 chemokine blockade (*Reshef et al. 2012*)), or protection of the gastrointestinal tract by transplantation of fecal microbiota (*Kakihana et al. 2016*). Recent pharmacological strategies include the use of substances able to limit transcription of pro-inflammatory genes (e.g. by inhibition of kinase signaling pathways) or agents targeting other pro-inflammatory pathways, multiple cytokine receptors and/or intestinal stem cells (*Zeiser et al. 2017a*).

These, and other, emerging approaches need to be validated in prospective clinical trials. Still, there is hope that the use of both standard and experimental treatment options can be used to further individualize allogeneic HSCT protocols, potentially reducing the incidence and severity of acute GVHD in future patients, especially if combined with relevant biomarkers to enable early intervention (*Hartwell et al. 2017, Zeiser et al. 2017a*).

6.1.2 Haploidentical HSCT

The development of new or adjusted immunosuppressive strategies during the last decade have made it possible to design allogeneic HSCT protocols with the potential to overcome HLA disparities (beyond the use of UCB HSCT). This have led to an increase in the number of performed haploidentical HSCTs in the last years (*Passweg et al. 2015, D'Souza et al. 2019*). This is of interest for several reasons. If haploidentical HSCTs provide similar clinical outcomes as URD HSCTs in prospective trials, it will significantly increase the available donor pool, offering patients lacking an available

HLA-identical sibling or URD to be transplanted with a graft from a related haploidentical donor. Haploidentical transplants may also accelerate the transplantation process and make it more flexible by reducing the need for URD searches and graft transportation. It could also facilitate a more rapid access to future adoptive immune modulatory measures post-transplantation, which may require additional donor cells. Haploidentical HSCT may also make an allogeneic HSCT available to patients currently not having access to the treatment modality. Due to limited donor availability and high costs, allogeneic HSCT is still primarily accessible to patients in high-income industrialized countries (*Giebel et al. 2010*). Safe haploidentical HSCTs could make the method available to additional patient populations worldwide in need of an allogeneic transplantation. Another argument for this hypothesis is the assumed cost-effectiveness of haploidentical HSCT, recently evaluated in a single-center study reporting it to be more cost-effective compared with URD HSCT in older patients with hematological malignancies (*Debals-Gonthier et al. 2018*).

It is important to stress that it is currently not clear how to best perform a haploidentical HSCT, and no definitive standard has been defined. Several methods exist, such as T cell depletion with different techniques, post-transplantation cyclophosphamide and "mega immunosuppression", all reporting good and possibly better results than the use of mismatched URD (*Bethge et al. 2008, Lang et al. 2008, Luznik et al. 2008, Bacigalupo et al. 2015, Locatelli et al. 2017*). In a recent retrospective analysis from the ALWP of the EBMT, the risk of relapse was the same in haploidentical HSCT and HLA-identical sibling HSCT for acute leukemia (suggesting a similar GVL effect), but leukemia-free survival was superior in matched sibling donors (*Ringden et al. 2016*).

6.2 ENHANCEMENT OF THYMIC FUNCTION AFTER ALLOGENEIC HSCT

Paper II and paper IV in this thesis indicate a need to develop strategies to enhance thymic function after allogeneic HSCT, given the facts that transplantation is increasingly used in older patients and that the ability of endogenous thymic regeneration and lymphocyte output decline with age and repeated insults (e.g. several lines of previous cytotoxic treatment). To facilitate T cell reconstitution, exogenous strategies are discussed in the allogeneic HSCT literature.

One approach is administration of keratinocyte growth factor to protect thymic epithelial cells and enhance thymic regeneration and T cell output after transplantation, previously evaluated in autologous HSCT (*Wils et al. 2012*) and now investigated in allogeneic HSCT (NCT01746849). Other strategies include exogenous administration of recombinant human IL-7 to enhance thymopoiesis and recovery of T cell lineages (*Perales et al. 2012*) or sex steroid inhibition to enhance T cell reconstitution and promote immune recovery (by inhibition of luteinizing hormone and follicle stimulating hormone release, NCT01338987).

It is not known whether these suggested advances can be translated from preclinical studies and animal models into clinical allogeneic HSCT therapy, but they all strive to enhance post-transplantation immune reconstitution.

6.3 GRAFT CELL CONTENT, IMMUNE RECONSTITUTION AND OUTCOME

In recent years it has been shown that graft composition, e.g. CD34+ cell subpopulations or immune cell subsets (T cells, B cells, NK cells, DCs, etc.) influence immune recovery and outcome after allogeneic HSCT. Higher numbers of NKT cells (*Malard et al. 2016*) and γδT cells (*Perko et al. 2015, Radestad et al. 2019*) in the graft have been associated with favorable immune reconstitution and positive clinical outcome in different transplantation settings. Partly, this impact is probably an effect of their potential to control or limit GVHD processes. The impact of specific cell subsets in donor grafts and their relation to outcome has also been studied (*Svenberg et al. 2019*).

Detailed graft cell analyzes, and subsequent graft manipulation, completing the rather standardized CD34+ and total nucleated cell counts, may still be difficult to implement in clinical routine. However, in a near future more detailed cell subset "graft engineering" could provide custom-tailored solutions to further control immune reactions and enhance immune reconstitution.

6.4 ENDPOINTS IN CLINICAL ALLOGENEIC HSCT RESEARCH

The overall aim of this thesis was to study outcome-related factors in patients undergoing allogeneic HSCT. Overall survival has been the gold standard for efficacy in clinical studies for decades, and all papers enclosed (I-IV) have survival analyses included as assessed endpoints (primarily OS). This can be justified given the hematological diseases being treated, but in the last years OS has to some extent given way to the composite endpoint disease-free survival (DFS), commonly defined as being alive without relapse or disease progression after transplantation (*Booth et al. 2009*). The downside with both OS and DFS is that they disregard complications like severe acute or chronic GVHD, closely linked to patient morbidity and an impaired quality of life (*Kurosawa et al. 2017*). In a survey by Kim and Armand in 2013, several publications comparing transplantation modalities (type of donor, stem cell source, or conditioning intensity) showed major differences in the incidence of chronic GVHD between studied transplantation groups (*Kim et al. 2013*). Such heterogeneity may lead to biases in the estimates of endpoints and may obstruct adequate comparisons of allogeneic HSCT studies, which is notable since such comparisons are a major tool to improve transplantation procedures.

A composite endpoint that combines both DFS and GVHD occurrence is desirable, and the use of GVHD-free, relapse-free survival (GRFS) as outcome parameter in allogeneic HSCT has been

proposed (*Holtan et al. 2015*). The GRFS endpoint may more accurately describe the patients' objective and subjective health status after transplantation and hence function as a proxy marker for quality of life. In 2016, the refined GRFS was defined in an ALWP-EBMT report as being alive without grades III–IV acute GVHD, no severe chronic GVHD, nor disease relapse (*Ruggeri et al. 2016*). Representing an outcome close to an ideal allogeneic HSCT recovery, it can be considered a valid endpoint in allogeneic transplantation research (*Holtan et al. 2015, Ruggeri et al. 2016*). Accordingly, when designing future (prospective) clinical trials in the field of allogeneic HSCT, GRFS may be a more desirable endpoint than OS and DFS.

A parallel to the reasoning above can be drawn from pediatric oncology; historically, the primary aim was "survival at all costs", but given the discovered risks of severe late complications in survivors (related to previous cancer therapy), the focus shifted and is now rather "survival at the most reasonable price" (*Hedlund 2016*).

6.5 CONCLUDING REMARKS

Like numerous publications during the last decades, this thesis presents allogeneic HSCT as valid treatment option for a number of otherwise lethal hematopoietic disorders, immune deficiencies and inborn errors of metabolism. Despite the evident risks of severe complications with a transplantation procedure, including fatal outcomes and life-long impairments of quality of life, thousands of patients make the choice to expose themselves to what is often the last curable opinion for their otherwise terminal diagnosis.

With the knowledge of these prerequisites, it is important to offer an allogeneic HSCT procedure as safe and validated as possible, while continuously re-evaluating and adjusting treatment protocols according to new research and the steadily increasing clinical experiences harbored from the field.

Since the first tentative experiments in transplantation, enormous progress has been made. Continuous refinements of procedures during the last fifty years have successively improved patient outcome and determined allogeneic HSCT to be a promising nexus between three expanding areas of contemporary clinical research: stem cell therapies, immune-modulating techniques and the individualization of cancer therapeutics (*Jenq et al. 2010*). Given its current potential, it is important to strive towards the key principles of availability, accessibility, adequacy, affordability and appropriateness. These terms will undoubtedly be important in the future era of allogeneic HSCT, not the least when an increasing number of advanced immune therapies, associated with escalating costs, are implemented in modern allogeneic HSCT protocols (e.g. chimeric antigen-receptor T cells, immune checkpoint inhibitors, bi-specific T cell engagers, etc.). This important issues have also been raised in a petition from the EBMT in 2017 (*Mohty 2017-12-13*).

The combined conclusions of the projects in this thesis do not present or suggest any revolutionary changes to current protocols or strategies in allogeneic HSCT. Nevertheless, some of the presented findings can act as the basis for minor changes to current practices or future research hypotheses.

The thesis may also add strength to a common implicit opinion among transplantation physicians; to be able to perform a successful and safe allogeneic HSCT, it is of utmost importance to be as careful and accurate as possible in all preparatory steps preceding the start of conditioning therapy. “As you sow, so you may reap”. The importance of selecting the right patient, the best possible donor, the most appropriate conditioning regimen, the most adequate cell dose and a specific MRD-marker to follow post-HSCT (if possible) cannot be underestimated in the endeavor to further improve patients’ outcome after treatment.

The scientific endeavor to develop, adjust and individualize the allogeneic HSCT modality will undoubtedly continue for many years to come as long as it can hold its position as the most successful and reproducible immune therapy in medical practice. Everything else would be a deception to the patients (and to their families) who expose themselves to one of the most dangerous clinical treatments modern medicine has to offer to survive their life-threatening diseases.

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