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CLINICAL ASPECTS OF CHRONIC GRAFT-VERSUS-HOST DISEASE

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Clinical aspects of Chronic Graft-versus-Host Disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedicated to Ninva, Isabella, Melissa and Nino Afram.
Mom, Dad and Malki.

Thank you for supporting me throughout all the nonsense

“The pendulum of the mind oscillates between sense and nonsense, not between right and wrong”-Carl Gustav Jung
ABSTRACT

Chronic graft-versus-host disease (cGVHD) remains one of the most severe complications after allogeneic hematopoietic stem cell transplantation (HSCT), affecting both the quality of life and mortality of long-term survivors. Its impact on morbidity and mortality varies depending on the severity and number of organs involved, allowing classification into mild, moderate, and severe cGVHD according to the National Institute of Health criteria (NIH). Chronic GVHD is associated with a graft-versus-tumor effect (GVT) that decreases the risk of relapse after transplantation. Treatments involve potent immunosuppressive modalities with side effects including infections and possibly relapse of the underlying malignancy.

The aim of this thesis is to increase the knowledge of cGVHD with emphasis on early detection of risk and prognostic factors in order to allow a more vigilant management of the syndrome as well as the evaluation of extracorporeal photopheresis.

In paper I we performed a retrospective study with emphasis on risk factors for the development of cGVHD. We showed a significantly higher incidence of severe cGVHD in patients with sibling donors compared to unrelated donors (URD). Relapse and Transplant-related-mortality (TRM) were similar in both groups. However, TRM was significantly higher in patients with severe cGVHD. The main findings were that despite HSCTs with sibling donors showing higher incidence of cGVHD they resulted in significantly better 5-year overall survival (OS) and relapse-free survival (RFS) compared to patients with a URD. Paper II is a multi-centre retrospective analysis with the aim to determine early detectable risk factors for the development of cGVHD. We found that risk factors for severe cGVHD include female donor to male recipient, reduced intensity conditioning and older patient age.

In 2005 the NIH formed consensus criteria for the diagnosis of cGVHD. The new scoring system proved time-consuming and difficult to manage during a standard out-patient visit. In paper III we aimed to determine the prognostic impact of the new NIH score and also of the newly implemented sub-categories of cGVHD, namely overlap syndrome and delayed acute GVHD. Our aim was to develop a simplified score with similar prognostic impact as that of the NIH score. We could show that factors adversely affecting prognosis upon diagnosis of cGVHD include ECOG, platelet count and, if present, severe gut involvement. In fact, by only using the combination of ECOG and platelet count we could identify the same prognostic risk groups.

The most well-established second line treatment for steroid-refractory, -intolerant or – dependent cGVHD to date is extracorporeal photopheresis (ECP). In paper IV we could conclude that ECP was a safe and well-tolerated treatment. Patients with severe skin cGVHD had the best response in terms of complete or partial response.

To summarize, this thesis provides new data regarding risk and prognostic factors for cGVHD which has led to perhaps a more-user friendly prognostic tool upon diagnosis of cGVHD. The findings help us to decide on immunosuppression for URD and what patient group would benefit the most from ECP treatment.
**Sammanfattning på svenska**


Syftet med denna avhandling är att öka kunskapen om cGVHD med inriktning på tidig upptäckt av riskfaktorer och prognostiska faktorer för att möjliggöra en mer vaksam hantering av syndromet liksom utvärdering av en väletablerad andra linjens behandling.

I det **första arbetet** utförde vi en retrospektiv studie med tonvikt på riskfaktorer för utvecklingen av cGVHD. Vi kunde visa en signifikant högre förekomst av svår cGVHD hos patienter med syskogivare jämfört med matchade obesläktad givare (URD). Överlevnad (OS) och transplantationsrelaterad mortalitet (TRM) var jämförbar i båda grupperna, syskon och URD. Oavsett donator var TRM signifikant högre i gruppen med svår cGVHD. De viktigaste resultaten var att patienter med syskondonator resulterade i ett signifikant bättre 5-års OS och överlevnad i avsaknad av återfall (RFS) jämfört med patienter med URD. Vi har nu därför minskat intensiteten av IS i URD-gruppen. Det **andra arbetet** är en retrospektiv multicenter studie med syfte att bestämma tidigt detekterbara riskfaktorer för utvecklingen av cGVHD. Riskfaktorer för svår cGVHD inkluderar kvinnlig givare till manlig mottagare, konditionering med reducerad intensitet och äldre patienter.

Det **tredje arbetet** syftade till att bestämma prognostiska värdet av den nya NIH-klassificeringen och även de nyligen införda underkategorierna av cGVHD, nämligen överlappssyndrom och fördrojj akut GVHD. Vårt mål var att utveckla en förenklad klassificering av cGVHD. Vi kunde visa att faktorer som påverkar prognosen vid diagnos av cGVHD inkluderar ECOG (funktionsstatus), nivå av blodplättar och förekomst av svår tarm-GVHD. Enbart ECOG och nivå av blodplättar är tillräcklig för att identifiera patienter med sämre prognos. För att kunna utnyttja den sistnämnda kombinationen måste vi först verifiera våra resultat i en prospektiv studie.

Den mest väletablerade andra linjens behandling för cGVHD är Extracorporeal fotoferes (ECP). **Fjärde arbetet** syftade till att utvärdera effekten av ECP behandling på vår klinik ur ett retrospektivt perspektiv och till att bestämma vilken patientgrupp som har den bästa responsen. Vi kunde dra slutsatsen att ECP var en säker och väl tolererad behandling. Patienter med svår hud cGVHD hade den bästa responsen med fullständigt eller partiellt svar.

Sammanfattningsvis ger denna avhandling nya uppgifter om riskfaktorer för cGVHD. Det har lett till ett mer användarvänligt prognostiskt verktyg vid diagnos av cGVHD. Fynden hjälper oss att bedöma lämplig IS för URD och vilken patientgrupp som mest kommer att dra nytta av ECP-behandling.
LIST OF SCIENTIFIC PAPERS


CONTENTS

1 HEMATOPOIETIC STEM-CELL TRANSPLANTATION ..............................................9
  1.1 BACKGROUND ..........................................................................................9
  1.2 HLA and DONORS ...................................................................................10
  1.3 STEM CELL SOURCE ...............................................................................12
  1.4 HSCT INDICATIONS ...............................................................................13
  1.5 TRANSPLANTATION PROCEDURE ............................................................13
    1.5.1 GVHD PROPHYLAXIS ......................................................................14
  1.6 IMMUNE RECONSTITUTION AND INFECTIONS .........................................16
    1.6.1 INNATE IMMUNITY .........................................................................16
    1.6.2 ADAPTIVE IMMUNITY ...................................................................16
    1.6.3 INFECTIONS ....................................................................................17

2 GRAFT-VERSUS-HOST DISEASE (GVHD) ......................................................20
  2.1 ACUTE GRAFT-VERSUS-HOST DISEASE ..................................................20
  2.2 CHRONIC GRAFT-VERSUS-HOST DISEASE .............................................22
    2.2.1 IMMUNOCELLULAR INVOLVEMENT OF cGVHD ..............................22
    2.2.2 PATHOMECHANISMS OF A 3-STEP MODEL .....................................25
  2.3 CLINICAL ASPECTS ..............................................................................28
    2.3.1 CLINICAL SIGNS OF ORGAN INVOLVEMENT ...............................29
    2.3.2 RISK AND PROGNOSTIC FACTORS ..............................................29
  2.4 FIRST LINE TREATMENT .....................................................................30
  2.5 SECOND LINE TREATMENT ..................................................................31
    2.5.1 EXTRACORPOREAL PHOTOPHERESIS ..........................................35
  2.6 EMERGING THERAPIES .......................................................................38

3 GVL .............................................................................................................39

4 AIMS ..........................................................................................................40

5 PATIENTS AND METHODS .......................................................................41
  5.1 PATIENTS ..............................................................................................41
  5.2 METHODS AND DEFINITIONS ............................................................42
  5.3 STATISTICS ..........................................................................................43

6 RESULTS .....................................................................................................45
  6.1 RISK FACTORS FOR cGVHD .................................................................45
  6.2 PROGNOSTIC FACTORS ........................................................................46
  6.3 EVALUATION OF SECOND LINE TREATMENT ......................................49

7 DISCUSSION AND CONCLUSIONS ..........................................................52
  7.1 CONCLUSIONS ........................................................................................57

8 FUTURE DIRECTIONS ................................................................................58

9 ACKNOWLEDGEMENTS .............................................................................60

10 REFERENCES .............................................................................................63
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACPA</td>
<td>Anti-citrullinated protein antibody</td>
</tr>
<tr>
<td>aGVHD</td>
<td>Acute graft-versus-host disease</td>
</tr>
<tr>
<td>ANA</td>
<td>Anti-nuclear antibody</td>
</tr>
<tr>
<td>ANCA</td>
<td>Anti-neutrophil cytoplasmic antibody</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ATG</td>
<td>Anti-thymocyte globulin</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-cell activating factor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BOR</td>
<td>Bortezomib</td>
</tr>
<tr>
<td>Bu</td>
<td>Busulphan</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>cGVHD</td>
<td>Chronic graft-versus-host disease</td>
</tr>
<tr>
<td>CIBMTR</td>
<td>Center for International Blood and Marrow Transplant Research</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNI</td>
<td>Calcineurin inhibitor</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>CyA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DLI</td>
<td>Donor lymphocyte infusion</td>
</tr>
<tr>
<td>EBMT</td>
<td>European society for Blood and Marrow Transplantation</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>Flu</td>
<td>Fludarabine</td>
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<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
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<tr>
<td>GVL</td>
<td>Graft-versus-leukaemia</td>
</tr>
<tr>
<td>GVT</td>
<td>Graft-versus-tumour</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
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1 HEMATOPOIETIC STEM-CELL TRANSPLANTATION

1.1 BACKGROUND

Hematopoietic stem cell transplantation (HSCT) is an effective treatment modality for several haematological malignancies of which the main group is patients with acute leukaemia. The transfer of the donor stem cells serves two main purposes. In part to restore a debilitated cellular and humoral immunity and to yield a graft-versus-leukaemia (GVL) effect. This facilitates an immunological elimination of residual cancer cells.

The role of transplanting a mixture of cells from blood forming organs, such as the bone marrow, was discovered in the aftermath of Nagasaki and Hiroshima. It was shown by Jacobsen et al that mice given lower doses of X-irradiation developed symptoms of bone marrow failure such as platelet deficiencies and infections. These symptoms were similar to individuals exposed to lower doses of fallout radiation from the atomic bomb. It was shown that shielding the spleen of mice with lead from this irradiation could significantly prevent the observed irradiation syndrome. Later, it was shown that the transfusion of healthy bone marrow to mice which had received lethal radiation could rescue the mice from irradiation syndrome. A clinical trial followed shortly thereafter. Six patients with terminal cancer received bone marrow from different sources. Such sources included foetus, deceased and living donors with varying blood types. The bone marrow was transfused after treatment either with chemotherapy or radiation. The study showed that the donated marrow cells were safe and in two patients a short “take” was observed. During the “take” patients had a significant but short-term increase in the number of cells of donor origin. These patients were irradiated extensively, to the point that they developed total bone marrow aplasia. All patients died but the study sparked the possibility of bone marrow transplantation as a treatment for leukaemia patients. Results from human bone marrow transplantations were dismal during the first decade and most patients died from infections, bleeding and/or from engraftment failure. Patients with successful engraftment without leukaemia died from a novel syndrome with skin rash, jaundice and severe diarrhoea soon after engraftment. This was coined as graft-versus-host disease (GVHD).

Bone marrow transplantation received a revival in the 1970’s due to the recognition of the major histocompatibility complex (MHC) and the human leukocyte antigens (HLA) as key elements of graft rejection and GVHD. Subsequently, Edward Donnall Thomas from the
Seattle group was awarded the Nobel prize in 1990 for his extensive research in the field of bone marrow transplantation. Thomas and his group produced convincing evidence regarding the notion of “graft-versus-leukaemia” effect and could efficiently dampen GVHD by treating patients with methotrexate before engraftment\(^6\). By 1977 one hundred patients with acute leukaemia had been treated with allogeneic bone marrow transplantation from HLA identical siblings and by 2013 the procedure had been conducted exponentially with over 400000 transplants having been performed worldwide\(^7,8\).

1.2 HLA AND DONORS

Major Histocompatibility Complex (MHC) are membrane bound proteins of which there are two major classes, I and II. Class I proteins are located on all nucleated cells except for erythrocytes and present intracellular protein fragments to the CD8+ cytotoxic T cell which requires MHC I, peptide fragment and co-stimulation for activation. Viruses are intracellular pathogens that hijack the protein synthesis of the cell to replicate. Therefore, viral peptides are also presented via MHC I. Class II molecules are found on all antigen presenting cells such as B-cells, dendritic cells and macrophages. These cells present extracellular proteins by internalizing them through phagocytosis. These proteins include peptides of bacteria among other foreign pathogens. CD4+ T helper cells can only respond to peptides presented by class II MHC molecules\(^9\). In humans, the genes for MHC are a linear array encoded on chromosome 6. They are called Human Leukocyte Antigens (HLA) because they were discovered as antigens of leukocytes when performing compatibility tests via the mixed lymphocyte culture methods in vitro\(^10\).

HLA Class I antigens include HLA-A, B and C. Class II antigens include HLA-DR, DP and DQ. They are inherited in a co-dominant Mendelian order in which one haplotype is inherited from the father and the other from the mother. This denotes that in siblings with unrelated parents, 25% of all siblings could be HLA-identical. A haplo-identical donor arises when a biological parent donates to its child or vice-versa. Other possible haplo-identical donors could exist such as the case in which two identical twins spawn children of their own. In that case a possible haplo-identical match could arise between the children who are considered cousins. Allelic variation, or polymorphism, accounts for differences to prevent a population from being completely eradicated by foreign pathogens. These differences originate from geographical patterns and perhaps due to functional selection in those regions\(^11\).
Interestingly, one study carried out in 1979 showed that patients with syngeneic (identical twin) transplantations did not develop graft-versus-host disease while those with HLA-identical donors did. One major finding in that study was that the relapse rate was higher in the syngeneic transplants. This finding formulated the theory that minor-histocompatibility antigens are the target of T-cell allogeneic response in HLA identical transplants, with the clinical benefit of graft-versus-leukaemia.

In general, increased HLA-disparity leads to increased risk of GVHD. Increased HLA-disparity in HLA-A,-B,-C, -DQ and –DR has an independently increased risk of GVHD and mortality. Certain HLA mismatches, such as residue (amino acid) mismatch at HLA-C level, increase the risk of acute GVHD and mortality. HLA-DPβ1 mismatch also increases the risk of acute GVHD and lowers the relapse risk, at least in patients with late stage acute leukaemia (beyond first remission) given a full match at HLA-A, B, C, DQ and DR. HLA-DPβ1 has frequently been mismatched in patient and donor pairs allowing analysis of its implications. Subsequently, a functional epitope-based algorithm was developed which classified permissive or non-permissive HLA-DPβ1 mismatches based on the developed immunogenicity towards shared epitopes. The great variation in non-MHC minor histocompatibility loci makes it difficult to predict their impact, however, one such minor antigen is accounted for in the clinical setting, namely, H-Y antigen where a female-to-male donor increases the risk of acute and chronic GVHD. At our centre we have not found adverse outcomes in RFS or OS upon HLA-C mismatch. In the first study, DPα1 was analysed separately for the first time without confounding class I or II mismatches. We could show reduced survival rates and RFS upon mismatch. At our centre we use high-resolution PCR to type for twelve alleles, namely, HLA-A, B, C, DPα1, DQβ1 and DRβ1. We initially match for HLA-A, B, C, DQβ1 and DRβ1. We then proceed to examine CMV serostatus followed by matching for HLA-DPα1 and DPβ1.

Donors graciously volunteer to be included in different national and international registries such as DKMS, NMDP and the Swedish Tobias registry. Once included the donor’s HLA-type is determined and registered for future solicitation. Registries try to recruit young adults as results have shown that matched donors in the age range of 18-32 yield a higher survival rate. For every 10-year increment in donor age there is a 5.5% increase in hazard ratio for mortality. In practice this can mean that using a matched URD would have better outcome than an older sibling donor. CMV serostatus is also a determinant in transplantation outcome with best outcomes seen in seronegative patients receiving seronegative grafts. Data have
suggested that stem cell source may impact the development of GVHD with higher incidence of chronic GVHD and faster hematologic recovery with peripheral blood stem cells compared to bone marrow stem cells from unrelated donors\textsuperscript{26–29}. One study has shown similar results for aGVHD in sibling donors\textsuperscript{30}. Finally a large meta-analysis showed that both sibling and URD transplants receiving peripheral blood stem cells developed higher incidence of cGVHD\textsuperscript{31}.

Since 2018 the classic criterion for HLA matching is 10/10 at HLA-A, HLA-B, HLA-C, HLA-DR\(\beta\)1 and HLA-DQ\(\beta\)1 for URD, all at high-resolution level, with mismatches associated with inferior patient outcome\textsuperscript{25}. The National Marrow Donor Program (NMDP) has demonstrated that approximately 75% of Caucasian patients are likely to identify an 8/8 HLA-A,-B,-C and –DR\(\beta\)1 matched URD. The rate is as low as 16% for ethnic minority and mixed-race patients. This is due to the higher genetic diversity of HLA haplotypes in African and certain Asian populations compared to Europeans as well as the lower representation and poorer availability of ethnic minority donors\textsuperscript{32}. HLA-identical siblings remain the golden standard while only prevalent in approximately 30% of transplants. Due to the increase in migration and the mixing of HLA-types, it is necessary to address this growing issue within the transplant community. During the past five years, a novel approach to the degree of matching related donors has been established. This has been developed to accommodate the difficulty in finding well-matched unrelated donors (URD) and well-matched related donors\textsuperscript{33,34}. The concept of haplo-identical matching has grown and at our institution we have employed this modality more frequently over the past two years with successful results. At our centre, we perform transplantation using non-manipulated bone marrow either from a haplo-identical sibling, parent or child. In general, transplantation with non-manipulated bone marrow has not been shown to cause more GVHD or relapse\textsuperscript{35}. However, a longer period of aplasia is observed.

1.3 STEM CELL SOURCE
Initially, the main source of hematopoietic stem cells (HSCs) was bone marrow from an HLA identical sibling\textsuperscript{36}. This has now expanded to granulocyte colony-stimulating-factor (G-CSF) mobilized peripheral blood stem cells (PBSC) and umbilical cord blood (UCB)\textsuperscript{37,38}, which in turn has extended transplant indications to benefit a larger group of patients. Traditionally bone marrow is harvested from the donor’s posterior iliac crest under general anaesthesia. This requires hospitalization for one night and possibly the transfusion of one to two units of blood. Safety of the donor is a major consideration in the pre-transplant work-up and there
are no clear age limits as to who can donate using this method providing the donor can tolerate general anaesthesia. In terms of adverse events there have been reports of pain at the collection site, longer rehabilitation periods post-operatively, haemorrhage and side effects that occur due to general anaesthesia. For PBSC-HSCT it seems that adverse events such as generalized bone pain and heart palpitations are more common, however, serious adverse events are more uncommon. UCB remains as an alternative source, especially due to advances in haplo-identical transplantation. The major advantage is the relative ease of procurement and the lack of adverse effects for infant and mother. The technique involves early clamping of the umbilical cord to ensure a large volume of product. However, early clamping may lead to iron deficiency in the new-born infant. One significant limitation remains the stem cell dose especially for an adult recipient. This leads to slower engraftment with a higher incidence of infections and higher TRM. Worldwide 71% of HSCT are performed using PBSC grafts, 22% from bone marrow and 7% UCB grafts.

1.4 HSCT INDICATIONS
The main indications for HSCT according to a survey from 2014 conducted by the EBMT shows AML 36%, ALL 16%, MDS/MPN 15%, lymphoma 12% (of which 3% are Hodgkin’s disease, 2% CLL, the remaining 7% are plasma cell disorders and other unclassifiable lymphomas), 22.7% non-malignant disorders and 0.3% solid tumors. The general use of HSCT has increased in the context of first remission AML, myeloproliferative neoplasia and bone marrow failure syndromes such as myelodysplastic syndrome in the past five years. Declining numbers of HSCT procedures are observed for CLL, perhaps due to the increased use of kinase inhibitors and other small molecule anti-neoplastic treatments. More recent figures from 2017 show similar trends with 57% for myeloid malignancies, 30% for lymphoid malignancies, 12.8% non-malignant disease and 0.2% solid tumors.

1.5 TRANSPLANTATION PROCEDURE
All patients are screened for co-morbidities before transplantation to predict non-relapse-mortality. The concept of HSCT is to administer a conditioning regimen to provide sufficient immunologic ablation to prevent graft rejection while reducing the tumor burden. Initially, these goals were achieved with otherwise supra-lethal doses of total body irradiation (TBI) and chemotherapeutic alkylating agents with non-overlapping toxicities to achieve a myeloablative result. It was however recognized that decreasing the intensity of the conditioning regimens reduced transplant-related-mortality and toxicity which permitted the transplantation of older patients with more co-morbidities. This was preceded by the finding that allogeneic hematopoietic cells not only rescued hematologic toxicity of high-dose...
conditioning regimens but, also contributed to the cure of malignant diseases through graft-versus-leukaemia effects. The definition of such reduced-intensity-conditioning (RIC) can be summarized as follows: any regimen that results in low non-hematologic toxicity and mixed donor–recipient chimerism in a substantial proportion of patients in the early post-transplantation period (around day +30). In addition to this, radiation dose < 500 cGy of total body irradiation as a single fraction or 800 cGy in fractionated doses, busulfan dose < 9 mg/kg, melphalan dose <140 mg/m², or thiotepa dose < 10 mg/kg should be considered as RIC regimens according to the CIBMTR. The EBMT has a similar definition of RIC except that the radiation dose should be 200cGy. In general the dose of alkylating agents and TBI is reduced by at least one third in RIC regimens compared to myeloablative conditioning (MAC) regimens. Usually RIC regimens require HSCT for haematological recovery to avoid prolonged cytopenias and most of them are based on the use of Fludarabine (Flu). There is also a non-myeloablative conditioning regimen group in which there is minimal hematologic damage and the hematopoietic recovery is not ubiquitous with the transplantation of stem cells. One such example is the conditioning regimen for aplastic anaemia with the use of 50mg cyclophosphamide/kg/day for 4 days.

The transplantation procedure starts with the above mentioned conditioning regimens, after which, donor stem cells are infused via a central line. Concurrent to these proceedings patients receive GVHD prophylaxis. At our centre we employ a regimen which is in line with the recommendations from the EBMT and the European Leukaemia Network (ELN).

1.5.1 GVHD PROPHYLAXIS

Even with a well matched donor and recipient, GVHD remains a problem unless post-HSCT methotrexate (MTX) is given, which slows donor lymphocyte replication. The comparison of a calcineurin inhibitor (CNI) combined with MTX to only MTX showed superiority for the prevention of GVHD. It was shown in the 80’s that the combination of a CNI and MTX was superior to use of only CNI to prevent GVHD. GVHD prophylaxis consists thereof also of a CNI to abrogate IL-2 production and thus allo-reactive T-cell activation and proliferation. Later, another CNI-based prophylactic regimen using tacrolimus (TAC) together with MTX was developed. Two randomized phase III trials were published after MAC in HLA-identical and URD, respectively. Both trials showed a significant decrease in acute GVHD grades II-IV however, neither could show improved survival compared to CNI/MTX. For RIC, two widely utilised regimens include the use of CNI in combination with mycophenolate mofetil (MMF, mechanism of action explained later in this thesis) or with MTX. One recent large comparative analysis between the two combinations failed to
show any differences in cGVHD incidence or OS61. MMF is administered as 30mg/kg/day however; this combination has yet to be evaluated in a randomized trial. Mammalian target of rapamycin (mTOR) more potently inhibits conventional T-cell expansion than regulatory T-cells due to higher dependency on the mTOR protein kinase-B pathway. A trial combining mTOR/CNI to CNI/MTX could not show a difference in incidence of acute GVHD grade II-IV nor did a trial conducted at our centre show any difference in incidence62,63. Thanks to randomized multi-centre trials, it has been known for the past two decades that pan-T-cell depletion by the addition of rabbit anti thymocyte globulin leads to a lower incidence of acute and chronic GVHD without increasing relapse64,65. Alemtuzumab, anti-CD52 antibody directed towards T- and B-cells, has shown similar results in terms of reduction of GVHD with perhaps an increase in fatal infections66. A phase II study of the proteasome inhibitor Bortezomib (BOR) compared the groups CNI/MTX, CNI/MTX/BOR and CNI/mTOR/BOR and showed similar incidence of aGVHD at day +18067. Finally, cell-based approaches by manipulating the donor graft, such as positive selection of CD34+ cells, enrichment of gamma-delta T-cells and reduction of alpha-beta T-cells, have shown promising results in reducing GVHD68–70. Allo-reactive T-cells give rise to GVHD and rejection in the unfavourable setting of haplo-identically matched donor and recipient71. Post-transplant cyclophosphamide given at a dose of 50mg/kg on days +3 and +4 is used successfully in the setting of haplo-transplantation and has selectively depletes allo-reactive T-cells72.

At our centre the calcineurin inhibitor is given at a lower dose and discontinued around three months post-transplant in related donor transplantation or six months if the donor is unrelated73,74. For non-malignant diseases CNI is continued for a minimum of twelve months. A short course of methotrexate is given early post-transplantation on days +1, +3, +6 and +11. Anti-thymocyte globulin (ATG) in the dose of 4-6mg/kg is administered in unrelated donor or non-malignant disease transplants. At our centre 2mg/kg is given to male recipients of grafts sourced from HLA-identical immunized female siblings. Supportive care including anti-bacterial, anti-fungal and anti-viral treatment is administered throughout the transplantation phase. Usually antibacterial and anti-fungal treatment is discontinued upon neutrophil recovery unless further treatment indication is present. The patient receives prophylaxis against pneumocystis jiroveci and varicella zoster virus for 6 and 12 months respectively unless further prophylaxis is warranted, for example in the case of concurrent cGVHD.
1.6 IMMUNE RECONSTITUTION AND INFECTIONS

1.6.1 INNATE IMMUNITY

The conditioning regimen often results in damage to epithelial surfaces including the mucosal membrane. This damage is greater in myeloablative conditioning compared to RIC, and in BM compared to PBSC. Moreover, it is observed more frequently in URD compared to that seen in matched sibling donors. Neutrophil recovery usually occurs during the first two to four weeks after transplantation. However, neutrophil function including chemotaxis and phagocytosis remains impaired for months especially in the presence of acute GVHD stage $> II$. During the pre-engraftment period the patient is susceptible to bacterial and invasive candida infections and later the patient is at risk of developing invasive mould infections mainly due to high doses of corticosteroid treatment for GVHD. Patients receive antibacterial, antifungal and antiviral prophylaxis during the pre-engraftment phase.

Neutrophil levels are first restored in the damaged tissue preceding that in the peripheral blood. The mucosal damage heals and marks the initial engraftment period. During the first few weeks post-transplant there is also a complete restoration of dendritic cells, macrophages and natural killer (NK) cells.

1.6.2 ADAPTIVE IMMUNITY

Innate immune cells reconstitute faster than adaptive immune cells because the latter require more extensive rearrangement and education processes to achieve full effector functions. Memory T-cells are the first to expand being either of host or donor origin. This is a thymic-independent pathway termed ‘homeostatic peripheral expansion’ (HPE) that involves expansion of mature T-cells which survive the preparative regimen and/or are contained within the allograft. These cells respond quickly to previously exposed pathogens and penetrate the tissues more readily. These cells account for a large pool of the CD8+ T-cells which keep viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) under control. To achieve a fully functional immunity, the pool of naïve T-cells must be replenished via the thymic-dependent pathway to gain a more diverse pathogen response. It seems that more HPE T-cells of donor origin are observed after myeloablative conditioning while more HPE T-cells of host origin are present after RIC/non-myeloablative conditioning. Even under favourable conditions it would require
weeks to months to produce naive T-cells from infused HSCs and a plateau level of thymic output is reached at 1 to 2 years after allogeneic HCT\textsuperscript{85}.

Regulatory T-cells (Tregs) are a subset of CD4\textsuperscript{+} T-cells whose function is to suppress immune responses and maintain self-tolerance. Tregs are a functionally mature subpopulation of T-cells and can also be induced (iTregs) from CD4\textsuperscript{+}CD45RA\textsuperscript{+} naive T-cells in the periphery under the influence of transforming growth factor beta (TGF\textbeta). Natural Tregs (nTregs) are derived from the thymus and are characterized by the co-expression of CD4, high expression of CD25 and FoxP3\textsuperscript{86}. Tregs can promote homeostasis and suppress effector T-cells after HSCT improving GVHD symptoms without hampering the GVL effect\textsuperscript{87,88}. The process of T-cell maturation is abrogated by impaired thymic function due to older age\textsuperscript{89} or GVHD\textsuperscript{90}.

The B-cell compartment is the slowest and may take up to 5 years to reconstitute\textsuperscript{82}. One reason may be the delayed T-cell reconstitution with a reversed CD4/CD8 ratio in which there are lower levels of T-helper cells in relation to levels of cytotoxic T-cells\textsuperscript{91} after HSCT. In the first 2 years following HSCT, B-cell function is compromised with transitional CD19\textsuperscript{+}CD21\textsuperscript{low}CD38\textsuperscript{high} B-cells detected in the peripheral blood after the first few months\textsuperscript{92}. These decrease in percentage and are replaced by more mature naïve CD19\textsuperscript{+}CD21\textsuperscript{high}CD27\textsuperscript{−} B-cells\textsuperscript{93}.

\textbf{1.6.3 INFECTIONS}

During the pre-engraftment period which stretches from day 0 to +30, patients are neutropenic and have varying degrees of mucosal barrier destruction depending on the conditioning regimen. In addition, humoral and cellular immunodeficiencies are coupled with functional asplenia in patients whom receive TBI. During this period, the patient is at their most vulnerable state and very susceptible to infections by gram-positive or -negative bacteria, herpes simplex virus and candida species\textsuperscript{94}. Most common clinical infections during the pre-engraftment period are bacteremia/sepsis and pneumonia.

In the post-engraftment period, which ranges from day +30 to +100, neutropenia and mucositis have resolved. However, central venous lines are usually still present at this stage and the patient has a continued adaptive immunodeficiency which is worsened by any GVHD and in its treatment or prophylaxis. This prolonged immunodeficiency leaves the patient at a high risk of developing de novo viral infections such as influenza, respiratory syncytial virus and adenovirus, as well as re-activation of latent viruses\textsuperscript{95}. HSV, CMV, EBV and varicella-
zoster (VZV) are common pathogens during the post-engraftment period and cause mucositis, gastroenteritis, post-transplant lymphoproliferative disorder and shingles respectively. CMV and EBV are routinely monitored for and all patients are given antiviral prophylaxis for at least 12 months post-HSCT against VZV.

The late post-transplant phase marks the period beyond day +100. Most infections are attributable to the presence of chronic GVHD and its treatment. Functional asplenia persists after TBI, cellular and humoral immunodeficiency may continue. Most common infections during this period include encapsulated bacteria (such as Streptococcus pneumoniae and Haemophilus influenzae) and invasive mould infections (IFI) of which aspergillus species remain the main pathogen. During this period reactivation of varicella-zoster-virus, CMV and infections caused by seasonal respiratory viruses such as influenza and RSV occur, especially in cGVHD and prolonged immunosuppression. Patients with chronic GVHD requiring high doses of corticosteroids are given antifungal prophylactic treatment to avoid IFI. Toxoplasma gondii in seropositive patients and pneumocystis jiroveci cause opportunistic infections predominantly if the patient has a low CD4+ T-cell count or ongoing chronic GVHD which requires immunosuppression. Both cause severe and life threatening infections with encephalitis caused by toxoplasma and pneumonia caused by pneumocystis. Patients routinely receive prophylaxis against both pathogens Trimethoprim/sulfamethoxazole is used for both pathogens in varying doses at our centre. Patients are routinely vaccinated against pneumococci, tetanus, diphtheria, pertussis and polio starting at three months post-HSCT. Other vaccinations are usually dependent on immune-reconstitution and whether the vaccine contains live attenuated particles such as measles. Vaccination for measles is not recommended within two years post-HSCT or if the patient is on immunosuppression.

Finally a wide array of airborne virus infections resulting in respiratory failure as well as reactivation of other herpes virus family members such as HHV-6 resulting in encephalitis can occur. A summary of the immune reconstitution, infections and prophylaxis is depicted in Figure 1.
### Time Period

<table>
<thead>
<tr>
<th></th>
<th>Pre-engraftment (&lt;30 days post HSCT)</th>
<th>Early post-engraftment (30-100 days)</th>
<th>Late post-engraftment (&lt;12 months)</th>
<th>Second year (&gt;12 months)</th>
<th>Late follow-up (&lt;5 years)</th>
</tr>
</thead>
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<tr>
<td><strong>Immune deficiencies</strong></td>
<td>Neutropenia</td>
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<td></td>
<td>NK Cell</td>
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<td>APC</td>
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<td></td>
<td>B-Cell</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Infections</strong></td>
<td>Bacterial</td>
<td>CMV, EBV, VZV</td>
<td>Longer if cGVHD</td>
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<tr>
<td></td>
<td>IFI</td>
<td>Longer if cGVHD</td>
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<td></td>
<td>Respiratory viruses</td>
<td></td>
<td>Longer if cGVHD</td>
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<tr>
<td></td>
<td>Pneumocystis</td>
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<td></td>
<td>Jiroveci</td>
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<tr>
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<td>Antibacterial</td>
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<td></td>
<td>Antifungal</td>
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<td></td>
<td>Antiviral:</td>
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<td></td>
<td>Aciclovir/Valaciclovir</td>
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<tr>
<td></td>
<td>Trim-Sulfa (Pneumocystis Jiroveci)</td>
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</tbody>
</table>

**Figure 1.** Overview of the immune deficiencies, infections and prophylaxis for patients. The risk of infections is prolonged if concurrent cGVHD and proper prophylaxis is required accordingly.

In the late phase, post-HSCT chronic GVHD and its treatment account for many infections. Encapsulated bacteria such as Haemophilus influenza, Streptococcus pneumoniae and Neisseria meningitidis become usual pathogens due to impaired opsonisation. There is also a higher incidence of late onset Pneumocystis jiroveci infection in the presence of IS due to cGVHD. Prolonged immunosuppression due to cGVHD and its treatment lead to an increase in the incidence of community acquired respiratory viruses such as RS-virus, parainfluenza virus, metapneumovirus and influenza. Invasive fungal and mold infections such as Candida and Aspergillus are more common in the late period due to corticosteroid treatment for chronic graft-versus-host disease than in patients with no corticosteroid.
In fact, a recent report states that patients with a history of cGVHD currently with or without IS had an elevated risk of developing late (up to 12 years post-HSCT) fatal infections. This highlights the negative impact of cGVHD on the development of functional immunity.

2 GRAFT-VERSUS-HOST DISEASE (GVHD)

2.1 ACUTE GRAFT-VERSUS-HOST DISEASE

GVHD was first recognized in murine models during the early 70’s. At that time not much was known about the HLA system and symptoms such as anorexia, reduced weight, diarrhoea and ruffled fur were termed “secondary” or “runt” disease.

The pathobiology is initiated during the conditioning regimen with substantial damage to mucous membranes and epithelial lining. This allows for bacterial translocation from the gut where host-derived antigen presenting cells recognize pathogens and recruit donor-derived T-cells causing them to proliferate. The developing inflammatory milieu persists even after the bacterial septicemia has resolved mainly due to up-regulation of inflammatory cytokines such as tumour necrosis factor, further recruiting donor-derived cells to the damage site. The gut seems to be the main propagating organ in which acute GVHD is both initiated and perpetuated.

Acute GVHD (aGVHD) is the major cause of short term mortality after HSCT and most commonly involves the skin with erythema (81% of aGVHD patients), gastrointestinal dysfunction (second most common organ affected with 54% incidence in aGVHD) and finally the liver with cholestasis (50% of aGVHD patients) arising within 100 days after HSCT. An interesting denominator is that all three organs involved are exposed to microbial pathogens through the intestine, epidermis and portal circulation. The overall severity grade is obtained by an accumulated scoring of the severity and number of organs involved (Table 1). Grade 1 aGVHD is considered to be mild, grade 2 moderate, grade 3 severe and grade 4 very severe. Of all HSCT patients 30 to 50% have aGVHD (grades 1–4) with 14% having severe aGVHD (grades 3–4). Risk factors for developing aGVHD include HLA disparity, the use of an unrelated donor, total body irradiation and female donor to male recipient. Protective factors include the use of anti-thymocyte globulin. Patients with acute GVHD grade 2-4 whom require systemic treatment usually with the addition of high dose methylprednisone 1-2mg/kg to concurrent CNI treatment have a response rate in
terms of full resolution of less than 40%. Those who do not respond after 3-14 days of corticosteroid treatment are defined as steroid-refractory aGVHD with poor response rates to second line treatments and high mortality rates\textsuperscript{123}. Second line treatment with focus on T-cell homing has shown promising results\textsuperscript{124}. Lower GI involvement is a strong predictor of treatment response\textsuperscript{125}. Previously, acute GVHD grade III-IV had an overall survival ranging from 10-25%. Improvements in supportive care have increased survival significantly with some centers reporting survival rates of up to 40% in grade IV aGVHD\textsuperscript{126}. Furthermore, at our centre we have shown that home care immediately after HSCT decreases the risk of aGVHD\textsuperscript{127,128}.

As mentioned, the primary target of aGVHD remains the gut and it is also highly depictive of its pathophysiology. REG3\textalpha{} is a bactericidal peptide contained within Paneth cells and ST2 is the receptor for IL-33, an alarmin released by stromal cells upon damage\textsuperscript{119,129}. The IL-33 binds to ST2 on donor T-cells which in turn release IFN\gamma{} to further inflammation. This is a prime example of the interaction between innate and donor-derived adaptive immunity in the post-HSCT setting. The discovery of ST2 and REG3\textalpha{} has permitted their role as biomarkers which can be analysed at 7 days after HSCT, convincingly predicting development of lethal GVHD and non-relapse-mortality (NRM)\textsuperscript{130–132}.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Skin</th>
<th>Liver</th>
<th>Intestinal tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maculopapular rash &lt; 25% of body surface</td>
<td>Billirubin 34–50 µmol/l</td>
<td>&gt; 500 ml diarrhea/d</td>
</tr>
<tr>
<td>2</td>
<td>Maculopapular rash 25–50% body surface</td>
<td>Billirubin 51–102 µmol/l</td>
<td>&gt; 1000 ml diarrhea/d</td>
</tr>
<tr>
<td>3</td>
<td>Generalized erythroderma</td>
<td>Billirubin 103–225 µmol/l</td>
<td>&gt; 1500 ml diarrhea/d</td>
</tr>
<tr>
<td>4</td>
<td>Generalized erythroderma with bullous formation and desquamation</td>
<td>Billirubin &gt; 255 µmol/l</td>
<td>Severe abdominal pain, with or without ileus</td>
</tr>
</tbody>
</table>

Table 1. Glucksberg criteria. Adapted from Przepiorka D et al\textsuperscript{120}. Copyright clearance approved under order number 4544740641172.
2.2 CHRONIC GRAFT-VERSUS-HOST DISEASE

Chronic graft-versus-host disease remains one of the most severe complications after HSCT, affecting both the quality of life and mortality of long-term survivors\textsuperscript{6,36,133,134}. Its impact on morbidity and mortality varies depending on the severity and number of organs involved, allowing the classification of patients into mild, moderate and severe cGVHD according to the National Institute of Health (NIH). This allows identification of those at low, intermediate or high risk of developing GVHD-related morbidity and mortality. Chronic GVHD is important due to its inherent GVL effect that decreases the risk of relapse after transplant\textsuperscript{135}. Appropriate management of cGVHD should be individualized according to the patients’ characteristics.

Chronic GVHD is an increasingly frequent complication after HSCT due, at least in part, to the more frequent use of peripheral blood stem cells, higher age of recipients/donors, and increased use of mismatched and unrelated donors. We have retrospectively classified a large cohort of patients in terms of cGvHD subtype and severity according to the NIH proposal\textsuperscript{136}. Various studies have attempted to identify the best strategy to prevent cGVHD and, to date, only the use of \textit{in vitro} or \textit{in vivo} T-cell depletion has been shown to reduce the risk of cGVHD, although its impact on survival has been relatively limited in unselected series of patients\textsuperscript{137}.

Chronic GVHD symptoms are reminiscent of a variety of autoimmune diseases such as Scleroderma, Sjögren syndrome, primary biliary cirrhosis, bronchiolitis obliterans, immune mediated erythema and cytopenias. Therefore, the diagnosis of cGVHD is based on different clinical manifestations as proposed by the NIH consensus conference\textsuperscript{138}.

2.2.1 IMMUNOCELLULAR INVOLVEMENT OF cGVHD

In 1966 Rupert E Billingham described necessary circumstances for the development of GVHD: The graft must contain immunologically competent cells. The recipient must express tissue antigens that are not present in the transplant donor and the recipient must be incapable of mounting an effective response to eliminate the transplanted cells\textsuperscript{139}.

\textit{T-Cells}

Chronic GVHD represents a syndrome in which the respective contributions of inflammation, innate and adaptive cell-mediated immunity, humoral immunity, abnormal immune regulation and fibrosis vary considerably from one patient to the next. Attempts to study this
have been made in mouse-models by grafting donor bone marrow cells into a recipient with the same MHC haplotype but from a different strain. The common finding of all immune models is the emergence of allo-reactive (recognizing host antigens as non-self) T and B-cell clones. The syndrome seems to rely more on CD4+ T-cells than on CD8+cells and is characterised by T-helper 2 cytokine expression (IL-4, IL-5, IL-6, IL-13 and IL-21)\(^{140}\). A sub-class of CD4+ T-cells, namely T-regulatory cells which are characterized as having membrane-bound IL-2Ra (CD25), lacking IL7-Ra (CD127) and expressing of transcriptional regulator FoxP3, have been implicated in the role of cGVHD. Previous data have been contradictory as to the role of these cells in cGVHD with both positive and negative correlations having been shown\(^{141-144}\). In autoimmune diseases it has been shown that these cells are down-regulated. Since cGVHD is defined by the loss of tolerance and the development of “autoimmune” symptoms, it has recently become more accepted that a deficiency in Treg cell reconstitution plays a distinct role in GVHD pathophysiology. In this respect, IL-2 deficiency (which is autocrine secreted to stimulate development of naïve T-cells to Treg) is noted with a subsequent deficiency in Treg numbers. It is generally accepted that Treg cells are vital for immune homeostasis. The proposed mechanisms include natural Treg that migrate to secondary lymphoid tissues and prevent allo-recognition by blocking interactions between dendritic cells and T-cells. Natural and induced Tregs inhibit activation of T-cells in the periphery via IL-10 and TGF-β secretion\(^{145}\). Not all FoxP3+CD4+ T-cells are Treg cells. To differentiate these cells CD45RA, present on most naive hematopoietic cells except for erythrocytes, is used. This creates a tri-linear division where CD45RA+CD25++FoxP3lo are resting Treg cells, CD45RA-CD25+++FoxP3hi are activated Treg cells and CD45RA-CD25++FoxP3lo are non-Treg cells\(^{146}\). At the onset of chronic graft-versus-host disease there is a deficiency in the number of Treg cells in a generalized fashion\(^{142}\). Adoptive transfer of Tregs in the allogeneic graft during HCT has been shown to prevent GVHD in the same mouse models, however, these Tregs only survive 2 weeks in vivo. Stimulation with low dose IL-2 has been shown to expand FOXP3+ Tregs\(^{147}\) and improve chronic GVHD symptoms\(^{148}\).

T-helper-17 cells (Th17) are pro-inflammatory T-cells that are characterized by their production of IL-17. After contact with pathogens, antigen presenting cells produce TGFβ, IL-6, IL-21 and IL-23 leading to differentiation of and proliferation of Th17. Th17 cells in turn produce IL-17, IL-21 and IL-22. Deletion of Th17 in mouse models increases Th1 and worsens acute GVHD\(^{149}\). IL-17 inhibition has been shown to impair CD4-mediated acute GVHD\(^{150}\). However, even when depleting Th17 cells in mice with aGVHD by inhibiting the
transcription factor RORyt, the severity or prevalence of aGVHD was not affected, demonstrating that TH17 could be sufficient but not necessary to induce or maintain acute GVHD\textsuperscript{151}. In patients with ongoing acute GVHD, IL-17 can be found in biopsy samples from the gut but not from the skin\textsuperscript{152}. Inhibiting IL-21 and IL-21 receptor signalling in vivo via anti-IL-21 antibodies in mouse models decreased acute GVHD in the gut by increasing Treg and decreasing Th17 in gut mucosa\textsuperscript{153}. It is important to recognize the immunopathology of acute GVHD since it remains the most persistent risk factor to develop subsequent chronic GVHD.

\textbf{B-cells}

B-cells are central in the humoral immune response. They produce antibodies and yield an immune defence against bacteria and viruses. They also have another property, namely, as antigen presenting cells (APC). Through their B-cell receptor they are able to internalize specific antigens. B-cells internalize antigens and present them by way of major histocompatibility complex II (MHCII). They also prime CD4+ and CD8+ T-cells. In patients with autoimmune diseases it is generally accepted that autoreactive B-cells evading tolerance check points are not deleted and are promoted by trophic factors such as B-cell activating factor (BAFF) which is part of the tumor necrosis factor family (TNF). It is thought that BAFF might support antigen independent expansion of activated memory B-cells (CD27+) in such a way that the activated B-cells continue antibody production without present antigen\textsuperscript{154,155}.

In patients with chronic GVHD, a disturbed B-cell homeostasis seems to exist in which there is a reduction of naïve B-cells and high number of erroneously activated transitional B-cells. This, together with the presence of allo-reactive CD4+ T-cells and elevated levels of BAFF, has been shown to exist at onset of cGVHD and with increasing severity of cGVHD\textsuperscript{156}. In addition to this, the prevalence of both known auto-antibodies (ANA, ACPA, ANCA) and certain allo-antibodies (H-Y antigen/Sex mismatched) exist in higher levels in patients with cGVHD, but their clinical relevance remains unclear\textsuperscript{157}. Pathogenic auto antibodies directed towards stimulating platelet-derived growth factor receptor (PDGFR) have been described in severe sclerodermatous cGVHD denoting higher levels of reactive oxygen species\textsuperscript{158}. Besides PDGFR, TGF-β1 is upregulated in patients with sclerodermatous cGVHD. Monocyte activation by allo-reactive T-cells lead to TGF-β1 release and up-regulation of collagen. Stimulation of PDGFR on fibroblasts is thought to increase extracellular matrix and collagen production. Both pathways lead to skin fibrosis in patients with clinically sclerodermatous cGVHD\textsuperscript{159}. 
**Natural Killer (NK)-Cells**

NK cells serve as the link between the innate and the adaptive immune system in part due to the release of IFNγ upon activation, activating tissue resident macrophages cascading the response to nearby T-cells. As mentioned earlier, NK cells are the first lymphocytes to reconstitute and their role has been studied in GVHD. Initially it was shown that donor derived and allo-reactive NK cells prevented graft rejection, severe GVHD and leukemic relapse in mouse models\textsuperscript{160}. HSCT in a mismatched setting yields NK cell alloreactivity due to “missing-self” especially in haploidentical transplants. This occurs when the recipient lacks one or more of the major inhibitory killer cell immunoglobulin-like receptor (KIR)--binding HLA motifs present in the donor\textsuperscript{161}. A sustained NK cell education, which is donor ligand driven in HLA-mismatched transplants without implicating the KIR mismatch as pivotal in the development of chronic GVHD. This does however suggest a sustained graft-versus-leukaemia (GvL) effect\textsuperscript{162,163}. Finally, it has been shown in both mouse models\textsuperscript{164} and in a recent human trial\textsuperscript{165} that the GvL effect remains intact upon NK cell infusion without enhancing acute or chronic GVHD.

**Innate lymphoid cells (ILC)**

ILCs are tissue-resident lymphocyte-like cells that produce cytokines and perform functions similar to those of T-cells but they do not express T-cell receptors. They are divided into three subgroups ILC1, ILC2 and ILC3 and thus mirror the CD+ T-cell groups of Th1, Th2 and Th17\textsuperscript{166,167}. They are always located in close affinity to epithelial cells and react to cytokines released by these cells upon infection or damage and subsequently guide the T-cell response. NK cell subsets with high IFNγ production are considered members of ILC1 group\textsuperscript{168}. While the role of ILCs in cGVHD needs to be further investigated, clear evidence exists as to how their respective cytokine production patterns influence cGVHD. ILC2 have been implicated in the formation of pulmonary fibrosis\textsuperscript{169}. In sclerodematous cGVHD it has been shown that both Th17 cells and IL-17a producing ILC3 cells contribute to the fibrotic development\textsuperscript{170}.

### 2.2.2 PATHOMECHANISMS OF A 3-STEP MODEL

**Damage**

To realise how cGVHD arises, one needs to understand that it begins already at the chemotherapy stage in which endothelial gut damage remains a major source of inflammation and involvement of innate immunity. Toll-like receptors (TLR) are membrane proteins on cells of the innate immunity such as macrophages and dendritic cells which activate these cells upon recognition of microbial proteins. TLR pathways are triggered when the gut
endothelial damage leads to a diminished barrier and translocation of bacteria to the blood yielding TLR pathway activation, as seen in Figure 2. This is confirmed by studies showing that inhibition of lipopolysaccharide, a bacterial endotoxin protein which TLR’s bind to, reduces acute GVHD. The activated dendritic cells of host origin then migrate to the nearest lymph node where they activate and imprint on naïve T-cells, committing them to a certain phenotype such as Th1, Th2 or Th17. These T-cells then up-regulate certain receptor molecules to home to the site of origin where the dendritic cell came from. The activation of the mentioned cells leads to increased IFNα production. This stimulates T-helper cell proliferation that secrete IFNγ driving a Th1 commitment leading to acute GVHD. The actual tissue damage can persist which leads to a progressive onset or overlap syndrome in about a third of chronic GVHD cases where the patient will present symptoms of both acute and chronic GVHD for a period of time. More evidence supporting persistence of tissue damage is the presence of IFNγ inducible chemokines such as CXCL9, which is up-regulated at diagnosis and remains elevated in severely affected cGVHD patients.

Figure 2. Pathomechanism of cGVHD. Cell damage upon administration of chemotherapy and/or radiation. Pathogens activate the innate immune system. During the first months B-cells may develop leading to adaptive immunity directed towards the host. All paths lead to end stage fibrosis.
Dysregulation and persistent inflammation

In a healthy person the immune response is regulated to avoid autoimmunity. However, in a patient that has undergone HSCT there is an over-production of inflammatory cytokines due to tissue damage which recruits donor-derived immune responses to damage sites. This phase involves the dysregulation of the immune system. The thymus selects proper T-cells through positive and negative selection to inhibit auto or in the case of HSCT, allo-immunity. It is thought that one contributing mechanism is that which is mainly employed as a prophylaxis of acute GVHD, namely calcineurin inhibitors. These are used to prevent acute GVHD, however, in doing so they also hamper the proper selection of non-alloimmune T-cells in the thymus by diminishing the binding to MHC and allowing T-cells to pass through undetected allowing allo-reactivity to arise\(^{176}\). In addition to this, aGVHD leads to thymic cell damage of both cortical and medullar cells leading to loss of both central and peripheral tissue tolerance such as that involved in chronic GVHD i.e skin, liver, salivary glands, lungs, eyes, lungs and gastrointestinal tract\(^{177}\). As mentioned before, another dysregulatory property of GVHD is the downregulation of Tregs and loss of B-cell tolerance. In this dysregulated immune milieu there is also loss of antibody titers to microbial patterns such as lipopolysaccharide which leads to chronic GVHD associated immune deficiency and hypogammaglobulinemia\(^{178}\).

Fibrosis

As the inflammatory response persists in chronic GVHD, tissue repair and recovery is hampered. The maintenance of tissue homeostasis is imperative for host-defence. Dysregulated immunity eventually leads to fibrosis (scarring). In normal wound healing the platelets are exposed to sub-endothelial tissue factor and anchor to the damage site via von Willebrand factor and glycoprotein anchors. Prothrombin, a coagulation factor present in the blood comes into contact with the platelet surface and is cleaved to thrombin. Thrombin is a serine protease which can cleave soluble fibrinogen to fibrin strands further strengthening the platelet-fibrin plug. Activated platelets release PDGF, a chemoattractant for inflammatory cells, and TGF\(\beta\) which in turn further stimulates local fibroblasts to produce more collagen and extracellular matrix (ECM). However, as the inflammatory response persists myeloid cells secrete soluble factors (TNF\(\alpha\), IL-1\(\beta\) and IL-6) further driving fibrosis. Tissue resident macrophages are also a major source of TGF\(\beta\). Adaptive immune cells such as Th17CD4+ T-cells are recruited to injury sites. All the above mentioned induce fibroblasts to produce more ECM\(^{179}\). Interactions between donor-derived T and B-cells in secondary lymphoid organs
(lymph nodes, tonsils, spleen, mucosa-associated lymphoid tissue and Peyers patches in the small intestine) further perpetuate the alloreactive nature to injury sites eventually leading to cGVHD. Reparative pathways remain insufficient.

Fibrosis is involved in all autoimmune disorders from rheumatoid arthritis to autoimmune hepatitis. Allo-reactivity and organ damage involved in cGVHD also seem to be largely due to fibrosis. One organ involvement is that of the lungs. A steady decline of lung function in the months to years after HSCT is termed as lung-GVHD and can be both obstructive, involving the peribronchiolar area, and restrictive, involving the interstitial area of the lungs. The most common is the obstructive presentation coined as Bronchiolitis obliterans Syndrome (BOS). Both presentations lead to fibrotic changes in the affected areas of the lung. This tri-phasic model of cGVHD (injury, persistent inflammation and fibrosis) can be applied to any organ manifestation of cGVHD.

2.3 CLINICAL ASPECTS

The reported incidence of cGVHD after HSCT is between 6% and 80% with a median of 50% and the syndrome seems to clinically manifest around three months after HSCT. Three types of onset exist: 1. De novo onset of cGVHD which is not preceded by acute GVHD, 2. Quiescent onset which is preceded by prior acute GVHD with full resolution of symptoms and 3. Progressive onset in which acute GVHD gradually develops into cGVHD with or without concurrent or residual aGVHD symptoms. Of these, quiescent onset is the most common, followed by de novo and finally progressive. The most common presentation of the syndrome is coined as “classic” cGVHD in which there is no clinical sign of co-existing aGVHD. The less common presentation is termed “overlap” cGVHD syndrome in which one finds co-existing signs of aGVHD. The most common sites involved at the initial diagnosis of chronic GVHD are skin (75%), mouth (51-63%), liver (29-51%), gastrointestinal tract/weight loss (23-45%) and eye (22-33%). Other less frequent manifestations include lung (4-10%), female genital tract (<5%) and joints/fascia (4-10%). Chronic GVHD is further classified according to activity into mild, moderate or severe cGVHD. The most common is moderate cGVHD (70%) followed by mild (10-20%) and severe (10%). Recently it has been shown that female genital cGVHD is highly underdiagnosed and the true incidence could affect well over 50% of women undergoing
HSCT\textsuperscript{189}. Male genital involvement which presents with lichenoid lesions, phimosis and contractures is also underreported\textsuperscript{190,191}.

2.3.1 CLINICAL SIGNS OF ORGAN INVOLVEMENT

All individual organ involvements are scored 0-3 depending on severity. The final global assessment of cGVHD is obtained by tabulating all individual organ scores to finally determine whether a patient has mild, moderate or severe cGVHD\textsuperscript{138,192}. Skin involvement ranges from lichen-planus-like lesions (violaceus and flat) to scleroderma with ulcerations. Oral involvement ranges from mouth dryness with lichenoid buccal plaques to ulceration with pain and fibrosis of buccal tissues causing decreased jaw range of motion and limited oral intake. Liver is characterized by elevated liver enzymes (ASAT, ALAT, ALP) and bilirubin. These biochemical signs can be very difficult to differentiate from toxicity due to medication or hemosiderosis and the diagnosis of cGVHD may warrant a liver biopsy. Gastrointestinal involvement presents with symptoms ranging from dysphagia, diarrhoea and nausea with weight loss of under 5% to significant weight loss of over 15% requiring nutritional supplement. Chronic GVHD of the eyes manifests with xerophthalmia and keratoconjunctivitis sicca requiring frequent eye drop administration and, in severe cases, special eyewear to read or work so as to relieve the pain that is present. Lung involvement can range from being asymptomatic with significant decrease of forced expired volume on a lung function test (<75%) to severe symptoms of shortness of breath during rest and requiring oxygen\textsuperscript{193}. The main consequence of chronic GVHD in joints and fascia is impaired range of movement in shoulders, wrists, fingers and ankles using the P-ROM scale\textsuperscript{194}. Finally, genital involvement ranges from dryness of the mucosa to strictures requiring dilator use by an experienced gynecologist\textsuperscript{195}.

2.3.2 RISK AND PROGNOSTIC FACTORS

Risk factors for cGVHD include high recipient age, prior acute GVHD, female donor to male recipient, HLA disparity between recipient and donor and use of peripheral blood as a source of stem cells\textsuperscript{181–183,196,197}. The conventional classification of limited versus extensive chronic GVHD was proposed in 1980 on the basis of only 20 cases\textsuperscript{134}. Studies have shown that after diagnosis of cGVHD according to the old Seattle criteria certain prognostic factors present at diagnosis of cGVHD could predict lower overall survival\textsuperscript{134,198,199}. These factors include a low platelet count, extensive skin involvement and low performance status at diagnosis.
The NIH constructed new criteria in 2005 in part due to the old criteria focusing firmly on a cut off of 100 days after HSCT\(^ {138} \). The old criteria were imprecise and oblivious to the distinct organ manifestations of acute or chronic GVHD. The new criteria were also developed as a means to define cGVHD severity and providing indication for systemic IS. However, the criteria were based upon expert opinion and required validation. While being very comprehensive, a full usage of all criteria during one out-patient visit proved very time consuming and demanding. To date, studies have validated the NIH criteria as significant parameters for overall survival and non-relapse mortality\(^ {200,201} \). A low platelet count and severity of cGVHD according to the NIH score has been confirmed to significantly impact overall survival\(^ {202} \). Overlap syndrome, defined as having cGVHD with concurrent clinical symptoms of aGVHD, is new to the NIH score and is associated with an adverse prognosis\(^ {203} \).

### 2.4 FIRST LINE TREATMENT

For mild cGVHD a local topical treatment such as corticosteroid cream/ointments is utilized. Other topical treatments include immunosuppressive eye drops, corticosteroid mouth wash, inhalations and corticosteroid vaginal creams. Moderate to severe cGVHD should be treated with potent and systemic immunosuppressive therapy\(^ {184} \). The mainstay of treatment has been corticosteroids at a dosage of 0.5-1mg/kg daily. This is gradually tapered if and when the clinical symptoms subside to prevent long term side-effects such as osteoporosis, cataract formation, risk for infection and diabetes. Patients with cGVHD and on IS receive preemptive treatment for fungal infection, viral infection and in some cases bacterial infection. This supportive care is site specific and varies. The corticosteroid treatment can be combined with calcineurin inhibitors to reduce the dosage and time of exposure to corticosteroids which are considered to have more detrimental side effects\(^ {204} \). It should be mentioned that a recent observational report has shown that a third of patients with moderate-severe cGVHD on IS are still alive after 5 years, half of which have discontinued IS. One third relapsed or died and one third remained on IS with up to 5 lines of treatment\(^ {205} \). Another larger review showed that 50% of patients have full resolution of cGVHD and are permanently off IS within 7 years from start of IS. Approximately 10% require continued systemic treatment for an indefinite period beyond 7 years and the remaining 40% have recurrent malignancy or die within 7 years during treatment of chronic GVHD\(^ {201} \).

Combination therapy with other immunosuppressive agents is often utilized in order to minimize toxicity caused by prolonged corticosteroid treatment\(^ {206} \). Randomized trials have thus far showed no benefit from adding other immunosuppressive agents such as
azathioprine, thalidomide, mycophenolate mofetil or hydroxychloroquine to corticosteroids\textsuperscript{207–210}. Finally a trial comparing cyclosporine plus prednisone versus prednisone alone for treatment of cGVHD did actually indicate a steroid sparing effect of cyclosporine without increased recurrence of underlying malignancy, however, the combination did not improve overall survival\textsuperscript{211}.

2.5 SECOND LINE TREATMENT
The median duration of IS for treatment of cGVHD is 2-3 years which warrants concern for risk of infections and relapse of underlying malignancy\textsuperscript{212}. Approximately half of the cGVHD patients respond to first line treatment but the remaining steroid-refractory (SR) cGVHD patients have a very poor prognosis with long term survival rates of less than 40%\textsuperscript{213–215}. The NIH consensus on chronic graft-versus-host disease (cGVHD) has defined SR-cGVHD as: Steroid-refractory chronic GVHD during first-line treatment may be defined when manifestations progress despite the use of a regimen containing prednisone at ≥1 mg/kg/day for at least 1 week or persist without improvement despite continued treatment with prednisone at ≥0.5 mg/kg/day or 1 mg/kg every other day for at least 4 weeks\textsuperscript{216}. Steroid-dependent chronic GVHD may be defined when prednisone doses >0.25 mg/kg/day or >0.5 mg/kg every other day are needed to prevent recurrence or progression of manifestations as demonstrated by unsuccessful attempts to taper the dose to lower levels on at least 2 occasions, separated by at least 8 weeks\textsuperscript{217}. Therefore, other seemingly less immunosuppressive alternatives have been employed of which the one with most evidence for positive results remains Extracorporeal Photopheresis (ECP)\textsuperscript{218}.

In ECP, mononuclear cells are collected and irradiated with UV-A light after addition of 8-methoxypsoralen (8-MOP). This can be performed in two different ways, either using an in-line discontinuous flow cell separator system (i.e. the Therakos system) where cell irradiation is incorporated, or by first collecting cells using conventional apheresis, adding 8–MOP and irradiating the cells in the laboratory prior to infusion\textsuperscript{219}. The cells are accessed via peripheral vein or more commonly a central venous line. Treatment with ECP has shown promising results in terms of response rates involving skin (70%), oral mucosa(70%), liver (50%) and lungs (50%) without increasing risk of infection even when used as a salvage treatment for patients who progress during standard IS or are steroid dependent\textsuperscript{220}. The treatment is well-tolerated, but is expensive and demands extra resources in terms of staff and apparatus. ECP seems to be a well-established second line treatment for cGVHD\textsuperscript{221}. This is important since 50-60% of cGVHD patients require second line treatment\textsuperscript{222}. The general perception remains
that those who do not respond to first line treatment have a poor response rate to second line treatment and high mortality\textsuperscript{223}.

Mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase which is downstream of the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (akt) pathway\textsuperscript{224,225}. mTOR is the catalytic subunit of mTOR complex 1 (mTORC1) and 2 (mTORC2) and, once activated, plays a major role in the activity of these enzymatically distinct complexes regulating cell metabolism, proliferation and survival\textsuperscript{226}. Dysregulation of either pathway can lead to malignant cell transformation and the mTOR inhibitor sirolimus, which solely affects mTORC, has potent antineoplastic properties used in breast cancer, neuroendocrine tumours and renal cell carcinoma\textsuperscript{227–229}. In addition to antineoplastic features, mTOR inhibitors also have immunosuppressive properties inhibiting T cell proliferation\textsuperscript{230}. It has been administered both to prevent and to treat aGVHD with the main drawback being side-effects such as hyperlipidemia, transplant-associated-microangiopathy (TMA), sinusoidal-obstructive-syndrome (SoS), hypercholesterolemia and cytopenias without improving overall survival\textsuperscript{231}. Patients receiving mTOR as prophylaxis for GVHD have lower incidence of aGVHD grades II-IV\textsuperscript{232}. Response rates of patients with steroid refractory aGVHD treated with mTOR are approximately 42%\textsuperscript{233}. mTOR inhibitors may also play a role in sclerodermatous cGVHD by inhibiting fibroblast proliferation via PDGF. However, it could also impair wound healing and should be used with caution in patients with manifest wounds\textsuperscript{234}. Phase II trials to treat steroid refractory cGVHD with sirolimus have reported varied results with response as high as up to 63%\textsuperscript{235} with an unacceptably high rate of cytopenias, renal insufficiency, TMA and infections due to the treatment combination of mTOR inhibitors with CNI\textsuperscript{236}. When examining the use of everolimus or sirolimus as a single agent or in combination with steroids to treat sclerodermatous cGVHD, one has shown a low incidence of TMA mainly related to high trough plasma concentrations of mTOR inhibitors with relatively high overall response rate of up to 76%\textsuperscript{237}. A similar study based on everolimus showed response rates in SR-cGVHD of 43% with side effects including infections and thrombocytopenia\textsuperscript{238}. Both studies were retrospective analyses and the first study had a high response rate with very few complete remissions. While mTOR inhibitors have been examined as second line treatments, the frequency and severity of side effects has limited their use nowadays and are mostly considered in the setting of relapsed lymphoma with concomitant cGVHD\textsuperscript{239}. 
Guanosine nucleotides are essential for the production of nucleic acids, both DNA and RNA. Mycophenolate mofetil (MMF) is a prodrug of mycophenolic acid and, is an immunosuppressant by inhibiting inosine-5’-monophosphate (IMP), an enzyme essential for the de novo synthesis of guanosine monophosphate (GMP). Lymphocytes are dependent on de novo synthesis of GMP rendering them especially susceptible to the actions of MMF.

The first publication from 1999 showing a 46% response rate in SR-cGVHD with a well-tolerated profile. Many phase II studies have shown promising results with response rates ranging from 40 to 75% and potentially resulting in steroid sparing effects. Side effects included diarrhoea which could result in drug discontinuation, CMV reactivation and leukopenia. All studies have reported varying levels of neutropenia and thrombocytopenia. One randomized control trial evaluating the addition of MMF to first line treatment of cGVHD has been conducted. The study failed to reach its primary end point of cGVHD resolution with complete discontinuation of immunosuppressive treatment within 2 years. However, it did show that relapse rates and infections were higher in the group treated with the combination of MMF/CNI/prednisone, although no statistically significant results could be reported.

As previously mentioned, an abnormal activation of PDGFR and TGFβ pathway is observed in patients with cGVHD indicating a pro-inflammatory and pro-fibrotic microenvironment. Tyrosine kinase inhibitors (TKI) are a class of drugs that inhibit oncogenic tyrosine kinase and the first generation drug Imatinib (Glivec®) was used to successfully treat chronic myelogenous leukaemia. TKI also have potent anti-fibrotic and anti-inflammatory effects inhibiting both aforementioned pathways. Second generation TKI (Dasatinib and Nilotinib) have been used to treat cGVHD. Imatinib has been tested in a phase II trial in 19 patients with steroid-refractory cGVHD and fibrotic skin lesions. The result showed overall response rate at 6 months was 79%, with 7 complete remission (CR) and 8 partial remission (PR). At a median follow-up of 17 months, 16 patients were alive, 14 still in CR or PR. As it was a phase II study, the primary end-point was safety was safety and the study could show that Imatinib was well tolerated as only 3 patients were discontinued due to toxicity.

The JAK family of non-receptor protein tyrosine kinases (JAK 1-3 and TYK2) are essential for transducing extracellular signals to intracellular mechanisms. They are key to cell growth, development, differentiation and survival of a variety of cells, in particular immune cells and hematopoietic stem cells. Germline JAK1 and JAK 2 deficiency is developmentally lethal. JAK3 is involved in the regulation of common gamma chain cytokines which are required by
both T and NK cell which leads to a functional deficiency, yielding the disease termed severe combined immunodeficiency syndrome\textsuperscript{252}. TYK2 mutations have been implicated in autosomal dominant hyperimmunoglobulin E or Job’s Syndrome, a condition in which the patient develops hypereosinophilia, eczema and recurrent pulmonary infections\textsuperscript{253}. Although there are ongoing attempts to inhibit JAK 1/2/3, the only clinical studies that have shown any benefit with regards to cGVHD are those where the drug ruxolitinib (FDA approved for intermediate to high risk myelofibrosis) was used to inhibit JAK1 and JAK2\textsuperscript{254–256}. Results showed promising partial remission and good tolerability. A recent larger single center retrospective study has confirmed the results of previous phase II studies for Ruxolitinib in cGVHD\textsuperscript{257}. Experience thus far shows good tolerability with main side effects including cytopenia and viral reactivation. A recent review highlights the use of JAK-inhibitors in one of the case reports and mentions that second line treatment remains a choice based on practical factors\textsuperscript{258}.

As previously discussed, there seems to be a deficiency in Treg numbers and consequently one can hypothesize that IL-2 is lacking. In an attempt to booster Treg numbers and alleviate symptoms in patients with steroid-refractory chronic graft-versus-host disease low dose Interleukin-2 was administered over a period of time to 29 patients\textsuperscript{148}. Normally CD4+CD25+FOXP3+ Tregs account for 5-10\% of circulating CD4+ T cells. The patients received daily injections for 12 weeks and the results showed that half of patients improved cGVHD symptoms with partial response whilst none progressed. The ratio of Treg:Tcon (conventional CD4+ T cell) increased to five times baseline value without decreasing Tcon numbers. The same group could show that extended low dose IL-2 treatment with 1\times10^6 IU Proleukin®/m\textsuperscript{2}/day could continue this trend and maintain response in cGVHD\textsuperscript{259}. In a more recent randomized trial, leukaemia patients either received low dose IL-2 prophylactically from day +30 for 14 days or they did not. The study demonstrated lower incidence of moderate-severe cGVHD in the IL-2 arm (33\% vs 57\%) with higher GVHD progression-free survival without lowering the cumulative incidence of leukaemia relapse. This study did show a non-significant higher relapse trend in the IL-2 arm\textsuperscript{260}.

In general, second line agents should include those with an adequate safety/toxicity profile in which treatment will not further abrogate quality of life and with well documented anti-GVHD activity from at least phase II studies.
2.5.1 EXTRACORPOREAL PHOTOPHERESIS

Preceding its clinical approval by the Food and Drug Administration in 1988 for cutaneous T-cell lymphoma (CTCL), studies had shown that both leukapheresis and methoxsalen (8-methoxypsoralen, 8-MOP) photochemotherapy (PUVA) could independently be used in a palliative fashion for CTCL. Edelson and colleagues based their new methodology on these observations and developed an ex-vivo method in which leukapheresis was combined with the DNA cross-linking effects of 8-MOP and ultraviolet-A (UVA) rays to activate 8-MOP ex-vivo. In the same study they showed that a few of their patients went into remission after treatment of less than 5% of their estimated total burden of malignant cells. This suggested that ECP had triggered a potent immune reaction against malignant CTCL cells. A total of 27 of the 37 refractory patients responded to ECP.

ECP relies on collection of mononuclear cells using either continuous or discontinuous cell separators and then ex-vivo exposing the buffy coat to a photosensitizing agent, 8-MOP, followed by photoactivation with UV-A irradiation and then re-infusing the photoactivated product. Apart from apoptosis of the irradiated mononuclear cells and preserved antigenicity, little is known about the definitive mechanisms of ECP in immune modulation. Other observations include induction of regulatory T-cells, maturation of dendritic cells and up-regulation of anti-inflammatory cytokine production. The hypothesis that ECP modulates other lymphocytic cells than those directly treated was confirmed in subsequent mouse models. A congenic derived ECP product was re-infused into other recipient mice with evident GVHD from the same congenic litter resulting in improved GVHD symptoms. This demonstrated that cells not exposed to ECP had been affected due to the evident improvement in GVHD status. Subsequent mouse model could demonstrate a three-fold decrease in IFNγ+CD8+ T cells that had not been exposed to 8-MOP or UVA, after ECP treatment. Regulatory T cells are induced in both mouse models and clinical trials. In the mouse model one could show stimulation with IL-12 (largely produced by dendritic cells in order to commit T cells to Th1) could prevent UVA-associated DNA damage. This finding shows that the ECP treated dendritic cells (DC) seem to be of most importance in the up-regulation of Tregs. DCs survive after infusion for 72-96 hours whereas T cells undergo cell death after 24 hours. During this period damaged but functional dendritic cells are loaded with self-antigen however; induce Tregs rather than T effector cells. In chronic GVHD the B-cells are of importance and ECP seems to decrease BAFF levels in patients with a clinical improvement. While the mechanism remains unclear, a main source of BAFF are DCs.
As the mechanistic knowledge increased so did the application of ECP in other areas of T cell mediated diseases that include rheumatoid arthritis, scleroderma, systemic lupus erythematosus, solid organ graft rejection and graft-versus-host disease\textsuperscript{218,272,273}.

The most commonly affected organ in chronic GVHD remains the skin. Responses to ECP treatment in patients with steroid-dependent or steroid-refractory cGVHD vary from 40 to 100\% in phase II studies\textsuperscript{273–276}. The same studies have shown favourable response rates for cGVHD of the liver, eyes, mouth and lungs. Gut and lung involvement have lower response rates of approximately 50\%. ECP has shown a beneficial steroid-sparing effect in several studies and is considered a well-tolerated, non-immunosuppressive second line treatment for cGVHD\textsuperscript{277–279}. Other second line treatments are depicted in Table 2.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Recommendation grade</th>
<th>Response rate</th>
<th>Side effects in more than 25% of treated patients</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroids</td>
<td>B</td>
<td>n.a.</td>
<td>Osteoporosis, osteonecrosis, diabetes mellitus</td>
<td>The main drug in cGVHD therapy; strategies to reduce use due to SEs very important</td>
</tr>
<tr>
<td>Ibrutinib</td>
<td>C-1</td>
<td>~50-75%</td>
<td>bruising, diarrhea, infections</td>
<td>FDA approved as 2nd line treatment of cGVHD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~15-7-25% CR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photophereses</td>
<td>C-1</td>
<td>~60-70%</td>
<td>Infections of the central venous access (if applicable)</td>
<td>Venous access required, steroid-saving effect, good tolerability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~30% CR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTOR Inhibitors (Sirolimus, Everolimus)</td>
<td>C-1</td>
<td>~60%</td>
<td>Transplant-associated microangiopathy, hyperlipidemia, cytopenia</td>
<td>Increased risk of microangiography when combined with CNI, regular examination of blood levels required</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~20% CR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMF</td>
<td>C-1</td>
<td>~50%</td>
<td>GI SEs, risk of infection (viral) and increased risk of relapse</td>
<td>Steroid sparing activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~10% CR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNI (Cyclosporin, Tacrolimus)</td>
<td>C-1</td>
<td>n.a.</td>
<td>Renal toxicity, hypertension</td>
<td>Reduces steroid use, examination of blood levels required</td>
</tr>
<tr>
<td>MTX</td>
<td>C-2</td>
<td>~50%</td>
<td>Cytopenia</td>
<td>Best results in mucocutaneous cGVHD, reduces steroid use, contraindicated in the presence of pleural effusions or ascites</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~10 - 20% CR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>C-2</td>
<td>~65% (PR only)</td>
<td>fever, malaise, and fatigue</td>
<td>Applied in sclerodermoid skin disease</td>
</tr>
<tr>
<td>Ruxolitinib</td>
<td>C-2</td>
<td>n.a. (retrospective analysis)</td>
<td>Increased risk for viral reactivation, bacterial infection, hepatotoxicity</td>
<td>Prospective data pending</td>
</tr>
<tr>
<td>Drug</td>
<td>Level</td>
<td>Use for</td>
<td>Side Effects</td>
<td>Other Effects</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------</td>
<td>---------</td>
<td>--------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>C-2</td>
<td>2nd line</td>
<td>Cytopenia, neuropathy</td>
<td>Trial was performed in first line treatment</td>
</tr>
<tr>
<td>High dose-Steroids (10mg/kg/day, 4 days)</td>
<td>C-2</td>
<td>50-75% (only PR)</td>
<td>Infections</td>
<td>Rapid control of cGVHD</td>
</tr>
<tr>
<td>Thoraco-abdominal Irradiation (1Gy)</td>
<td>C-2</td>
<td>~ 50%</td>
<td>Cytopenia</td>
<td>Best results for fasciitis and mucocutaneous cGVHD</td>
</tr>
<tr>
<td>Hydroxychloroquin</td>
<td>C-2</td>
<td>~ 25%</td>
<td>GI side effects</td>
<td>Best results for mucocutaneous and hepatic cGVHD</td>
</tr>
<tr>
<td>Pentostatin</td>
<td>C-2</td>
<td>~ 50%</td>
<td>Cytopenia, risk of infection</td>
<td>Best results in children</td>
</tr>
<tr>
<td>Rituximab</td>
<td>C-2</td>
<td>~ 50%</td>
<td>Risk of infection</td>
<td>Effective in manifestations associated with autoantibodies and scleroderma cutaneous involvement</td>
</tr>
<tr>
<td>Imatinib</td>
<td>C-2</td>
<td>~ 50%</td>
<td>Fluid retention</td>
<td>Efficacy demonstrated mainly in scleroderma cGVHD and bronchiolitis obliterans</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>C-3</td>
<td>~ 20-30% (only PR)</td>
<td>Neurotoxicity, drowsiness, constipation</td>
<td>Treatment for simultaneous cGVHD and recurrent multiple myeloma</td>
</tr>
<tr>
<td>Azathioprin</td>
<td>C-3</td>
<td>n.a.</td>
<td>cytopenia, risk of infection, secondary malignancies</td>
<td>Increased risk of malignant disease of the oral mucosa</td>
</tr>
<tr>
<td>Retinoids</td>
<td>C-3</td>
<td>~ 60% (only PR)</td>
<td>Skin toxicity, hyperlipidemia</td>
<td>Effective in scleroderma cutaneous involvement</td>
</tr>
<tr>
<td>Abatacept</td>
<td>C-3</td>
<td>~ 40%</td>
<td></td>
<td>Effective in mucocutaneous and pulmonary involvement</td>
</tr>
<tr>
<td>Regulatory T cells</td>
<td>C-4</td>
<td></td>
<td></td>
<td>Currently explored in several clinical trials</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>C-4</td>
<td>n.a.</td>
<td></td>
<td>Repetitive application required</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>C-4</td>
<td>n.a.</td>
<td>Infectious risks</td>
<td>Last resort for refractory cGVHD</td>
</tr>
<tr>
<td>Etanercept</td>
<td>C-4</td>
<td>n.a.</td>
<td>Infectious risks</td>
<td>May be used to treat mixed acute and chronic GVHD or pulmonary or GI manifestations of cGVHD</td>
</tr>
</tbody>
</table>

**Table 2.** Current second line treatments as adapted from the EBMT 2019 handbook and Wolff D et al. B: should generally be used; C-1: use in second-line therapy justified; C-2: use after failure of second-line therapy justified; C-3: should only be used in specific circumstances, due to unfavorable risk profile; C-4: experimental, should only be used in clinical trials and individual cases; II: Evidence from >1 well-designed clinical trial without randomization, from cohort or case-controlled analytic studies (preferable from >1 center) or from multiple time series; III-1: several reports from retrospective evaluations or small uncontrolled clinical trials; III-2: only one report from small uncontrolled clinical trial or retrospective evaluations; III-3: only case reports available MMF: mycophenolate mofetil; CNI: calcineurin inhibitors; MTX: methotrexate; CR: complete remission; GI: gastrointestinal; SE: side effect; n/a: not available.
2.6 EMERGING THERAPIES

Pre-clinical mouse models have shown that stimulation with G-CSF leads to elevated IL-17 levels due to commitment to TH17 lineage. These cells quickly sequester into target organs such as lungs, liver, skin and intestine due to chemotherapy induced cytotoxicity and inflammation which they home to\textsuperscript{170}. Both bronchiolitis and scleroderma have been shown to be IL-17 dependent\textsuperscript{216}. The IL-17/IL-17R blocking monoclonal antibodies (secukinumab, brodalumab, ixekizumab) are used to treat psoriasis\textsuperscript{282}. In murine model studies they have shown promising results with the decrease of pulmonary cGVHD\textsuperscript{283}.

More clinical trials attempting to inhibit inflammatory cytokines (IL-1B and IFNα) in rheumatic patients are ongoing and could implicate future therapeutic options for cGVHD. Proteasome inhibitors such as bortezomib or carfilzomib, normally used in the treatment of multiple myeloma, have a positive effect on sclerodermatous cGVHD in murine models\textsuperscript{284}. Brutons tyrosin kinase and IL-2 inducible kinases are both inhibited by the drug ibrutinib which is used in CLL treatment. This denotes its effect in blocking both B and T cell signaling and studies have shown promising clinical effect in cGVHD\textsuperscript{285}. Finally in the field of cellular immunotherapy, several ongoing trials for donor origin Treg infusions are listed at Clinicaltrials.gov.
3 GVL

Chronic GVHD remains the most relevant cause of non-relapse mortality and morbidity in approximately 25% of patients that have undergone allo-HSCT\textsuperscript{286}. Chronic GVHD incidence has increased over the past decades perhaps due to higher patient age, PBSC, more frequent utilization of unrelated donors and RIC regimens\textsuperscript{287}. However cGVHD does have a protective anti-leukaemic property labelled as “graft-versus-leukaemia or GvL” in which relapse incidence is lower in patients who develop cGVHD\textsuperscript{288}. This protective effect of cGVHD is hampered in more severe stages of cGVHD due to the inherent morbidity and mortality from severe cGVHD\textsuperscript{289}. It is therefore important to be able to control the severity of cGVHD and perhaps to induce it during certain clinical scenarios such as impending relapse of underlying malignancy.

A central mechanism responsible for GvL is the interplay between donor and host which also controls the extent of cGVHD. It is well known that at the time of transplant, all DCs are of recipient origin. When activated by danger signals due to tissue damage and pathogens, DCs will present endogenous antigens as well as cross-present antigens derived from the non-hematopoietic tissues and pathogens. The presentation will primarily be directed towards donor-derived T-cells (naïve, αβ and γδ T cells)\textsuperscript{290,291}. Due to this knowledge donor lymphocyte infusions (DLI) are readily utilized for prophylactic scenarios, pre-emptive scenarios and in combination with other chemotherapeutic drugs upon clinically manifest relapse\textsuperscript{292,293}. Of the T-cell subsets, it is evident that donor-derived αβ cells yield a more severe GVHD reaction with more GvL and the γδ subset seems more efficient in suppressing CMV reactivation\textsuperscript{294}.

Timing of DLI taken into consideration the maturing immune system is also an important factor. During the first 6 months most of the DCs are replaced by donor-derived DCs and this is reflected by the ability to administer much higher doses of αβ T cells at 6 months post-HSCT (1x10\textsuperscript{6}/kg) compared to at 3 months (1x10\textsuperscript{5}/kg) without developing severe cGVHD\textsuperscript{295}.

At our centre, DLI is mostly utilized pre-emptively when a patient has a consistently positive MRD, usually from underlying AML or MDS. We have also administered DLI together with other drugs such as azacytidine (a hypomethylating agent) in the setting of MDS/AML relapse.
4 AIMS

The principal aim of this thesis is to expose the clinical intricacies of chronic graft-versus-host disease and to improve the understanding of the syndrome in order to provide better health care for patients. In this pursuit the following specific aims were the subject of the research presented in this thesis:

1. **To identify risk factors for developing chronic GVHD:** For decades an HLA-identical sibling donor has been the gold standard. In *paper I* we aimed to identify significant risk factors for the development of cGVHD comparing the URD and sibling groups. In *paper II* we continued the search for risk factors from a multi-centre perspective. The purpose of this study was to obtain clinical tools which could be used early on, and perhaps before development of cGVHD, to identify patients at risk and therefore employ a more vigilant surveillance.

2. **To evaluate prognostic factors of cGVHD using NIH criteria:** In 2005 the NIH developed an internationally accepted consensus criteria for the diagnosis of cGVHD. This set of clinical criteria was the first of its kind and the reception was positive. Although being very detailed and encompassing all aspects of cGVHD, the main drawback was the complexity and the time consumption when using these criteria. In *paper III* we attempted to evaluate the different NIH criteria with focus on NRM. We also attempted to simplify the diagnostic criteria to obtain a clinically more user friendly diagnostic tool which could pertain to the survival prognosis of cGVHD patients.

3. **To evaluate ECP treatment for cGVHD:** The prognosis of steroid refractory cGVHD remains poor in terms of overall survival and NRM. The most extensively utilised second line treatment internationally and at our own centre is ECP. We still did not know based on the 2005 NIH criteria which patients have the best response. In *paper IV* we aimed to evaluate ECP treatment in our own patient cohort to stratify response based on cGVHD organ involvement. This knowledge would allow us to properly select patients for ECP.
5 PATIENTS AND METHODS

5.1 PATIENTS

**Paper I** we studied 537 consecutive adult patients transplanted for a malignant disease between the years 2000 and 2014 either with an HLA-identical sibling donor not receiving ATG (n=187) or with an HLA-A, -B, and -DR matched unrelated donor (URD) receiving ATG [Thymoglobulin, Genzyme, 4-8 mg/kg (n=350)]. Median age of patients was 49 (18-72) and median follow up was 5.65 years (0.3-15.3). Data was collected from the patient medical records at our centre. Acute and chronic GVHD were classified according to previously mentioned criteria\textsuperscript{120,138}.

**Paper II** and **III** was a retrospective multicentre study that utilized the same joint data-base. Eight hundred and twenty patients undergoing HSCT at three different centers from January 2000 to December 2006 were included. The analysis was restricted to patients surviving more than 100 days after HSCT (n=747). The number of patients from each center as follows: Karolinska = 425, Salamanca = 162 and Sant Pau = 160. Median age at the time of transplantation was 50 years. Median follow up was 41 months. The most common diagnosis was acute myeloid leukaemia in 24% of patients, acute lymphoblastic leukaemia in 11% and myelodysplastic syndrome in 11%. Twenty-seven percent of patients were in 1st or subsequent complete remission at the time of transplant. Eighty-four percent received hematopoietic stem cells from a related donor and 71% received reduced intensity conditioning regimens. Sixty percent of the patients received cyclosporine plus methotrexate and 27% received in vivo T-cell depletion. Chronic GVHD was retrospectively classified according to the NIH criteria and based on data gathered from patient charts\textsuperscript{138}.

**Paper IV** was a retrospective single centre analysis of ECP treatment as second line for cGVHD. During the period of 1998-2011 a total of 34 patients received ECP. In total, during this period, 881 patients underwent HSCT and 134 patients developed moderate to severe cGVHD. Chronic GVHD was retrospectively categorized according to the NIH consensus criteria\textsuperscript{138}. Of the 34 treated patients, 7 had moderate and 27 had severe cGVHD. Other patients that did not respond to first line treatment with corticosteroids and CSA received either MMF or research oriented drugs, treatment was selected at the discretion of the attending physician. A representative age- and disease-matched control group was selected from the local HSCT quality registry based on similar cGVHD status. The controls and
patients were matched based on the global NIH score, in which the controls had to have the same index organ severity as the patients.

All studies were conducted in accordance with the Helsinki declaration and approved by the regional ethical committee in Stockholm and Comité Ético CEIM (Comité de ética de la investigación con medicamentos) in Spain.

5.2 METHODS AND DEFINITIONS

Due to the retrospective nature of this thesis with limitations such as the re-evaluation of cGVHD according to the new NIH criteria we had to be systematic in our approach. Most patients were classified in their medical charts according to the old Seattle criteria\textsuperscript{134}. This had to be converted to the NIH criteria and the method used was a systematic review of all medical charts, blood analyses, lung function tests, radiological examinations, pathology reports and performance scores. At our center we used the Karnofsky performance score or the Eastern Cooperative Oncology Group (ECOG) score\textsuperscript{296,297}.

According to the NIH scoring system, mild cGVHD was diagnosed when only one or 2 organs or sites (except the lung) were involved, with no clinically significant functional impairment (maximum score 1 in all affected organs or sites). Moderate cGVHD involved at least one organ or site with clinically significant impairment but no major disability (maximum score 2 in any affected organ or site), or 3 or more organs or sites with no clinically significant functional impairment (maximum score 1 in all affected organs or sites). A lung score of 1 was also considered moderate cGVHD. Severe cGVHD was indicated by a major disability caused by cGVHD (score 3 in any organ or site). A lung score of 2 or over was also considered ‘severe cGVHD’\textsuperscript{138}.

Patients who received prednisone, or who were still on a therapeutic dose of cyclosporine due to prior aGVHD that had evolved into cGVHD without the resolution of symptoms, were considered as having ‘progressive cGVHD’. Patients who were on cyclosporine taper with a resolution of symptoms, or who were free from immunosupression at the time of diagnosis, were categorized ‘quiescent’, while those without a prior history of aGVHD were diagnosed with ‘\textit{de novo} cGVHD’\textsuperscript{138}. Otherwise, acute and limited \textit{versus} extensive chronic GVHD were graded by established criteria. Assigning patients to the various categories for the different classifications was done based on organ involvement observed within the first month of cGVHD diagnosis. The disease response was generally evaluated five weeks after the
introduction of steroids and then every three months until the end of treatment. In papers II and III overlap syndrome and delayed acute GVHD was defined according to the NIH criteria\textsuperscript{138}.

All patients received antibacterial, antifungal and antiviral prophylaxis according to standard procedures.

The new composite end point, GRFS, was used in paper I and is defined as survival without relapse, severe acute and severe chronic GvHD after HSCT\textsuperscript{298}. Non-relapse mortality (NRM) was defined as “death due to causes unrelated to the underlying disease” and relapsing patients were censored at the time of relapse. GVHD-related mortality (cGVHD-RM) was calculated from the time to cGVHD onset until cGVHD related death and, defined as, “death due to causes directly related to GVHD according to primary physician criteria”. More specifically, among patients diagnosed with cGVHD, those deaths attributed to complications or failure in cGVHD-target organs as well as deaths related to immunosuppression, such as infectious complications in patients requiring treatment for cGVHD, were considered as cGVHD related mortalities. Overall survival (OS) was calculated from transplant until death from any cause, and surviving patients were censored at the last follow up. In addition, overall survival from cGVHD onset (OS-cGVHD) was also calculated from the time of cGVHD diagnosis until death from any cause.

5.3 STATISTICS

Mean and median values, as well as their 95% confidence intervals (CI) and ranges, were calculated for each continuous variable. The $\chi^2$ test was used to establish differences in the distribution of discontinuous variables, whereas Mann-Whitney’s U test was applied to compare continuous variables. All reported $P$ values are two-sided. $P<0.05$ was considered statistically significant. Overall survival was calculated using the Kaplan-Meier estimate. The log rank test was used for univariate comparisons. Patients who survived more than 100 days were evaluable for cGVHD. The incidences of cGVHD and its different subtypes were calculated from the time of transplantation using cumulative incidence estimates, taking competing events into consideration. Transplant-related mortality (TRM) and relapse were estimated using cumulative incidence curves.
In paper I univariate and multivariate risk-factor analyses for chronic GvHD were performed using the proportional subdistribution hazard regression model developed by Fine and Gray. Only patients surviving beyond 100 days after HSCT were included in the cGVHD analysis.

For papers II and III the starting point (Day 0) for the landmark analysis of the incidence of cGVHD-RM and NRM was the time of onset of cGVHD. The competing events were disease progression and death not related to cGVHD. Patients who were still alive and progression-free at the time of analysis were censored at the last follow up or at five years post-transplant. Univariate analyses of the variables that influenced cGVHD-RM and NRM were performed using proportional hazards models for competing risks (Gray’s test). The variables that showed at least a trend in univariate analysis \( (P<0.1) \) were used in a multivariate Cox’s proportional hazards regression analysis, checking for the assumption of proportional hazards over time for each tested variable. All factors that significantly or marginally \( (P<0.1) \) influenced the incidence or outcome of cGVHD in the univariate analysis were included in a multivariate analysis using a forward step Cox’s regression model. The statistical analyses were performed using SPSS version 18.0 (SPSS, Chicago, IL, USA), with the exception of the cumulative incidence plots which were carried out with NCSS 2004 (Number Cruncher Statistical System, Kaysville, UT, USA) and the univariate Gray’s test which was carried out using the Cmprsk package R software (The R Foundation, Vienna, Austria).

In paper IV Overall survival (OS) was calculated using the Kaplan-Meier method and compared by the log-rank test. Transplant-related mortality (TRM) was estimated using a nonparametric estimator of cumulative incidence curves, taking competing events into consideration. Categorical parameters were compared using the chi-square test, and continuous variables were compared using the Mann-Whitney test. When comparing parameters before and after ECP the Wilcoxon matched pair test was used. Analysis was performed using the cmprsk software package (developed by Gray, June 2001), Splus 6.2 software (Insightful, Seattle, WA) and Statistica software (StatSoft, Tulsa, OK).
6 RESULTS

6.1 RISK FACTORS FOR CGVHD

In paper I comparing between the HLA-identical sibling donors not receiving ATG \((n=187)\) with HLA-A, -B and –DR identical URD receiving ATG [Thymoglobulin, Genzyme, 4–8 mg/kg \((n=350)\)] showed that HSCTs with sibling donors resulted in significantly better 5-year OS (66 vs 58\%, \(P<0.05\)) and relapse-free survival (60 vs 50\%, \(P=0.025\)) for patients with sibling donors. However, TRM (17 vs 21\%, \(P=0.48\)) and relapse (24 vs 30\%, \(P=0.13\)) were similar.

The cumulative incidences of acute GvHD grades II–IV and III–IV were 47 and 19\% in patients with a sibling donor and 38 and 7\% in patients with an URD \((P=0.04\) and \(P<0.001\)), respectively. The cumulative 5-year incidence of chronic GvHD was higher in the sibling donor cohort (57 vs 28\%, \(P<0.001\)). The incidence of severe chronic GvHD was higher after sibling donor HSCT (12.4 vs 2.5\%, \(P<0.001\)). GRFS was 49\% in the sibling cohort and 48\% in the URD cohort, \(P=0.80\). During the first 3 months, the target blood CsA levels were 100 ng/mL in patients with a sibling donor and at 200–300 ng/mL in patients with a URD. In the absence of GvHD, CsA was discontinued at 3 months if a sibling donor was used and at 6 months if a URD was used. Factors associated with severe chronic GvHD within the sibling donor group were older patient age up to 50 years, \(\text{CD34}^+\) cell-dose >9.5 × 10⁶/kg and female donor to male recipient.

In paper II the use of ATG was a protective factor for cGVHD development. In multivariate analysis the following variables significantly influenced the risk of overall cGVHD: use of ATG (HR=0.41), higher patient age (in 10-year increments), prior acute GVHD and reduced intensity conditioning (RIC). When analysing risk factors for cGVHD after correcting differences between patients receiving RIC or MAC, it is apparent that RIC patients still have higher cGVHD incidence. The analysis was done to eliminate confounding factors. The overall incidence of severe cGVHD was 14\%. In multivariate analysis, female donor to male recipient, RIC and prior aGVHD significantly increased the risk of severe cGVHD while ATG remained a protective factor (HR=0.21). Based on our findings we developed a scoring system including significant risk factors from multivariate analysis with regard to severe cGVHD incidence.
Figure 3. Risk factor score for developing severe cGVHD including risk factors from multivariate analysis with female donor to male recipient, reduced intensity conditioning (RIC), anti-thymocyte globulin (ATG) and prior acute GVHD.

We continued with a multivariate analysis including only significant risk factors present at the time of transplantation. We found that older patient age, female-to-male donation, and RIC increased the risk of severe cGVHD, while ATG remained a protective factor. When two to three of these risk factors were present, there was a significant protective effect from ATG on the incidence of severe cGVHD at 5 years post-transplant. Relapse-free survival at 5 years was similar in patients developing mild or moderate cGVHD [59% vs. 64%, respectively], but significantly higher in these groups compared to patients without cGVHD or with severe cGVHD [RFS of 39% and 46%, respectively. ATG had no effect on RFS.

6.2 PROGNOSTIC FACTORS
Results were similar among the 3 centers in terms of survival (53%, 52% and 49% at five years, respectively) and NRM (26%, 21% and 30% at five years, respectively). The cumulative incidence of cGVHD was 48%. Incidence of mild, moderate and severe cGVHD
was 14%, 22% and 17% respectively. Fourteen percent of patients developed overlap syndrome while 13% developed delayed aGVHD. At the time of cGVHD diagnosis, 51% had involvement of 3 or more organs. We could show an onset of de novo, quiescent and progressive cGVHD in 18%, 22% and 8% of patients in this cohort.

Chronic GVHD-related mortality (cGVHD-RM) includes deaths due to causes directly attributed to complications or failure in cGVHD target organs. Deaths related to immunosuppression such as infectious complications were also considered cGVHD related mortalities. Causes of NRM included cGVHD-RM in 21 patients, invasive fungal infections in 10 patients, viral infections in 3 patients, respiratory failure in 3 patients, brain haemorrhage in 2 patients, finally, internal bleeding and heart failure in one case each.

Progressive type onset, overlap syndrome, 3 or more organs involved, severe cGVHD, ECOG≥2, platelets<100x10^{9}/L and higher NIH score in index organ at the time of cGVHD diagnosis all negatively affected cGVHD-RM in univariate analysis. It is worth mentioning that a high percentage of patients categorized as having overlap syndrome had a progressive type of onset so that both variables were highly correlated. In the multivariate analysis, performance status at the time of cGVHD diagnosis, platelet count and severe gut involvement significantly impacted cGVHD-RM.

Overall survival from cGVHD onset (OScGVHD) was also calculated from the time of cGVHD diagnosis until death from any cause. The same factors as those analysed for cGVHD-RM remained significant.

From our analysis we devised a simplified and prognostically significant scoring system which is less time consuming by utilizing the three factors of ECOG, platelet count and gut involvement. The new variable was developed by allocating a score of 0 or 1 for platelets over or below 100x10^{9}/L plus score 0, 1 or 2 for ECOG 0, 1 or 2 or over. A severe gut involvement was given 3 points. In fact, one could discern the group with the highest cGVHD-RM (score 3) by only combining ECOG and platelets. The point allocation was based on the best predictive value obtained by the multivariate analysis for each factor.
Figure 4. Landmark plot illustrating the impact of the new composite variable on the incidence of cGVHD-related mortality. The composite variable has a value of 0 to 3 based on the sum of platelet count < 100x10⁹/L (1 point) and the ECOG performance status (0, 1 or 2 points for ECOG of 0, 1 ≥2). In addition, patients with severe gut involvement were assigned 3 points irrespective of platelets and ECOG.
Figure 5. Overall survival from cGVHD diagnosis depending on the variable which resulted from combining ECOG, platelets and gastrointestinal involvement. The four graphs show overall survival for patients with a score of 0 (84%), 1 (64%), 2 (43%) and ≥3 (0%).

Furthermore, the combined variable ECOG with platelets also identified different subgroups of patients in terms of survival within the different NIH categories. Therefore, for patients with mild cGVHD, the combined variable identified patients with 90%, 61%, 57% and 25% OS (P<0.001). The corresponding values for patients with moderate cGVHD were 93%, 65%, 44% and 0% OS (P=0.001). For patients with severe cGVHD the OS rates were 66%, 58%, 38% and 0% (P<0.001).

6.3 EVALUATION OF SECOND LINE TREATMENT

Most patients in paper IV had cGVHD involving 1 (n = 9) or 2 (n = 8) organs. Furthermore, 8 patients had 3 organs involved, 4 patients had 4 organs involved and 5 had 5 to 7 organs involved. Skin cGVHD was seen in 18 patients. Seven patients had moderate cGVHD and 27 patients had severe cGVHD. Most patients (80%) had been on immunosuppressive treatment with prednisone (1-2 mg/kg) and CsA before starting ECP. The remaining 20% had different regimens including, among others, sirolimus, tacrolimus and MMF combined with prednisone.
ECP was performed every week on two consecutive days until a clinical response was achieved, and then tapered by slowly extending the treatment intervals (every other week and eventually every four weeks). Evaluation of ECP treatment was based on medical records and NIH cGVHD classification at set time points: 8 weeks and 6 months after initiation of ECP. A complete response (CR) to ECP was defined as full resolution of cGVHD. If improvement in cGVHD was observed with a decrease of ≥ 1 point on the organ-specific NIH cGVHD score, this was defined as a partial response (PR). The definition of stable disease (SD) included no observed change in cGVHD activity and progressive disease (PD) was defined as progressing cGVHD activity during or up to 8 weeks after cessation of ECP treatment.

Overall survival for all 34 patients in this study was 82% at one year and 58% at 5 years after initiation of ECP. Median time to cGVHD onset was 200 (range 67-1222) days after HSCT. ECP was initiated within median 161 (range 10-1421) days after the onset of cGVHD. The median number of ECP treatments was 22 over median 26 weeks. The ECP treatments were well tolerated and no side effects were reported during cell infusions. Responders for CR, PR and SD were 15%, 53% and 24% respectively and in line with a recent longitudinal follow-up. Three patients (9%) suffered from progressive disease during ECP. OS was higher and TRM lower in CR/PR group compared to SD/PD and control group. Platelet counts and albumin levels were significantly higher in responders after ECP treatment compared to those with SD/PD. Patients in the CR/PR group received treatment for a shorter time period than those with SD/PD. There was a transient drop in leukocyte count in both groups which normalized upon cessation of ECP.

The most common organ involvement in partial responders was skin cGVHD with an OSS of 2-3 (n = 13). The majority of such patients had a combination of skin and liver involvement. Patients with GI, liver and pulmonary cGVHD were most frequent in the SD/PD group. When looking only at those with PD the most frequent organ involvement was lungs in the form of bronchiolitis obliterans. The highest frequency of severe cGVHD was found in patients with skin cGVHD, of the 18 patients 3 had mild, 6 had moderate and 9 had severe cGVHD with hidebound sclerodermatous changes. One patient presented with a sentinel lesion in the form of a wound on both arms. Patients with CR/PR were predominantly those with cGVHD of the skin and/or oral mucosa. Skin responses included softening of the skin, decreased erythroderma and less hidebound sclerosis.
In the data analysis stage of the current study we chose to analyze corticosteroid treatment before, at 8 weeks and 6 months after cessation of ECP treatment to obtain a more defined trend of sustained and decreased need of corticosteroids even after ECP treatment was stopped. There was a significant decrease in corticosteroid doses both early (+8 weeks) and late (+6 months) after ECP treatment. In the CR/PR group, the corticosteroid dose was significantly lower 8 weeks after ECP than at the start \( (p < 0.001) \) and at 6 months after ECP a further decrease was seen \( (p = 0.02) \). In the SD/PD group, no significant decrease was detected.
7 DISCUSSION AND CONCLUSIONS
In our first risk factor study we could show a higher incidence of cGVHD in sibling transplants without higher TRM or relapse compared to URD. However, OS and RFS was higher in the sibling group. We could show that the incidence of all grades of cGVHD was higher in the sibling donor group.

The URD group received immunosuppression for 6 months compared to 3 months for the sibling group with a higher target concentration of CyA (200-300ng/ml compared to 100ng/ml). These findings allowed us to make adjustments to propagate the GVL effect in the URD group. We changed our ATG strategy by lowering the existing dose to 4mg/kg for all URD with malignant underlying diseases. Our findings also led us to reduce the target concentration of CyA in the URD patient group to 150-200ng/ml. This has led to less nephrotoxicity. Although no new data exist, the clinical experience is that neither severe aGVHD nor severe cGVHD incidence has increased. Of note, the incidence of severe cGVHD was higher in the sibling group as was TRM for patients with severe cGVHD, perhaps due to more fatal infections. We must keep this in mind since the purpose of any transplantation remains remission with a good quality of life and unhampereed survival. The changes done to the immunosuppressive regimens for the URD group have so far not shown an increasing trend in severe cGVHD.

In our multi-center study we identified risk factors with regard to development of severe cGVHD. These were female donor to male recipient, prior aGVHD and use of reduced intensity conditioning. The first two risk factors have previously been described, the latter requires further clarification. Finding a higher risk of cGVHD among patients receiving RIC is somewhat surprising since previous studies have not shown a difference in cGVHD incidence with less intense conditioning\textsuperscript{299,300}. Mechanisms involved in the development of acute and chronic GVHD are not entirely congruent. In this regard, cGVHD is not simply the end stage of acute GVHD\textsuperscript{122}. In accordance with this hypothesis, use of RIC might, in fact, decrease the risk of acute and increase the risk of chronic GVHD. It could be speculated that acute GVHD is mostly dependent on the cytokine storm mediated by the tissue injury induced by the high doses of chemoradiotherapy which is avoided in RIC. On the other hand, chronic GVHD would be more dependent on the persistence of host–derived APCs that might trigger an alloresponse in donor T cells. The use of RIC favours the persistence of a mixed chimerism for a longer period post-transplant compared to myeloablative conditioning. It could also be argued that since many centers show a preference toward treating older patients
with RIC mainly due to comorbidities, the median age in this patient group is higher compared to those treated with myeloablative conditioning (MAC). Older age is a risk factor for cGVHD incidence and the biological aspect could be the lack of functioning thymic tissue to properly implement non-alloreactive thymocytes. We show that older age up to the age of 50 years is a risk factor to develop cGVHD. After that cut off, age does not have impact, this is congruent with the notion of thymic involution which is highly present around that age.

Previously the dose of ATG was 10mg/kg administered to patients with a malignant disease receiving grafts from URD. A dose finding study showed that the relapse rate was higher with that dosage. The dose was dropped to 8mg/kg without affecting the incidence of acute or chronic GVHD. With this knowledge the dose was lowered to 4mg/kg with the adversity of increased acute GVHD and therefore we settled on 6mg/kg. In a prospective study conducted at our center the routine was to differentiate between the intensity of the conditioning regimen and based on MAC or RIC administer 6mg/kg or 4mg/kg respectively. The study did not show any difference in the incidence of acute or chronic GVHD for the two groups, the analysis was restricted to patients with malignant disease receiving grafts from unrelated donors.

In our study, OS was similar for patients with severe cGVHD or no cGVHD. To stimulate GvL, the most desirable is mild-moderate cGVHD. We developed a scoring system for cGVHD including risk factors known at the time of transplant: RIC, female-to-male donation and patient age>45 years. Patients with these three risk factors had an incidence of severe cGVHD of 40% at 5 years, while the same patients had a cumulative incidence of 7% at 5 years when they received ATG. We also identified RIC, female-to-male donation, prior aGVHD and not receiving ATG as risk factors for developing severe cGVHD. Studies have shown a protective effect of ATG on the incidence of severe acute or chronic GVHD in URD without any effect on OS. A prospective study has actually shown lower OS in the ATG-Fresenius (20mg/kg) treated group as compared to those not having received ATG despite lower incidence of severe cGVHD. Studies have shown a correlation between low absolute lymphocyte counts (<0.1x10^9/L) and decreased OS upon administration of ATG. A later prospective study investigated the addition of lower dose ATG-Fresenius (10mg/kg) in sibling transplants receiving MAC. The study showed significantly lower incidence of cGVHD with similar RFS and OS for patients that received ATG. ATG seems to have a dose-dependent impact on the balance between GVL and GVHD. A recent study has shown lower incidence of severe cGVHD and higher OS for patients receiving ATG-
Fresenius in the URD setting together with MAC\textsuperscript{307}. The study had three arms including HLA-matched without ATG, HLA-mismatched and HLA-matched with ATG (6mg/kg and 4.5mg/kg respectively). There is a lack of randomised, controlled trials comparing the different ATG products. At our centre we also utilise rabbit-ATG, however from a different manufacturer, Thymoglobulin by Sanofi. Our findings would be of greatest use to modify immunosuppression during the conditioning regimen where the addition of ATG seems beneficial for older male patients receiving grafts from female donors. Due to toxicity, it would be difficult to motivate a more intensive regimen for an older patient where RIC remains most appropriate. Furthermore, keeping in mind that RIC regimens vary in intensity and have been shown to give different outcomes in terms of TRM and OS\textsuperscript{308}.

Prognostic impact of the NIH score was a novel idea and we could exclude delayed acute GVHD as having any adverse effect on outcome. On the other hand overlap syndrome, which is more common in the group with progressive onset, did have a negative impact on OS which is in line with other publications\textsuperscript{309,310}. We carefully tried to differentiate between patients who had signs or symptoms of aGVHD that were resolving when cGVHD appeared, with emphasis on those who developed aGVHD symptoms during or after diagnosis of cGVHD. In this study we focused on developing a simplified and more user-friendly score. We confirmed that NIH criteria are the most important variables in predicting outcome in multivariate analysis. Among those variables included in the NIH, performance status according to ECOG score and platelet counts at the time of cGVHD had the highest impact on outcome, both in terms of cGVHD-RM and survival and is confirmed by other studies\textsuperscript{213,311,312}. In addition, gastrointestinal involvement significantly influenced outcome in multivariate analysis. Although the other organ manifestations only had impact upon univariate analysis, in fact, skin (r=0.16, P=0.012) and lung involvement (r=0.29, p<0.001) had a high correlation with ECOG and, accordingly, are responsible for the performance score of the patients at the time of cGVHD.

Interestingly, NIH proposed a cut off of 3 organs involved in order to distinguish mild from moderate cGVHD so as to indicate systemic treatment with immunosuppressants\textsuperscript{184}. Since ours is a retrospective study, we cannot draw any conclusions about the best treatment for patients with involvement of three organs. According to our data, these patients had a similar survival to those with 1-2 organs involved and a significantly better survival than those with 4 or more organs involved. Thus, further studies will be required to confirm the best cut off for systemic treatment. According to our findings we were able to develop a powerful and
simplified scoring system. Greater ECOG score, a low platelet count and severe gastrointestinal involvement are strong prognostic factors for cGVHD-RM and OS. Interestingly, the simple combination of ECOG plus platelet count allowed us to discriminate the same subgroups of patients suggesting that this combination has prognostic impact and could be applied irrespective of organ involvement. This needs to be confirmed in larger prospective studies.

The methodology to assess ECP response varies between different studies but most use organ-specific measurements as is the case in our study. We show that the highest response rate was seen in patients with skin cGVHD. The majority of these patients were only partial responders however; they consisted of the patient group with the highest frequency of severe cGVHD. The group of patients with the highest CR rate was those with liver involvement. Only one patient had isolated liver involvement; the rest had concurrent skin and/or visceral involvement. Perhaps this is due to the fact that the liver is a highly immunologically active organ and one can speculate that the vast majority of ECP-treated cells would be found in the liver and spleen; accordingly, immune modulatory effects should be highest in these organs. In the current study most patients with skin cGVHD had scleroderma before ECP treatment was started. This may have contributed to the low response rate in these patients.

As mentioned previously, a low platelet count has been shown to be a poor prognostic indicator for cGVHD and is associated with a lower response rate to ECP. Our results show that ECP may directly or indirectly induce increased and normalised levels in ECP responders. Furthermore, we also show that responders would have significantly increased albumin levels. Due to the increase in albumin, it is noteworthy that the second-largest group of responders was those with GI-GVHD after skin-GVHD. The observed differences in platelet counts and albumin levels between the two groups after ECP treatment may be used prospectively as essential parameters in evaluating treatment response before more evident clinical changes manifest such as skin softening, which can take up to a year to six months to appear.

Patients with CR/PR have higher survival and less TRM than the SD/PD group. Unexpectedly, when compared to the control group, the CR/PR patients had better results in terms of TRM and survival. This would indicate a beneficial effect of ECP in terms of clinical outcome. To date these findings have been novel, however as mentioned before the
initiation of ECP has been at the discretion of the treating physician taking into account limiting medical and practical issues. A limiting factor has been the size of this study population and the long accrual time.
7.1 CONCLUSIONS

1. Risk factors for severe cGVHD include female donor to male recipient, RIC and older patient age, of which, RIC had previously not been reported. RFS was impaired in patients with severe cGVHD or with no cGVHD. ATG had a protective effect against severe cGVHD without hampering RFS. In addition, there was higher incidence of overall cGVHD and severe cGVHD in sibling transplants when comparing sibling and URD transplants.

2. Factors affecting prognostic impact upon diagnosis of cGVHD include ECOG, platelet count and, if present, severe gut involvement. In order to only utilise ECOG and platelet count we must verify our findings in a prospective setting.

3. ECP seems to be the most well-established second line treatment. Patients with severe cGVHD involving primarily the skin followed by oral involvement, had the best response rates to ECP treatment. In this single center setting we can conclude that ECP is a safe treatment option for patients with steroid-refractory cGVHD. We did not find convincing response rates in patients with severe pulmonary cGVHD, however perhaps treatment was started at a late phase in these patients.
8 FUTURE DIRECTIONS

Despite nearly 40 years of research we are still faced with the major cause of morbidity in our patients, namely, cGVHD. Many studies have been conducted throughout the years to find a treatment which can inhibit GVHD without hampering the GvL effect, and to date, we still revert back to our most widely utilized treatment which is corticosteroids. In and of itself the treatment is effective however with a great deal of serious side effects including both physiological and psychological.

Most studies have been single center phase II studies testing novel therapies with very little definitive results. We have even exhausted the search for risk factors in terms of minor histocompatibility complexes, which from a clinical perspective, prove to be too complex for implementation. With increased knowledge and capabilities in the field of genetics, we have started to identify both gene mutations (e.g. lower VAMP8 expression in ocular GVHD) and micro-RNA patterns pertaining specifically to the inflammatory milieu in GVHD, we start seeking targeted therapies for GVHD.

The fact remains that our ability to prevent cGVHD remains poor at best and our in depth knowledge of the syndrome is not cohesive by any means. We are still unable to properly identify those at risk of developing severe cGVHD and, beyond first line treatment, we still struggle to reach remission without excessive suffering for the patients.

Chronic GVHD is a very complex syndrome which involves, as stated in this thesis, a multitude of happenings and pathomechanisms. It is a self-fulfilling syndrome which, if left uncontrolled, leads to major suffering and death. It is because of these facts that we need to employ a more pragmatic approach to cGVHD.

We know that the incidence of cGVHD is approximately 50% post-HSCT. What is truly interesting is not the fact that it occurs in half of the transplanted patients but rather, why it does not occur in all of our transplanted patients. Obviously our efforts within the transplant community have not been in vain, since we succeed in knowingly or unwittingly preventing it somehow. My future suggestion for managing cGVHD is based on the following question: “Isn’t it odd that it does not occur in all patients?”
Since the syndrome is so complex and heterogeneous, it is necessary to attempt a multi-center biomarker finding study. To do so, we would all have to view and diagnose it in the same way and that is where the new NIH classification would facilitate such a project. In terms of biomarkers, we could expand on those already brought forth by MAGIC consortium such as ST2 and REG3a and involve more biomarkers pertaining to the adaptive arms of the immune system, especially plasma and B-cells.

Severe cGVHD, as defined by the NIH criteria, has an incidence of 10-15% with 2-year overall survival up to 60\%. The fact that there seemingly is a minority of patients developing the most detrimental form of cGVHD prompted us to develop a multi-disciplinary team at our center. This would allow a more focused and dedicated team to be exposed to greater volumes of these patients rather than the scarcity prevalent at each individual physician’s out-patient clinic.

Finally, from a clinical perspective the direction forward is to keep evolving a multi-disciplinary cGVHD outpatient approach. The future of cGVHD does not lie within the different second-line treatments available but more so in the attempted prevention of it with the preservation of the GvL effect.
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