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Gwendolyn van Gorkom

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Improving Outcome of Haematopoietic Stem Cell Transplantation

a possible role for haploidentical donors and ascorbic acid

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General introduction

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Cancer

Cancer is a disease resulting from an uncontrolled division of cells. It is a major problem worldwide given the high lifetime incidence of cancer. Treatments are costly and intense, and the curation chances are limited, making cancer one of the leading causes of death. In 2020, there were 19.3 million new cases of cancer and 10 million cancer deaths globally. Because of population ageing and growth the numbers of cancer patients are still increasing worldwide. It is predicted that by 2030 the number of new cases of cancer will be 22.2 million[1]. In the Netherlands, as in other western countries, cancer is the main cause of death. In 2020, according to GLOBOCAN, there were 132.014 new cases and 49.008 cancer related deaths in our country. The risk of developing cancer before the age of 75 is 35.6% in men and 31.4% in women. Localized malignancies can be treated curatively with surgery or high dose radiotherapy. When cancers are more spread, they are treated systemically with chemotherapy, endocrine therapy, immunotherapy and/or targeted therapy. Around 45% of cancer patients will survive more than 10 years. The success of treatment depends highly on the type of cancer and the stage at diagnosis. Chemotherapy can kill cancer cells almost everywhere in the body, but the chances of curation are limited and strongly depend on the sensitivity of the tumour cells for chemotherapy. In some cases, high dose chemotherapy can be used to optimize the treatment. To limit bone marrow toxicity, autologous stem cells can be administered after high dose chemotherapy (autologous haematopoietic stem cell transplantation). Targeted therapy is increasingly available for specific types of cancer, but it needs specific receptors or pathways to work. With some types of cancer, especially in certain aggressive haematological malignancies, it is possible to use immune cells to find and destroy cancer cells. We call this immunotherapy. One of the most common and oldest form of cellular immunotherapy is the allogeneic haematopoietic stem cell transplantation (HSCT), a procedure in which haematopoietic stem cells from a healthy donor are administered in order to replace the patients' bone marrow cells. A more recent and very promising form of cellular immunotherapy is the use of chimeric antigen receptor (CAR) T cells. CAR T cells are genetically engineered to produce artificial T cell receptors that are able to recognize antigens specific for certain cancer cells. In this way, these cells are able to effectively target and destroy the tumour cells without damaging healthy cells[2]. Finally, a third form of immunotherapy is the use of

antibodies. Monospecific antibodies target antigens expressed on the surface of tumour cells and promote their destruction by antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP). Bispecific or trispecific antibodies bind both tumour cells and cytotoxic cells, and thereby stimulate the patients' own immune cells directly to destroy the cancer cells[3].

Autologous haematopoietic stem cell transplantation

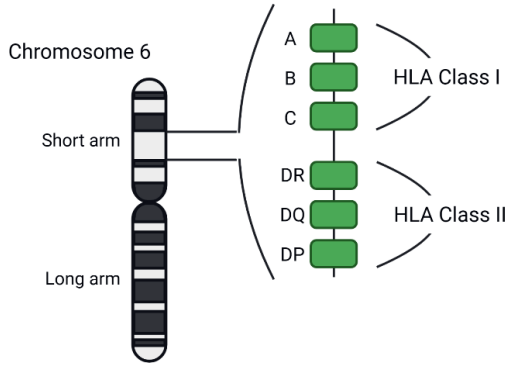
Autologous HSCT is a procedure in which previously harvested and frozen stem cells are returned to the same individual to improve cellular reconstitution after high-dose myeloablative (bone marrow destructive) chemotherapy[4]. In this type of transplantation, the primary and only tool to treat the malignant disease is high dose chemotherapy or radiotherapy. The resulting myeloablation could be lethal if autologous stem cells were not provided as rescue. Research on autologous HSCT started in the early 80s and nowadays it is regularly performed (European Society of Blood and Marrow Transplantation (EBMT): around 60% of all HSCT)[5]. It is considered standard first-line treatment for fit multiple myeloma and favourable risk acute myeloid leukaemia patients, and as second-line treatment for various types of relapsed or refractory lymphoma patients. Furthermore, it is used in some forms of solid cancer and in autoimmune disorders. The major complications of this type of transplant are infections due to severe neutropenia in combination with mucosal damage.

Allogeneic haematopoietic stem cell transplantation

Allogeneic HSCT is an important curative treatment option for many, especially malignant, haematological disorders[6]. After World War II, many scientists worked on ways to counteract radiation damage inflicted by nuclear weapons to the haematopoietic system which is extremely vulnerable to radiation. In mice and guinea pigs, infusion of bone marrow cells led to the survival of otherwise lethal irradiation[7]. Later, while working

on the concept of using irradiation to cure leukaemia, the use of bone marrow transplantation was introduced. The first allogeneic HSCT in humans with leukaemia after conditioning with radiation and chemotherapy was performed as early as in 1957 by Thomas and colleagues[8]. All 6 of his transplanted patients, however, died within months after treatment, probably because of lack of knowledge about human leukocyte antigen (HLA)-types. These were discovered in the 1960s by van Rood and colleagues and are cell-surface proteins responsible for the regulation of the immune system. They present foreign antigens to immune cells[9]. HLA genes inherited from both parents are located on chromosome 6 (**Figure 1.1**). As there is a very wide variation in HLA genes, combinations of HLA are very specific for an individual.

Figure 1.1 The distribution of HLA alleles on the short arm of chromosome 6.



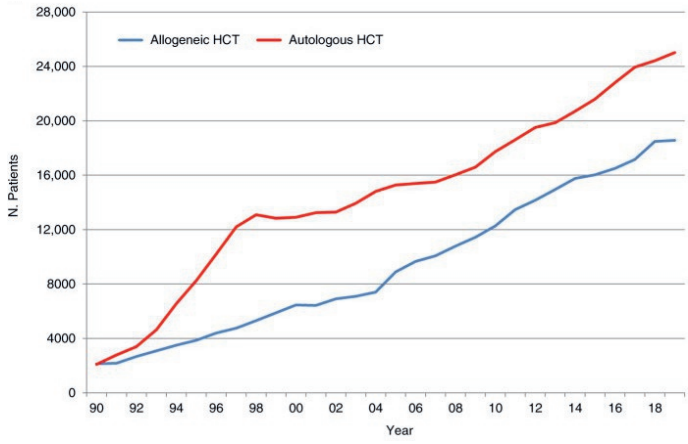
HLA class I alleles –A, –B and –C are expressed on all nucleated cells, while class II alleles –DR, –DQ and –DP are expressed only on antigen-presenting cells.

In 1977, Thomas published an important article, in which he described the HSCT of 100 acute leukaemia patients treated with an HLA-matched donor: a donor that shares several HLA genes with the patient[10]. Even though only a small part of these patients showed long-term disease-free survival, it was the proof of principle that donor immune cells can be used to battle cancer cells. In 1990, Thomas received the Nobel Prize for this important contribution to the development of allogeneic HSCT.

Already in the 1970s donor availability was a major concern, given the limited number of patients having an HLA-identical sibling. In 1979, the first successful bone marrow transplantation using a HLA-identical unrelated

donor was performed, increasing the number of possible donors[11]. Over the last decades, to make HSCT accessible for a larger group of patients (older and with more comorbidities), transplantation procedures have been adapted. One example is the use of non-myeloablative conditioning. The number of HSCTs in Europe is rising exponentially (**Figure 1.2**) as it is considered standard treatment for many high-risk haematological malignancies[12]. However, the treatment is still complex, intensive, dangerous, and costly[13-15].

Figure 1.2 Number of patients receiving autologous and allogeneic HSCT from 1990 to 2019 in the EBMT community[12].



HCT: haematopoietic stem cell transplantation.

Donor T-lymphocytes are thought to be important in finding and fighting cancerous cells in allogeneic HSCT. However, these donor lymphocytes can be harmful as they are also able to attack healthy cells, and thereby cause graft versus host disease (GVHD). There are 2 different forms of GVHD. Acute GVHD appears within 100 days of the HSCT, and is mostly localized in the skin, intestinal tract and/or liver. Severe forms can even be fatal. Chronic GVHD appears later, sometimes even several years after transplant, has a more gradual course, and can affect every organ system. This type of GVHD is difficult to treat and leads to a significant loss of the quality of life[16,17].

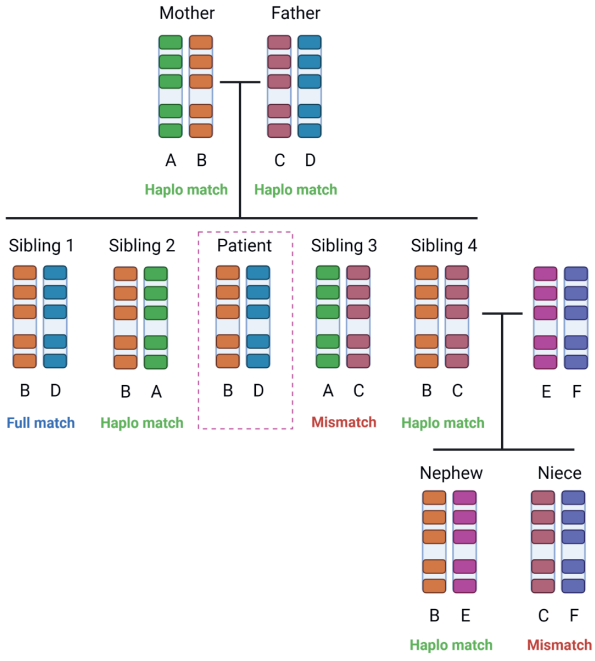
To reduce the incidence of GVHD, it is necessary to treat patients with immunosuppressive therapy after the HSCT, or to manipulate the graft itself

to limit the activity of the donor lymphocytes. This makes patients very susceptible to infectious complications. Additionally, the immunosuppressive treatments impair the donor immune cells' ability to prevent relapse of the original haematological malignancy. Because of these potential problems, the allogeneic HSCT still offers many challenges, and improvements are necessary.

Donor availability

The ideal donor for an allogeneic HSCT with regards to anti-tumour response, engraftment, and a low incidence of GVHD is an HLA-identical family donor[18,19]. HLA-identical means that a donor is identical in both alleles of the HLA-A, -B, -C, -DR, and –DQ genes. We call this a 10/10 match (**Figure 1.3**).

Figure 1.3 Potential family donors for allogeneic HSCT.



Displayed is the HLA-profile in colour of the patient and different family members. For the patient shown in the middle, each sibling has a 25% chance of being a full match based on the inheritance of the same maternal (B) and paternal (D) alleles as the patient. Each sibling has 50% chance of being a haploidentical match by inheriting one identical allele (B or D) from the parents. Even second- or third-degree family members can still be haploidentical to the patient.

As people nowadays have fewer siblings than before, the availability of this type of donor is decreasing. Currently, in only 30% of cases, an HLA-identical family member can be found, and they frequently have to be rejected due to high age or comorbidities. It is expected that this number will decrease in the future because of demographic changes[20]. Therefore, there has been an increase in the use of alternative donors[5]. Alternative donors can be HLA-identical matched unrelated donors (MUD), partial non-HLA-identical matched unrelated donors (MMUD), unrelated umbilical cord blood (UCB) donors, or related haploidentical donors. Each of these alternative donor choices has its advantages and disadvantages (**Table 1.1**).

Table 1.1 Donor options for an allogeneic HSCT and their potential advantages and disadvantages.

Donor	Pro	Con
HLA-identical family donor	Rapid Small chance of GVHD and graft failure	Only available for 30% of patients
HLA-identical unrelated donor (MUD)	Larger number of potential donors Small chance of graft failure	High costs Not available for everybody Time consuming
Partial non- HLA-identical unrelated donor (MMUD)	Large number of donors	Time consuming Higher chance of GVHD and graft failure Intensive conditioning More infections
Cord blood donor (UCB)	Big diversity Readily available Small chance of GVHD	Small transplant Slow immune reconstitution High chance of graft failure High costs No possibility for cellular therapy
Haploidentical donor (without optimisation)	Large number of donors Rapid	Intensive conditioning Slow immune reconstitution High chance of graft failure High chance of GVHD High chance of infections
Haploidentical donor (after optimisation*)	Large number of donors Rapid Low chance of GVHD	Intensive conditioning Slow immune reconstitution High chance of rejection with donor specific HLA-antibodies

* Optimization means with post-transplantation cyclophosphamide, T-cell depletion or GIAC method (G: G-CSF priming; I: intensified immune-suppression; A: ATG application; C: combination of peripheral blood stem cell and bone marrow as graft).
Abbreviations: HSCT: haematopoietic stem cell transplantation, HLA: human leukocyte antigen, GVHD: graft-versus-host disease, MUD: matched unrelated donor, MMUD: mismatched MUD, UCB: umbilical cord blood.

When searching for an alternative donor a HLA-identical donor can be found in the extensive unrelated donor registries in only 50 to 60% of patients worldwide. For patients from non-European descent, this percentage is even much lower[20]. Other disadvantages of MUD procedures are that they are time-consuming and costly.

Another option is UCB. The chance of finding suitable UCB is higher, since HLA similarity is less important with this type of HSCT. However, this type of transplant is also costly and since the amount of stem cells in the graft are small, it is only possible for children and adults with a relatively small body surface. In most adults, two UCBs are necessary, which makes the procedure even more expensive. In all patients after an UCB HSCT, the immune reconstitution is slow, and there is an increased chance of graft failure[21]. Another disadvantage is that after the transplantation cellular therapy, such as donor lymphocyte infusions (DLI), is not possible.

A haploidentical family donor seems a better alternative. A haploidentical donor is a family donor that shares one haplotype with the patient and differs with a variable number of HLA-genes in the other, unshared haplotype. This means that every parent or child can be a suitable donor, and 50% of the siblings. Even second-degree family members still have 25% change to be a haploidentical match (**Figure 1.3**).This means that for almost all patients (>95%) a haploidentical family donor can be found[22]. Because they are widely available, they can, mostly, be selected and approved for transplantation within 1 month. After transplantation they are readily available for additional cellular therapy when necessary. A disadvantage of a haploidentical donor is an intense bi-directional alloreactivity that can lead to a high incidence of stem cell rejection and GVHD. In the last decade, several very effective methods have been utilized to prevent these problems and currently this type of donor is increasingly used.

History of haploidentical HSCT

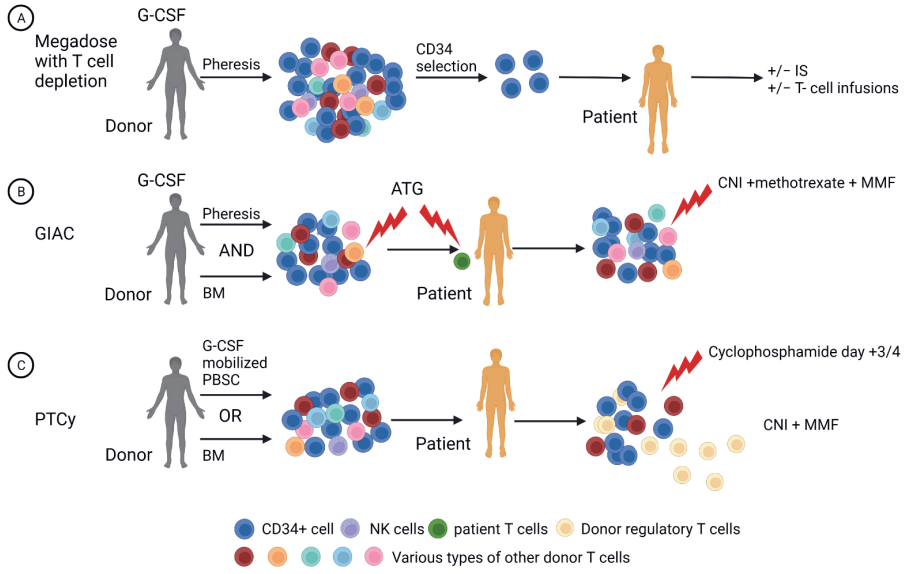
The haploidentical HSCT has caused lots of turbulence over the past few decades. It was first introduced in the late 1970s in children with acute leukaemia[23]. Despite side effects the first results were promising, and it seemed a good therapeutic alternative for patients for whom no HLA-

identical family donor could be found. Several years later the first larger studies were published which showed that haploidentical HSCT had a slower engraftment, a higher incidence of graft failure (20%), and more acute GVHD (70%) than HLA-identical HSCT[24]. These negative results notwithstanding, there continued to exist worldwide interest in this type of transplant and several attempts were made to optimize treatment. In Italy, a clinical study with *in vitro* T cell depletion of the graft to prevent GVHD improved outcomes[25]. It even showed a survival benefit in patients with acute myeloid leukaemia (AML) when donors were used that also had a natural killer (NK) cell alloreactive effect[25]. Reproducing these results, however, proved to be difficult. Studies using the same transplantation procedure were performed worldwide and showed an unacceptably high rate of non-relapse mortality (NRM) and morbidity due to both acute and chronic GVHD as well as opportunistic infections[26]. In the Netherlands, the Maastricht and Leiden university hospitals tried the transplant method from Italy[27]. In Maastricht, 11 high-risk AML patients have been transplanted. Two of these patients are still alive with a long-lasting complete response, the other patients all died of opportunistic infections early after transplant because of the intense T cell depletion of the graft. In Leiden, only 4 patients have been transplanted; 2 died of opportunistic infections, 1 of relapse. One patient has a long-lasting response. Comparable results were seen by others[28]. Many efforts were undertaken to optimize haploidentical HSCT, for instance with the reintroduction of selected or altered T cells post-transplant, CD34 positive cells selection and reduced intensity conditioning. These led to a decrease in early transplant mortality and development of GVHD but did not overcome other problems such as late immune reconstitution, graft failure, and early relapse[29]. Promising results were seen with a donor-derived T cell-enriched preparation depleted of recipient alloreactive T cells through the use of photodepletion, but a phase III trial was stopped prematurely by the sponsor[28] (unpublished results).

Even though there were problems, the urgent shortage of donors ensured that the interest in haploidentical HSCT never went away. In recent years several methods are emerging for safe transplant of haploidentical donors. The three most used regimens are T cell depletion with megadose CD34 positive stem cells, the GIAC method (G: G-CSF priming; I: intensified immune-suppression; A: ATG application; C: combination of peripheral blood stem cell and bone marrow as graft), and post-transplantation high dose cyclophosphamide (PTCy) (**Figure 1.4**). These regimens have not been

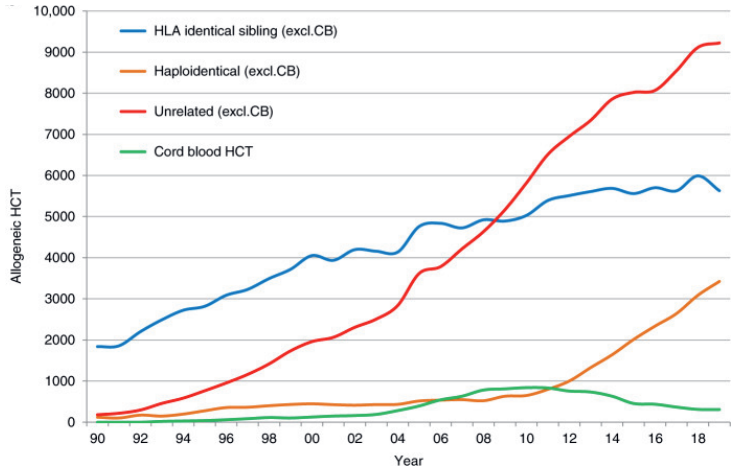
compared head-to-head, but the outcomes are comparable. Thereby, the use depends foremost on the experience and possibilities of the transplant centre. The method that nowadays is used most frequently is PTCy, developed in Baltimore in 2008, and T cell depletion has faded to the background somewhat[30,31]. In the PTCy regimen, on day +3 and +4 of HSCT high dose of cyclophosphamide is administered to eliminate activated alloreactive T cells present in the graft, which are responsible for GVHD. In this way, part of the T cells that have to fight against pathogens but have not been activated yet will survive and protect the patient against opportunistic infections[32]. Studies using this method show a low incidence of graft failure, grade III/IV GVHD and infections[31,33]. Since the introduction of PTCy, the use of haploidentical donors worldwide has been rising exponentially, and transplantation methods are increasingly being perfected (**Figure 1.5**).

Figure 1.4 The three most used regimens in haploidentical HSCT.



A. T cell depletion with megadose CD34+ cells.
B. GIAC method with a G-CSF primed bone marrow and peripheral blood stem cell graft, with ATG targeting both the donor and the patient. GVHD prophylaxis is a CNI + methotrexate + MMF.
C. PTCy impairs proliferating graft derived T cells while favouring regulatory T cell recovery (here depicted in light yellow).
IS: immunosuppression. G-CSF: granulocyte-colony stimulating factor, BM: bone marrow, ATG: anti-thymocyte globulin, PBSC: peripheral blood stem cell, CNI: calcineurin inhibitor, MMF: mycophenolate mofetil, PTCy: posttransplant cyclophosphamide.

Figure 1.5 Distribution of donor type among allogeneic HSCT recipients from 1990 to 2019 in the EBMT community[12].



HLA: human leukocyte antigen, HCT: haematopoietic stem cell transplantation, CB: cord blood.

Nowadays the disadvantages of haploidentical donors have been fully countered and most data show the results of HSCT with haploidentical donors are similar to those with HLA-identical donors[34-37]. Using the haploidentical HSCT also opens the exciting possibility to select donors with an NK cell alloreactivity, which results in both T and NK cell mediated anti-tumour responses.

Natural killer cells

After B and T lymphocytes, NK cells are the most prominent lymphocyte subset as they make up to 20% of the blood lymphocyte population and are important for the immunity against pathogens (especially viruses) and tumour surveillance. They are large granular lymphocytes arising from the same lymphoid progenitors as T and B lymphocytes and are primarily formed in the bone marrow. NK cells were first identified in the 1970s as part of the innate immune system. They are unique in that they are able to detect and kill abnormal cells without prior sensitization. With this capacity they are very effective in preventing the development of many cancers[38,39]. They exhibit direct cytotoxic effects, secrete cytokines and chemokines, and

regulate other immune cells. NK cell cytotoxicity is based on a delicate balance between inhibitory and activating signals that come from receptors on the cell membrane[40]. This cytotoxicity is inhibited when the killer cell immunoglobulin-like receptor (KIR) that is expressed on NK cells binds to its ligand, their own major histocompatibility complex (MHC) class I. So, the presence of self-MHC class I is used to discriminate between normal and diseased cells, and only under the influence of "missing self" are NK cells licenced to kill (**Figure 1.6**). Killing of these target cells that miss MHC class I can only be initiated after simultaneous detection of activating signals, such as stress signals on the surface of tumour or infected cells[41]. Incidentally, tumour cells are killed even though they carry the right MCH class I. This happens when the tumour cell has highly upregulated its stress receptors, so that the activating signals overrule the inhibitory signal. This is called "induced-self". These special qualities make NK cells attractive as an effector cell for cellular immunotherapy.

KIR-ligand mismatch in donor stem cell transplantation

KIRs are a family of highly polymorphic activating and inhibitory receptors that serve as key regulators of human NK cell function. KIR genes are randomly expressed and the distribution of KIRs differs per NK cell. In addition, the expression of KIRs is independent of HLA expression since KIR genes are located on chromosome 19 while HLA genes are located on chromosome 6. This gives rise to an extra opportunity in haploidentical HSCT: NK alloreactivity. NK alloreactivity means that the transplant has donor NK cells for which the recipient lacks a KIR ligand (HLA-molecule)[42]. This is also called a KIR-ligand mismatch (**Figure 1.7**). Because of the lack of "self" HLA on the recipient cells, alloreactive NK cells are theoretically able to kill these cells, if NK cell-activating signals are expressed. Thereby, they are able to kill tumour cells of the recipient.

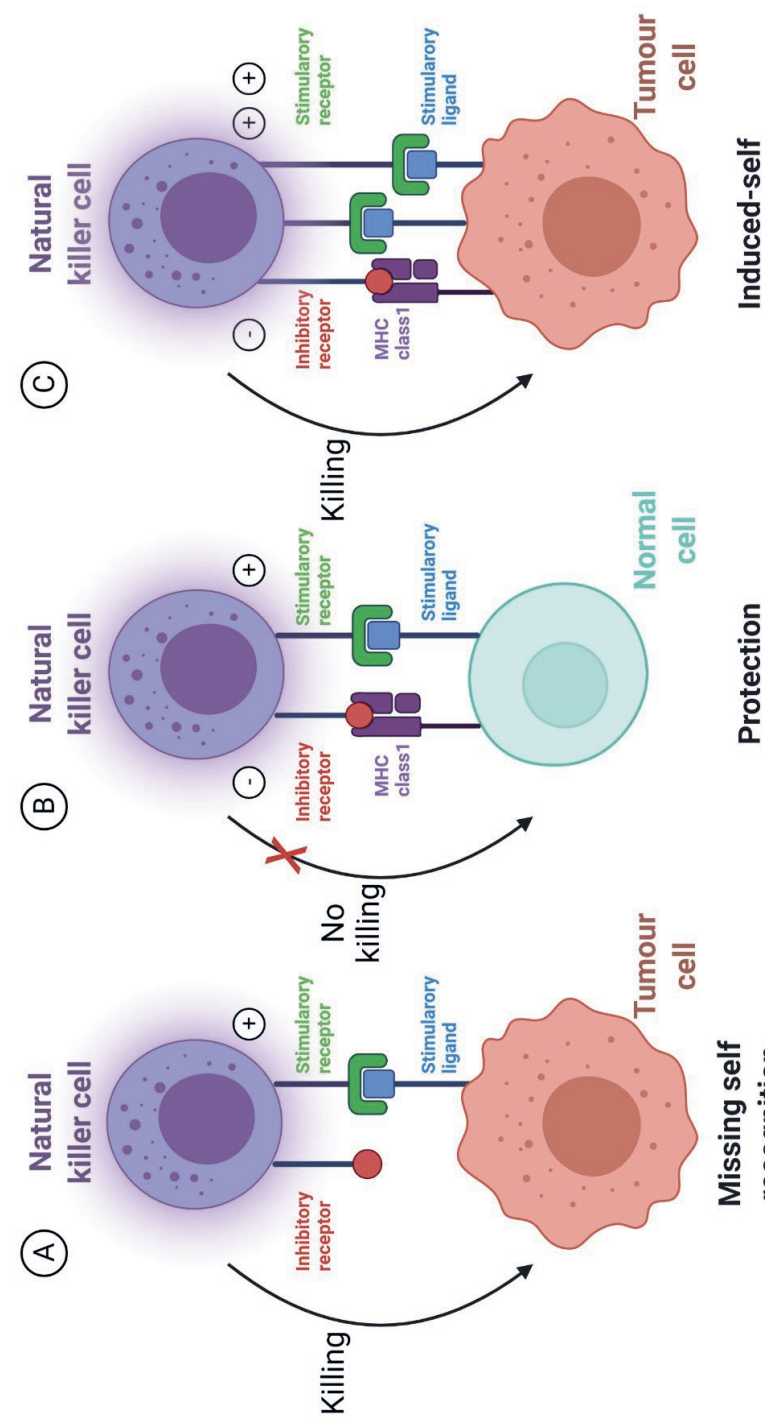
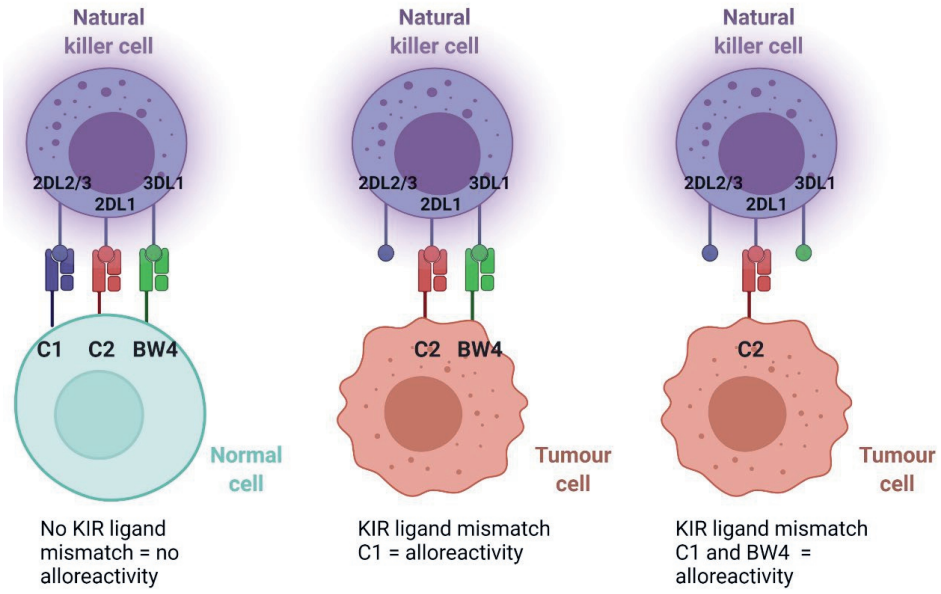


Figure 1.6 Summary of effects of AA on lymphocytes.

Figure 1.7 NK cell alloreactivity.



The donor NK cells have a KIR receptor for which the tumour cells lack a MHC class I. This creates NK cell alloreactivity and enables more killing of the target cell.

The role for such KIR-ligand mismatches was demonstrated in patients with AML, receiving haploidentical T cell-depleted HSCT[43]. In this study, a KIR-ligand mismatch was associated with a significantly increased overall survival, better engraftment, and a reduced incidence of GVHD. The proposed explanation for these clinical observations was NK cell-mediated clearance of residual leukaemia cells (less relapse), host T cells (better engraftment) and host dendritic cells (DC) (less GVHD). Moreover, the decreased risk of relapse after KIR-ligand mismatched transplantation correlated with expansion of donor derived NK cell clones capable of killing recipient tumour cells, thereby constituting indirect evidence for the involvement of alloreactive NK cells. However, this effect was only seen in AML and not in acute lymphocytic leukaemia.

In preclinical studies in multiple myeloma, a haematological malignancy of plasma cells, our research group observed an increased degranulation of NK cells in KIR-ligand mismatched setting compared to KIR-ligand matched

setting[44]. Moreover, even in breast cancer cell lines, we observed the same effect[45]. We aim to explore the use of KIR ligand mismatch further in haploidentical HSCT or NK cell immunotherapy in other cancer types.

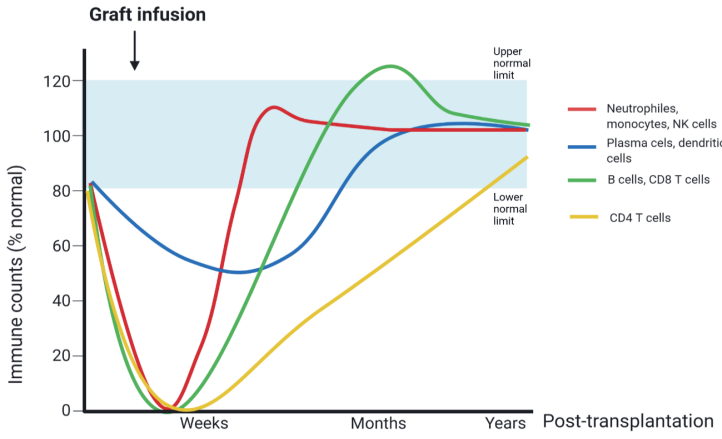
Immune reconstitution after haematopoietic stem cell transplantation

Slow immune reconstitution after high-dose chemotherapy with autologous or allogeneic HSCT is a major clinical problem. Chemotherapy is a frequently used treatment modality that eliminates cancer cells, but also destroys many blood cells. While red blood cells and platelets can be substituted temporarily though transfusions, leukocytes that constitute the cellular immune system cannot be replaced. This leads to a severely impaired immunity causing infections. These infections lead to (longer) hospital stays and increased costs, and sometimes are lethal. In the early post-transplant period, even the innate immunity (like neutrophils and epithelial barriers) is affected making patients especially vulnerable. Epithelial barriers consist of the skin, and the respiratory and digestive mucosa and are directly damaged by the chemotherapy and radiotherapy. This increases the chance of penetration of pathogens. Luckily, recovery of neutrophils starts quickly after HSCT. It takes only 2 to 3 weeks after the reinfusion of granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood stem cells for neutrophils to increase to $\geq 0.5 \times 10^9/l$[46] (**Figure 1.8**). The number of stem cells infused and, in some situations, the use of G-CSF after HSCT can affect the speed of the neutrophil recovery[47].

NK cells recover within 3-4 weeks after HSCT, but until 6 months post-transplant there is an imbalance between mature and immature NK cells in the peripheral blood. However, as the first reconstituted lymphocyte subset after transplantation, early NK cell reconstitution plays a critical role in controlling potential relapse in allogeneic HSCT[48,49]. Recovery of the adaptive immunity takes much longer. This means that patients are still vulnerable to infections after their leukocyte cell count has normalised. The recovery of CD4 T cells is especially slow and it can take years before numbers have normalised[50]. The regeneration is slower in adults than in children, probably due to the natural involution of the thymus[51-53]. The T cell repertoire is limited in the beginning, but versifies when counts are rising[54]. CD8 T cells

are low during the first months after HSCT but reach normal levels one year post-transplant. T cells are necessary to fight viral infections. B cells are not present in the first months after HSCT but increase to supra-normal levels 1 to 2 years after HSCT[55]. Slow immune reconstitution is seen both in autologous as well as in allogeneic HSCT. In allogeneic HSCT, the presence of GVHD and the use of immunosuppressive therapy can impair immune recovery even more creating a risk factor for the development of infectious complications.

Figure 1.8 Immune reconstitution after myeloablative HSCT.



Approximate immune cell counts are expressed as percentages of normal counts. The red line represents the innate immune cells like neutrophils, monocytes and NK cells. Their recovery is influenced by graft type. The green line represents the recovery of CD8 T cells and B cells. Their counts may transiently become supra-normal. The blue line represents the recovery of relatively radio/chemo-resistant cells like plasma cells and tissue dendritic cells. The yellow line represents CD4 T cells. Their recovery is influenced primarily by T cell content of the graft and by patient age. HSCT: haematopoietic stem cell transplantation, NK: natural killer.

At our research laboratory, we looked at ways to improve T cell recovery after cancer therapy like HSCT. For this, we investigated factors that influence human T cell development and found by coincidence that vitamin C (ascorbic acid) acts as a factor that promotes maturation of T cells. For *in vitro* T cell development, ascorbic acid (AA) even proved to be indispensable[56]. Additionally, we showed that NK cells regenerate faster in the presence of AA[53]. Because of these findings, we decided to explore the clinical use of AA in HSCT.

Ascorbic acid and cancer

AA is an important water-soluble vitamin which is found in various foods, mainly but not exclusively citrus fruits, kiwifruit, guava, broccoli, and red peppers. AA is an essential micronutrient for humans with a diversity of functions in the human body. It is an antioxidant and a free radical scavenger and serves as an essential cofactor for many enzymatic reactions through iron and 2-oxoglutarate-dependent dioxygenases. These dioxygenases are important in epigenetic regulation by catalysing the hydroxylation of methylated nucleic acids (DNA and RNA) and histones[57]. While most mammals use the enzyme gulono-gamma-lactone oxidase to synthesize AA in the liver, many primates and humans, carry a non-functional copy of the *GULO*-gene and consequently depend on dietary sources of AA[58,59]. Furthermore, there is a low storage capacity for AA in the human body, so a regular intake of AA is required to prevent hypovitaminosis C.

When investigating the effects of AA and AA deficiency *in vivo* in animal models, the non-functional copy of the *GULO*-gene is a complicating factor. Guinea pigs, like humans, also have a defect in the GULO-gene and are thus often chosen for AA deficiency studies. Alternatively, there are two knockout mouse models in which biosynthesis of AA in the liver is blocked.

AA has often been linked to cancer treatment. Already in the 1970s, Cameron and Pauling reported that AA increased the survival time of terminal cancer patients by more than four times[60], but this finding could not be confirmed in other studies[61,62].

Ascorbic acid and the immune system

The immune system consists of a sophisticated network of organs, tissues, cells, proteins, and chemicals. It is specialized to protect the host against a wide range of pathogens, such as viruses, bacteria, and fungi, and against the development of cancer cells. Just like other organs, the immune system requires appropriate energy and nutrient supply. Therefore, it is not surprising that immunocompetence is significantly influenced by the intake of micronutrients[63]. One of the most studied micronutrients in this context is AA. AA plays a multitude of roles in the immune system, though not all

molecular mechanisms are fully understood. The concentration in immune cells is 10- to 100- fold higher than the concentration in plasma, suggesting that AA is vital to those cells[63-65].

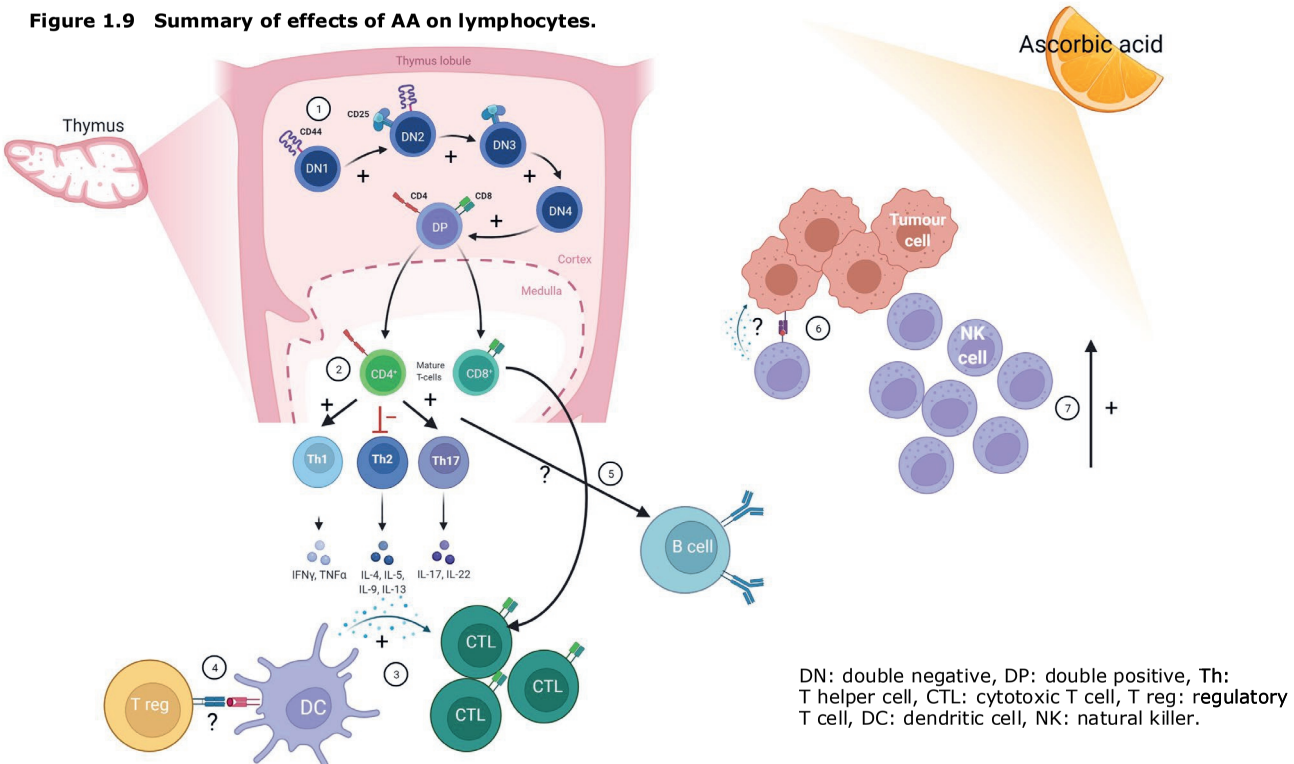
The role of AA in infections has been studied extensively *in vivo*. There are clues that AA supplementation might be beneficial in reducing the duration of symptoms of the common cold[66]. Furthermore, there might be an effect of AA supplementation on pneumonia, especially in elderly patients, but these results are not consistent over different studies[67]. Currently, there has been renewed interest for the potential role of AA in COVID-19 pneumonia. There were indications, that AA supplementation could lead to better outcomes, but further randomised trials are warranted[68].

The role of AA in phagocytic cells, such as neutrophils, has been investigated thoroughly. In summary, AA enhances neutrophil migration in response to chemoattractants (chemotaxis), enhances phagocytosis of microbes, and stimulates reactive oxygen species (ROS) generation and killing of microbes. Furthermore, AA supports caspase-dependent apoptosis by macrophages, enhancing uptake and clearance of microbes, and it inhibits necrosis. Thereby it supports the resolution of the inflammatory response[69].

Ascorbic acid and lymphocytes

The role of AA in the different subsets of lymphocytes is less clear. Since lymphocytes actively transport AA via sodium-ascorbate co-transporters (SVCT) and hexose transporters (GLUT) (reviewed in[70]) and have intracellular AA concentrations that are 10-100-fold higher than plasma levels[64,71], it is likely that AA has an essential function in these cells.
It is known that AA has multiple effects on the development, proliferation, and function of some types of lymphocytes (**Figure 1.9**). The relationship of AA in T cells has been studied most extensively, and overall AA positively influences T cell development and maturation, especially in the case of AA deficiency[56,72-75]. For B lymphocytes, responsible for the production of antigen-specific immunoglobulin (Ig) directed against invasive pathogens (antibodies), the effect of AA has not been unravelled yet. The reported effects of AA on NK cells are mostly positive, but there is no definitive conclusion yet. Under the influence of AA, mature NK cells are proliferating better, and the development of immature NK cells is improved[76-79].

Figure 1.9 Summary of effects of AA on lymphocytes.



DN: double negative, DP: double positive, Th: T helper cell, CTL: cytotoxic T cell, T reg: regulatory T cell, DC: dendritic cell, NK: natural killer.

1. T cell development: Enhanced T cell development due to fast transition from DN to DP stage.
2. Th cell differentiation: skewing towards Th1 and Th17, with inhibition of Th2 polarization.
3. CTL induction: Increased induction of CTLs due to production of IL-15 and IL-12 by DCs.
4. Treg induction: Current data are conflicting.
5. B cell: No conclusive data.
6. NK cell function: No conclusive data.
7. NK cell proliferation: increased NK cell proliferation.

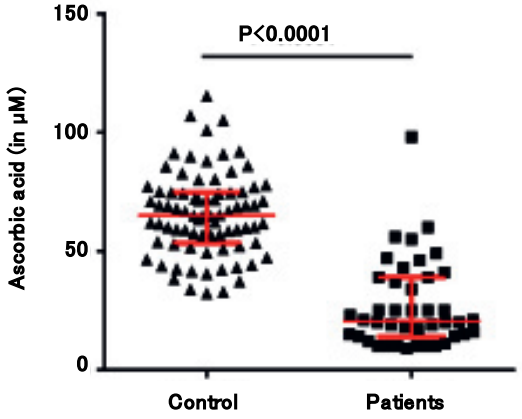
Dosage of AA seems important, since restoration to physiological levels results in mostly positive results, whereas supraphysiological doses are sometimes toxic for the cells[80-82]. Interpretation of *in vivo* data is limited regarding the ideal dosage since little information is available on the plasma levels and/or intracellular levels of lymphocytes in most of the intervention studies.

There is limited knowledge about the mechanisms that lead to the effect of AA on lymphocytes. Most of the time the effects seem to be due to epigenetic regulation through effects on DNA and histone demethylation. Other effects are probably more related to the antioxidant role of AA. It is likely that the epigenetic regulation is the most important pathway in developmental stages of lymphocytes[72,79].

Ascorbic acid and haematopoietic stem cell transplantation

We discovered that in haematological cancer patients serum AA levels are often severely decreased compared to healthy controls (20.5 ± 12 µM versus 65 ± 4 µM, respectively)[83] (**Figure 1.10**).

Figure 1.10 Serum AA levels in healthy volunteers and patients admitted for haematological malignancies[83].



Serum ascorbic acid concentrations (in µM) in healthy volunteers (n=79) and in patients admitted on a haematology ward with various diseases and treatments at a random moment (n=42). Significance was tested with a Mann-Whitney U test.

Serum AA levels were even undetectable in 19% of patients with a haematological malignancy, irrespective of the treatment[83]. Low serum AA levels were also seen in patients with autologous and allogeneic HSCT[83,84]. Since AA is a cheap and readily available drug with a safe profile, it is attractive to speculate that these cancer patients, who need to regenerate their immune system after HSCT, may benefit from the effects of AA on immune reconstitution. By supplementation AA during treatment, we hypothesized that mortality and morbidity resulting from opportunistic infections could be reduced in both autologous and allogeneic HSCT. It could also be that NK cells regenerate and mature faster. Thereby, they will be able to kill cancer cells more effectively in allogeneic HSCT. Perhaps the slow T cell regeneration after HSCT is even partly due to the AA-deficient state of these patients. Furthermore, AA supplementation could be used in cellular therapies, in which *in vitro* proliferated and adapted subtypes of lymphocytes are used to eliminate tumour cells *in vivo*.

Outline of the thesis

The main aim of the research performed in relation to this thesis is to improve the outcome of patients after haematopoietic stem cell transplantation, as it is still suboptimal. For allogeneic HSCT one of the problems is donor availability, but hope is that this problem might be solved by using haploidentical donors that are more widely available due to the introduction of PTCy as GVHD prophylaxis. For both autologous as well as allogeneic HSCT another major problem is slow immune reconstitution after the transplantation, which leads to infectious problems. Furthermore, it can delay a graft-versus-tumour effect in the allogeneic setting for a prolonged period. We discovered that patients during allogeneic and autologous HSCT have low AA levels. AA is important for the proliferation and differentiation of immune cells as previously described in this introduction. We hypothesized that supplementation of AA leads to faster immune reconstitution. This would lead to fewer infections, a faster graft-versus-tumour effect and thereby fewer relapses, and better overall survival.

In **chapter 2** we described a retrospective study investigating if a haploidentical donor is suitable as an alternative donor for patients in need of an allogeneic stem cell transplantation for chronic lymphatic leukaemia

(CLL). CLL is a malignant disease of the B- lymphocytes that is seen in the bone marrow, spleen or lymph nodes that is seen frequently in especially the elderly. On rare occasions, in fit patients with an aggressive disease course, there can be an indication for an allogeneic HSCT in order to cure these patients.

The outcome of haploidentical donor HSCT with PTCy after the introduction of this modality in our centre was assessed in **chapter 3**.

In **chapter 4**, we evaluated the cost effectiveness and resource utilization of haploidentical donor HSCT. We compared these costs with matched-related and matched unrelated donor HSCT.

The platform of haploidentical HSCT with PTCy was used to study the effect of a KIR-ligand mismatch in patients with multiple myeloma (MM) in **chapter 5**.

To investigate the effect of supplementation of AA on immune reconstitution after HSCT, we developed a method to measure intracellular AA in peripheral blood mononuclear cells (PBMCs) to reflect the total body storage. This method is described in **chapter 6**.

In **chapter 7**, we performed a systematic review of trials in which AA supplementation was used in cancer to investigate if there were any negative effects. The aim was to establish if AA supplementation in our HSCT patients would be safe.

The research protocol of the ViCAST study is outlined in **chapter 8**: a double blind, randomised, placebo-controlled study on the effect of AA supplementation on immune reconstitution in autologous stem cell transplantation in MM and lymphoma patients.

Finally, in **chapter 9**, we summarize all the results of our research in a general discussion. We speculate on how the findings described in this thesis will improve the outcome of allogeneic HSCT patients in the future. Finally, we philosophise on the possibilities of AA to improve immunity and to use it in cancer treatments.

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Part I

Haploidentical stem cell transplantation as alternative donor strategy

2

Outcomes of haploidentical stem cell transplantation for chronic lymphocytic leukaemia

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Bone Marrow Transplant. 2018 Mar;53(3):255-263.

Abstract

Allogeneic haematopoietic stem cell transplantation (HSCT) may result in long-term disease control in high-risk chronic lymphocytic leukaemia (CLL). Recently, haploidentical HCT is gaining interest because of better outcomes with post-transplantation cyclophosphamide (PTCy).

We analysed patients with CLL who received an allogeneic HSCT with a haploidentical donor and whose data were available in the EBMT registry. In total 117 patients (74% males) were included; 38% received PTCy as graft-versus-host disease (GVHD) prophylaxis.

For the whole study cohort OS at 2 and 5 years was 48% and 38%, respectively. PFS at 2 and 5 years was 38% and 31%, respectively. Cumulative incidences of non-relapse mortality in the whole group at 2 and 5 years were 40% and 44%, respectively. Cumulative incidences of relapse at 2 and 5 years were 22% and 26%, respectively. All outcomes were not statistically different in patients who received PTCy compared to other types of GVHD prophylaxis.

In conclusion, results of haploidentical HSCT in CLL seem almost identical to those with HLA-matched donors. Thereby, haploidentical HSCT is an appropriate alternative in high-risk CLL patients with a transplant indication but no available HLA-matched donor. Despite the use of PTCy, the cumulative incidence of relapse seems not higher than observed after HLA-matched HSCT.

Introduction

High-risk relapsed/refractory (R/R) CLL patients can nowadays be treated with novel agents targeting various kinases downstream of the B-cell receptor. The median progression-free survival (PFS) on the Bruton's tyrosine kinase (BTK) -inhibitor ibrutinib for R/R CLL patients with del (17p) is 26 months (95% CI 18-37) with an estimated 1-year PFS of 80%, while for all other patients median PFS is at least twice as long[1,2]. Two-third of these progressive patients have only CLL, the remainder have a Richter's transformation at the time of progression. These latter patients nowadays still have a very poor prognosis[3]. Patients failing ibrutinib with CLL can subsequently be treated with idelalisib which results in an overall response of approximately 35% and a median PFS of 9 to 12 months[4,5], or the BCL2 inhibitor venetoclax which results in an overall response of approximately 70% and a median PFS that had not been reached yet in two studies although with a relatively short follow-up (<12-14 months)[5,6]. Treatment options in case of refractoriness of ibrutinib, idelalisib and venetoclax are scarce and the outcome is poor.

Allogeneic haematopoietic stem cell transplantation (HSCT) may be a valuable option for fit high-risk patients with CLL with del(17) or TP53 mutation or when refractory to one or more of the new agents. An allogeneic HSCT is currently the only treatment able to achieve longer-lasting PFS especially in the many patients that achieve minimal residual disease (MRD)-negativity[7] and its effect is not influenced by the presence of del (17p), TP53 or other high risk molecular abnormalities[8,9]. The fact that MRD-negativity typically occurs during or after tapering of post-transplantation immunosuppression and/or in the context of chronic graft versus host disease (GVHD) suggests an actual graft-versus-leukaemia effect[10-12].

In several retrospective registry studies, 5-year PFS and overall survival (OS) after allogeneic HSCT for CLL were 36 to 46%[13,14]. In most patients in these studies, HLA-identical donors were used.

Approximately 15 to 20% of patients lack an HLA-identical sibling or fully HLA-matched unrelated donor, but very few publications addressed the issue of using alternative donor sources for allogeneic HSCT in CLL. The use of HLA-mismatched unrelated donors for CLL patients reduces 3- and 5-year overall

survival when compared to HLA-matched related or unrelated donors as a result of increased non-relapse mortality (NRM)[15], while umbilical cord blood transplantation resulted in almost comparable 3-year overall survival compared with HLA-matched transplantations[16]. These effects on outcome of a lower degree of HLA matching between donor and patient are seen in various other haematological malignancies too[17].

In recent years the use of haploidentical donors has substantially increased because the use of post-transplantation cyclophosphamide (PTCy) after T-cell replete stem cell transplantation effectively prevents severe acute GVHD in the majority of patients while retaining T-cell mediated anti-infectious immunity resulting in an acceptably low NRM (11-14% after 1-4 years) and PFS in various haematological malignancies[18,19]. These results seem comparable or only slightly inferior to outcome after matched unrelated donor transplantations[20] and matched sibling donor transplantations[17], even though prospective randomized trials are lacking. Up to now, no study specifically focused on the outcome of haploidentical HSCT in CLL patients, while some larger series reporting on haploidentical HSCT with PTCy contained only a few patients with CLL[17,21-23].

We now report for the first time on the results of allogeneic HSCT with haploidentical donors in CLL with special emphasis on the effect of PTCy on relapse given the requirement of active donor immune cells for an operative graft-versus-leukaemia effect.

Methods

Study design: patients and definitions

All patients aged 18 years or older with CLL who received a first allogeneic HSCT with a related haploidentical donor and whose data were available in the European Society for Blood and Marrow Transplantation (EBMT) database between November 1984 and February 2016 were included in this study. All patients provided informed consent for the registration and the allogeneic HSCT, according to the Declaration of Helsinki.

The primary endpoint was to describe progression-free survival (PFS) after haploidentical HSCT, defined as time from allogeneic HSCT to relapse, disease progression, or death. The 2 and 5 years after allogeneic HSCT landmarks were selected for reporting point-estimates. Secondary endpoints were the probability of OS, defined as the probability of survival regardless of disease state in any point of time; acute GVHD at day 100; relapse or progression; and NRM defined as death without previous relapse or progression.

The intensity of the conditioning was based on the reported dosage of drugs and total body irradiation (TBI) and categorized according to published guidelines[24].

Statistical analysis

Median values and ranges are reported for continuous variables and percentages for categorical variables. The probabilities of OS and PFS were calculated using the Kaplan-Meier method and 95% confidence intervals (CI) are given. A log-rank test was used for univariate comparisons. P-values <0.05 were considered statistically significant.

Relapse/progression, NRM and acute GVHD were analysed in a competing risk framework[25]. The outcomes of the patients with and without PTCy were compared using a Gray's test. Statistical analyses were performed using SPSS 23 and R 3.1.0, packages "survival" and "cmprsk".

Results

Baseline patient and disease characteristics

Patient and disease characteristics are summarized in **Table 2.1.** One-hundred-seventeen patients with CLL (74% males) underwent a mismatched related donor transplantation between 1984 and 2015 (1984-1999: 10, 2000-2004: 18, 2005-2009: 23, 2010-2016: 66). Median follow up of patients alive after HSCT in the whole cohort was 37.6 months (range 2-187 months); the median follow-up of the patients alive after HSCT with PTCy was 30.5 months (range 2-67 months). Median age at transplantation was 54 years (years) (range 27-71 years). Median time from diagnosis to HSCT was 67 months (range 4-207 months). Eighteen patients (15%) had previously undergone

autologous HSCT. Disease status at HSCT was complete remission (CR) in 16%, partial remission (PR) in 39%, stable disease (SD) in 13% and progressive disease (PD) in 32%. Karnofsky score was known for 98 patients (84%), of these 96% had a score of 70 or more at the time of HSCT.

Table 2.1 Patient characteristics.

Parameter	Classification	All patients (n=117) N (%)	PTCy (n=40) N (%)
Patient gender (n=117/n=40)	Male	86 (74)	27 (68)
	Female	31 (26)	13 (32)
Median Age at allogeneic HSCT [years] (n=117/n=40)	Median (range)	53.5 (27-71)	56.5 (27-68)
Karnofsky Index	≥70	94 (96)	35 (97)
(n=98/n=36)	<70	4 (4)	1 (3)
Interval CLL diagnosis – allogenic HSCT [months] (n=117/n=40)	Median (range)	67 (4-207)	59 (5-156)
Previous autologous HSCT	Yes	18 (16)	6 (15)
(n=116/n=40)	No	98 (84)	34 (85)
Remission Status at allogeneic HSCT	Complete Remission	17 (16)	10 (26)
(n=110/n=38)	Partial Remission	43 (39)	15 (40)
	Stable disease	15 (13)	5 (13)
	Progressive disease	35 (32)	8 (21)

Abbreviations: PTCy: posttransplantation cyclophosphamide, HSCT: haematopoietic stem cell transplantation, CLL: chronic lymphatic leukaemia.

Baseline transplantation characteristics

Transplantation characteristics are summarized in **Table 2.2.** Fifty-eight percent of patients received reduced-intensity conditioning, 42% myeloablative conditioning. Peripheral blood (PB) stem cells were used in 68% of patients, bone marrow (BM) in 32%. The HSCT was sex-matched in 59% of recipient-donor pairs. Forty patients (38%) received PTCy as GVHD prophylaxis. The characteristics of those patients are specified in **Table 2.1 and 2.2**. Confounders were equally spread amongst patients with and without PTCy, except all the patients with PTCy were transplanted in the latest period. In the other 77 patients various other methods of GVHD prevention were used, for example *in vitro* T cell depletion, anti-thymocyte globulin (ATG) and alemtuzumab.

Table 2.2 Transplantation characteristics.

Parameter	Classification	All patients N (%)	PTCy N (%)
Year of allogeneic HSCT	1984-1999	10 (9)	0
(n=117/n=40)	2000-2004	18 (15)	0
	2005-2009	23 (20)	0
	2010-2016	66 (56)	40 (100)
Stem cell source	PB	79 (68)	17 (42)
(n=117/n=40)	BM	38 (32)	23 (58)
Conditioning regimen	MAC	47 (42)	17 (44)
(n=112/n=39)	RIC	65 (58)	22 (56)
Recipient-Donor sex-match	Patient male – Donor male	54 (46)	15 (38)
(n=116/n=39)	Patient male – Donor female	31 (27)	11 (28)
	Patient female – Donor male	16 (14)	8 (21)
	Patient female – Donor female	15 (13)	5 (13)
Recipient CMV-status	Patient negative	19 (20)	4 (11)
(n=93/n=37)	Patient positive	74 (80)	33 (89)
Donor CMV-status	Donor negative	37 (40)	15 (41)
(n=92/n=37)	Donor positive	55 (60)	22 (59)

PB: peripheral blood, BM: bone marrow, MAC: myeloablative conditioning, RIC: reduced intensity conditioning, CMV: cytomegalovirus.

Transplantation outcomes

GVHD

The cumulative incidence of acute GVHD at 100 days was 32% for grade II-IV and 16% for grade III-IV with a median time of onset of 23 days (range 4-57 days). The cumulative incidences of acute GVHD at 100 days with or without PTCy were similar for grade II-IV (28% vs. 31%, p=0.59). The cumulative incidences of severe acute GVHD (grade III/IV) were also not significantly different with or without the use of PTCy (11% vs. 17%, p=0.37).

NRM

Forty-seven patients experienced NRM, causes of death were mostly transplantation-related (26 died of infection, 11 of GVHD, 2 of organ failure, 2 of toxicity, 2 of myocardial infarction, 1 of a secondary malignancy, 1 of a cerebral lesion of unknown origin, 1 of bleeding, 1 unknown HSCT related). The cumulative incidence of NRM at 2 years was 40% (95% CI 30-50%) and at 5 years 44% (95% CI 34-54%). The cumulative incidences of NRM and at 2 and 5 years were not statistically different in patients who received PTCy compared to other types of GHVD prevention (39% (95% CI 23-55%) vs.

42% (95% CI 29-55%) at 2 years, 43% (95% CI 26-60%) vs. 47% (95% CI 33-60%) at 5 years, p=0.72).

Relapse or progression

The cumulative incidences of relapse or progression at 2 and 5 years were 22% (95% CI 13-30%) and 26% (95% CI 16-35%), respectively. Median time to relapse or progression was 3.4 months (range 0.5-118.2 months).

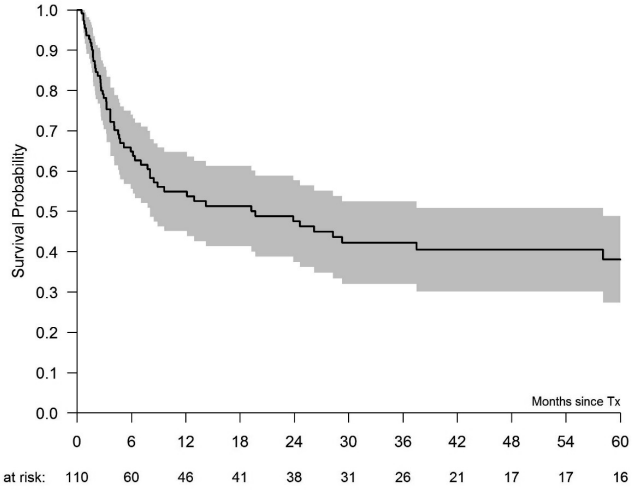
The cumulative incidence of relapse at 2 and 5 years were not statistically different in patients who received PTCy compared to other types of GVHD prevention (17% (95% CI 4-30%) vs. 25% (95% CI 13-36%) at 2 years, 17% (95% CI 4-30%) vs. 30% (95% CI 17-43%) at 5 years, p=0.34).

Overall, 25 patients relapsed or progressed after mismatched related HSCT. Of these patients, 8 were alive at last follow-up and 17 died.

OS and PFS

The probabilities of OS at 2 and 5 years were 48% (95% CI 37-58%) and 38% (95% CI 27-49%), respectively (**Figure 2.1**). PFS at 2 and 5 years was 38% (95% CI 28-48%) and 30% (95% CI 20-41%), respectively (**Figure 2.2**). Overall, 73 of the 117 patients died or relapsed.

Figure 2.1 Overall survival according to use of PTCy.



In univariate analysis, the use of PTCy vs. other GVHD prophylactic methods did not have a significant impact on PFS (44% (95% CI 27-61%) vs. 34% (95% CI 20-47%) at 2 years, 40% (95% CI 22-57%) vs. 24% (95% CI 11-36%) at 5 years, p=0.27) (**Figure 2.3**) and OS (48% (95% CI 31-65%) vs. 45% (95% CI 32-59%) at 2 years, 43% (95% CI 26-61%) vs. 36% (95% CI 22-50%) at 5 years, p=0.63) (**Figure 2.4**).

Figure 2.2 Progression-free survival after mismatched related HSCT for CLL.

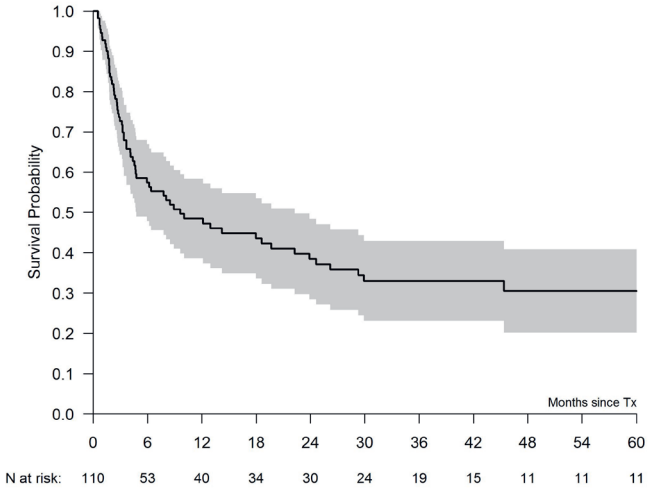


Figure 2.3 Progression-free survival according to use of PTCy.

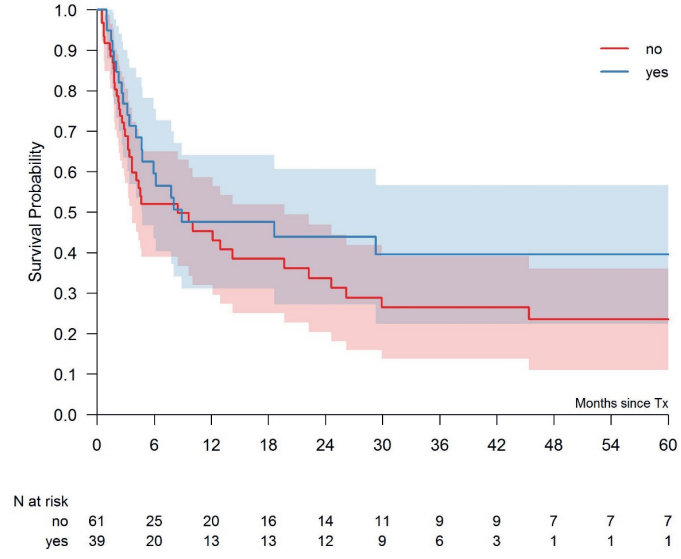
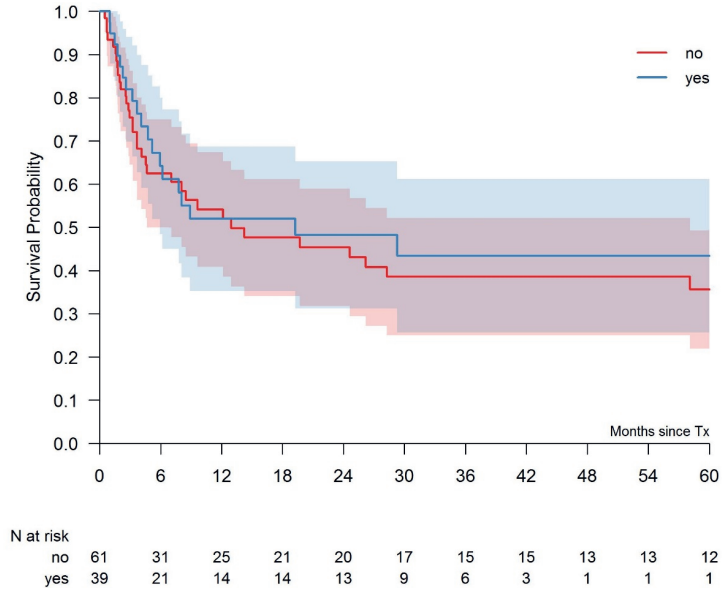


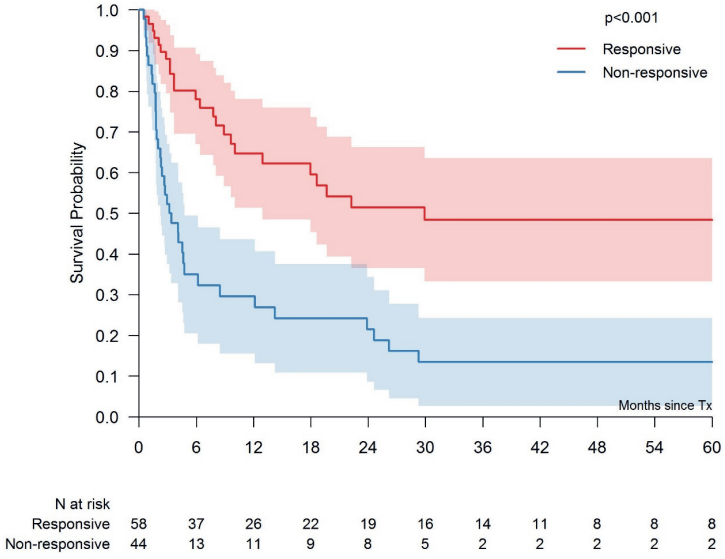
Figure 2.4 Overall survival according to use of PTCy.



Univariate analysis did not show a statistically significant impact on 2-year PFS and OS of patient age (≤53 vs. >53 years old), type of conditioning (myeloablative vs. reduced intensity conditioning), recipient donor gender match (female donor for male patients vs. all others), patient cytomegalovirus (CMV) status, stem cell source (PB or BM) and autologous transplantation before HSCT.

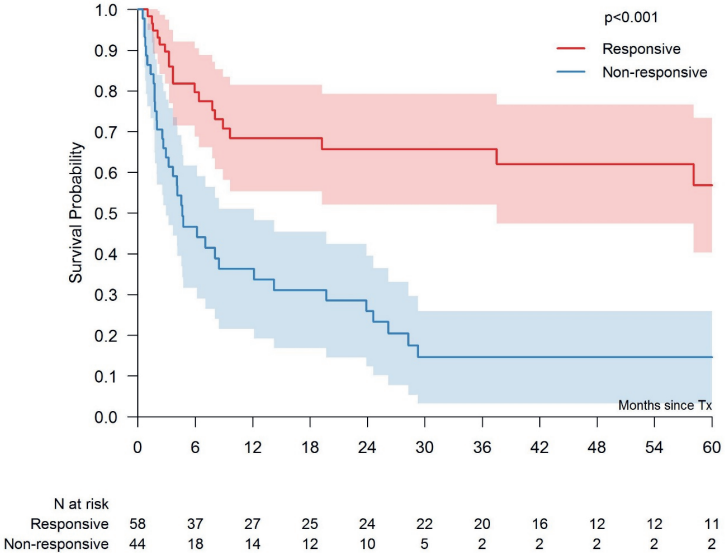
Disease status (responsive disease (CR/PR) vs. non-responsive disease (SD/PD) at HSCT had a very significant impact on PFS and OS. PFS at 2 years was 51% (95% CI 36-66%) for responsive disease and 22% (95% CI 9-34%) for no-responsive disease, PFS at 5 years was 48% (95% CI 33-64%) for responsive disease and 13% (95% CI 3-24%) for non-responsive disease ($p<0.001$) (**Figure 2.5**). OS at 2 years was 66% (95% CI 52-79%) for responsive disease and 26% (95% CI 12-40%) for non-responsive disease, OS at 5 years was 57% (95% CI 40-73%) for responsive disease and 15% (95% CI 3-26%) for non-responsive disease ($p<0.001$) (**Figure 2.6**).

Figure 2.5 Progression-free survival according to disease status.



N at risk											
Responsive	58	37	26	22	19	16	14	11	8	8	8
Non-responsive	44	13	11	9	8	5	2	2	2	2	2

Figure 2.6 Overall survival according to disease status.



N at risk											
Responsive	58	37	27	25	24	22	20	16	12	12	11
Non-responsive	44	18	14	12	10	5	2	2	2	2	2

Discussion

This is the first study that focused on outcomes of haploidentical HSCT for CLL patients.

Long-term outcome (5-year PFS/OS) of haploidentical HCT in CLL was in the same range to other publications on allogeneic HSCT in CLL patients where mostly HLA-identical donors were used[13,14] and this holds also true for the 40 patients that received PTCy as GVHD prophylaxis.

The use of PTCy seemed not to have a negative impact on the cumulative incidence of relapse, as it seems identical to the reports on outcome after HLA-matched HSCT where patients had similar baseline characteristics[13,14].

NRM was higher in this study than in the publications on allogeneic HSCT in CLL where mostly HLA-identical donors were used[13,14], but comparable to the results with umbilical cord blood transplantation (UCBT) and mismatched unrelated allogeneic HSCT[15]. The NRM in our study was also higher than described in other publications about haploidentical HSCT using PTCy[18,19,26], but in those studies patients were younger and were mostly in complete remission. NRM found in our study was comparable with studies on haploidentical HSCT with in vivo T-cell depleted grafts[27-29].

The incidence of both grade II-IV and grade III-IV acute GVHD was comparable with other studies on HSCT in CLL[13,15,30] and slightly lower than with UBCT in CLL[16]. The incidence of severe acute GVHD after PTCy was slightly higher than described in other publications on haploidentical HSCT with use of PTCy[18,19], but this could be just a coincidence due to the small amount of patients in this group.

In other studies, several risk factors have been identified for a poor outcome of allogeneic HSCT in CLL patients[13-15]. These include higher age, lower performance status, unrelated donor type, unfavourable sex-mismatch (female donor to male patient), prior autologous transplantation and remission status at transplantation[13]. In our study, both PFS and OS were significantly better in patients with responsive disease compared to patients with non-responsive disease at transplantation. This was also the risk factor that had the highest hazard ratio (HR) for relapse and poor outcome in earlier

studies[13]. Other risk factors (prior autologous HSCT, lower performance status, unfavourable sex-mismatch and CMV status of patients) regarding the prognosis could not be identified in this small group of patients, but in earlier studies the impact of these factors was much lower than disease status at transplantation.

The availability of BTK and BCL2 inhibitors resulted in a decrease in the number of transplants because of their efficacy and relatively good tolerability. A subgroup of patients with del(17p)/TP53 mutation or being refractory to one or more of the new drugs may still benefit from allogenic HSCT. The timing of allogeneic HSCT in these patients depends on the expected benefit from it, especially with respect to NRM, above that of continuing the (sequential) use of the new drugs. The results of allogeneic HSCT appeared to be better for fit younger patients in remission and having an HLA- and, in case of males, sex-matched donor[13,14]. A higher risk of NRM after allogeneic HSCT is however acceptable when patients become refractory or intolerable to two or more of the new drugs as the response rate and PFS seems lower when applied sequentially[5,6]. In this case, the use of alternative donors, including haploidentical, seems appropriate based on the results of this study. The best moment to perform the allogeneic HCT is when the patient is in remission, since the results of patients with refractory or stable disease at HSCT are dismal.

In conclusion, this retrospective multi-centre analysis shows reasonable outcomes of CLL patients when transplanted with a haploidentical donor. Thereby, allogeneic HSCT with a haploidentical donor may be considered in patients with high-risk CLL and otherwise good risk transplantation characteristics that are refractory on BTK inhibitors and/or a BCL2 inhibitor. The use of PTCy as GVHD prevention seems not to compromise outcome in this setting.

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Part I

Haploidentical stem cell transplantation as alternative donor strategy

3

Introduction of T cell replete haploidentical transplantations in a single centre

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Abstract

Objectives
The aim of this study was to describe outcomes of T cell replete haploidentical haematopoietic stem cell transplantation (HSCT) after the introduction of this modality in our centre and to compare them with different donor types.

Method
Outcomes of 30 consecutive patients with haematological malignancies that received T cell replete haploidentical HSCT with post-transplantation cyclophosphamide (PTCy) from 2016 to 2018 in our centre were analysed and compared to the outcome of HLA related and unrelated matched donor HSCT (n=97) and to a historical cohort of T cell depleted (TCD) haploidentical HSCT (n=11).

Results
One year graft-versus-host-free relapse-free survival (GRFS) in haploidentical HSCT was comparable with other donor types (haploidentical 40%, matched related donor (MRD) 33%, matched unrelated donor (MUD) 25%, p=0.55). Non relapse mortality was high in haploidentical HSCT (50%), mostly due to infectious complications. However, relapse rates were only 3%, and OS and PFS after 1 year were 47% and thereby also similar to HLA-matched HSCT in our centre (MRD 53%, MUD 48%).

Conclusion
Our data show that T cell replete haploidentical HSCT has similar outcomes to HLA identical HSCT after introduction in our centre. Thereby, it is a feasible option when lacking an HLA-identical donor. This option has advantages over an unrelated donor as it brings less logistical challenges than MUD transplantations.

Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is recommended in some patients with high-risk haematological diseases. Historically, the preferred stem cell sources are human leukocyte antigen (HLA) matched related donors (MRD) or 10/10 HLA-matched adult unrelated donors (MUD) in most transplantation centres including ours. Many patients lack such donors. Alternative hematopoietic stem cell donors can be haploidentical donors. The HLA of these donors matches minimally 50% with the HLA type of the patient and they can often be easily and rapidly found among family members. However, HLA disparity between patient and donor induces a high incidence of severe graft-versus-host disease (GVHD) and graft rejection. Special measures are needed to prevent this. One strategy to prevent GVHD is to extensively deplete donor T cells from the graft (TCD). The key publication and subsequent updates from Perugia on haploidentical HSCT with TCD demonstrated that haploidentical HSCT can be effective in patients with acute myeloid leukaemia (AML) and that TCD resulted in a very low incidence of GVHD[1-3]. Non-relapse mortality (NRM) with this strategy remained very high and resulted mainly from lethal infections due to the prolonged T cell deficient state caused by the extensive TCD by the *in vitro* CD34+ cell selection. We, and others, performed this type of TCD haploidentical HSCT in the past and were not able to duplicate the results from Perugia. We observed an even higher rate of NRM and accordingly very low survival rates and have abandoned this type of transplantation[4,5]. Interesting strategies have been developed to overcome this drawback of TCD haploidentical HSCT, for instance by only removing specific cell types *in vitro*[6]. Different types of TCD are explained more in detail by Or-Geva et al.[7].

Since the development of *in vivo* T cell depletion with posttransplantation cyclophosphamide (PTCy), the use of haploidentical donor cells is universally increasing[8,9]. The biological concept of this technology is to functionally impair alloreactive donor T cells, activated by day 3 to 5 after transplant, and spare non-alloreactive T cells[10]. These unmanipulated T cell replete haploidentical HSCTs are characterized by an encouraging overall survival (OS) and low incidences of GVHD and relapse[8,11-14].

Several retrospective studies indicate that the results of transplantations with haploidentical grafts are comparable to HLA identical grafts[11,15-18], and

perhaps even better than those after an umbilical cord blood (UCB) graft or a MUD graft[1,11,17,19-21]. Even a large meta-analysis comparing haploidentical HSCT (n=1410) to MRD HSCT (n=6396) showed no significant differences between both treatment groups with regards to OS, relapse and GVHD-free, relapse-free survival (GRFS)[22]. T cell replete haploidentical HSCT has several other benefits: limited costs for the donor transplant (compared to MUD or UCB), rapid availability for almost all patients, and the possibility to collect additional cells for cellular therapy at the time of transplant or thereafter[23].

Given this opportunity, we decided to re-introduce haploidentical HSCT in our hospital in 2016 for patients without HLA-identical donors, however this time with PTCy. We decided to retrospectively evaluate the outcomes and compare them with outcomes from HLA-identical HSCT in the same period and with our historical cohort of TCD haploidentical HSCT, as a quality control for our institute to see if our transplant data are comparable to those published.

Methods

Patients

This is a retrospective study of 127 consecutive patients (30 T cell replete haploidentical, 36 MRD, 61 MUD) undergoing an allogeneic HSCT between 1th of January 2016 and 21th of September 2018 at the Maastricht University Medical Center, Maastricht, the Netherlands and a cohort of 11 patients receiving a TCD haploidentical HSCT from 2005 to 2011 in the same centre; a procedure we stopped because of the poor clinical results. We excluded UCB transplantations as we performed too little of them to draw any conclusions. All patients signed consent forms allowing analysis and dissemination of their outcome data. The follow-up and analyses were performed with November 2020 as last data point for all patients.

Donor selection criteria

In our institute, the following hierarchy for donor choice applied: the first donor choice for every patient was an HLA-identical donor (MRD or 10/10 MUD) but when not available a haploidentical family donor was chosen. Both patient and donor HLA typing were performed using sequence-based typing for HLA-A, -B, -C, -DRB1, and -DQB1 loci. In case a haploidentical donor was

available, patients were tested for the presence of donor-specific antibodies (DSA). When DSA were present in high levels (mean fluorescence intensity>4000) the donor was excluded.

Conditioning regimens

Haploidentical transplantations

T cell replete

Chemotherapy-based myeloablative conditioning regimens included thiotepa, busulphan and fludarabine (TBF) in most of these haploidentical stem cell transplantations according to the myeloablative conditioning regimen used in Genua, Italy[24]. In some indications, a radiotherapy-based conditioning was used with total body irradiation (TBI) consisting of 10 Gray (Gy) in fractionated doses, combined with fludarabine (Flu-TBI) ± cyclophosphamide before transplantation.

TCD

In all cases a myeloablative conditioning regimen was used that included thiotepa, fludarabine and TBI (8 Gy).

MRD and MUD

A non-myeloablative conditioning regimen with a combination of low dose TBI (2 Gy) and fludarabine was most often used in HLA-identical SCT. Other utilized regimens can be found in **Table 3.1**.

Stem cell source

The preferred source of stem cells in T cell replete haploidentical HSCT was bone marrow (BM). Peripheral blood (PB) was preferably used in HLA-identical transplantations. In these transplantations, unmanipulated BM and PB stem cells were given on day 0. Only in cases of ABO major or minor incompatibilities, red blood cell or plasma depletion of the harvested product was performed.

In TCD haploidentical HSCT, PB stem cells were used after ex vivo CD34 selection and cryopreservation.

GVHD prophylaxis

In recipients of T cell replete haploidentical grafts PTCy 50 mg/kg intravenously on days +3 and +5 was given to create in vivo T cell depletion, and they received cyclosporine (CyA) from day 0 until day +180 and mycophenolate mofetil (MMF) from day +1 to day + 28[24].

Most recipients of HLA-identical stem cell transplantations received CyA from day -3 to day +180 and MMF from day +1 to day +85. Only patients diagnosed with myelofibrosis also received anti-thymocyte globulin (ATG).

Recipients of TCD haploidentical grafts received ATG on days -6 to -2, but no GVHD prophylaxis after stem cell transplantation.

Supportive care

During neutropenia, ciprofloxacin and fluconazole were given as selective digestive tract decontamination. Anti-microbial prophylaxis furthermore consisted of valacyclovir, and after one year experience on haploidentical HSCT cotrimoxazole was added to prevent pneumocystis infections.

Endpoints

The primary endpoint was GRFS at 1 and 2 years. This was defined as time from transplantation until grade III-IV acute GVHD (aGVHD), chronic GVHD (cGVHD) requiring systemic immunosuppressive treatment, disease relapse or death, whichever occurred first[25]. Secondary endpoints were OS, progression-free survival (PFS), relapse rate, NRM, incidence and severity of aGVHD and cGVHD and time to engraftment.

Statistical method

Categorical variables are expressed as number and proportion, and continuous variables as median and range. The Kaplan–Meier method was used for the OS, PFS and one-year GFRS analyses.

The cumulative incidences of acute and chronic GVHD were estimated considering death not related to GVHD as a competing event. For the calculation of NRM, disease relapse or progression was treated as a competing event and for the calculation of relapse; NRM was treated as a competing event. Outcomes were calculated from the day of transplantation.

Comparisons between all groups were made using log-rank and Gray's tests, and p-values of these comparisons were given.

Analyses were performed using SPSS, version 25 and R software.

Results

Patient characteristics are summarized in **Table 3.1**.

In the T cell replete haploidentical group, the median age at transplant was 61 years, 70% of patients were older than 55 years. The most common diagnosis was AML (53%). The other groups (MRD, MUD, TCD haploidentical) were well matched for the indicated demographics, with two exceptions: patients in the TCD group were younger and, in that group, there were hardly any other transplant indications than AML (73%). Most patients were in remission at the time of transplantation and there were no significant differences between the treatment groups.

Most T cell replete patients received a TBF-based, myeloablative conditioning regimen (97%), while this was only around 27% in HLA-matched transplantation. In 90% of the T cell replete haploidentical HSCT the stem cell source was BM. MUD donors were generally younger and better sex matched.

Table 3.1 Patient characteristics.

Parameter	Haplo + PTCy (n=30) N (%)	MRD (n=36) N (%)	MUD (n=61) N (%)	T-deplete haplo (n=11) N (%)	p
Median age (range)(years)	60.3 (19-74)	61.3 (23-71)	58.3 (21-76)	43.8 (19-61)	0.002
Sex					0.70
Male	20 (67)	24 (67)	35 (57)	6 (55)	
Female	10 (33)	12 (33)	26 (43)	5 (45)	
HCT-CI score					0.26
0	19 (63)	17 (47)	32 (52)	10 (91)	
1 or 2	7 (23)	12 (33)	18 (30)	1 (9)	
≥3	4 (13)	7 (19)	11 (18)	0 (0)	
Diagnosis					0.06
AML	16 (53)	16 (44)	19 (31)	8 (73)	
ALL	6 (20)	3 (8)	6 (10)	0 (0)	
MDS/MPN	6 (20)	5 (14)	16 (26)	1 (9)	
NHL/HL/CLL	2 (7)	8 (22)	16 (26)	0 (0)	
Other	0	4 (11)	4 (7)	2 (18)	

Table 3.1 (continued)

Parameter	Haplo + PTCy (n=30) N (%)	MRD (n=36) N (%)	MUD (n=61) N (%)	T-deplete haplo (n=11) N (%)	p
Disease risk index					0.34
Low	0 (0)	5 (14)	4 (7)	0 (0)	
Int	17 (57)	19 (53)	36 (59)	7 (64)	
High	13 (43)	12 (33)	21 (34)	4 (36)	
Number allogeneic transplantation					0.33
First	29 (97)	33 (92)	58 (95)	9 (82)	
Second	1 (3)	3 (8)	3 (5)	2 (18)	
CMV status recipient/donor					0.90
+/-	10 (33)	10 (28)	17 (28)	4 (36)	
Other	20 (67)	26 (72)	44 (72)	7 (64)	
Recipient/donor sex match					0.009
M/F	10 (33)	13 (36)	6 (10)	2 (18)	
Other	20 (67)	23 (64)	55 (90)	9 (82)	
Donor age, years					<0.001
≤40	15 (50)	4 (11)	52 (85)	5 (45)	
>40	15 (50)	32 (89)	9 (15)	6 (55)	
Regimen type					<0.001
MAC	29 (97)	10 (28)	16 (26)	11 (100)	
Bu-based	27	6	10	0	
TBI-based	2	3	6	11	
Other	0	1	0	0	
RIC	1 (3)	10 (28)	14 (23)	0	
Flu/Cy	1	6	11	0	
Cy/TBI	0	2	1	0	
Other	0	2	2	0	
NMA	0 (0)	16 (44)	31 (51)	0	
Flu/TBI	0	16	31	0	
Stem cell source					<0.001
PBSC	3 (10)	35 (97)	58 (95)	0 (0)	
BM	27 (90)	1 (3)	3 (5)	11 (100)	

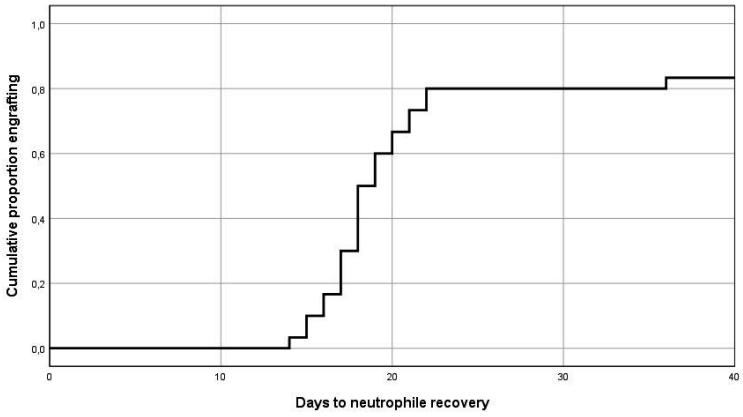
Abbreviations: Haplo: haploidentical, PTCy: posttransplantation cyclophosphamide, MRD: matched related donor, MUD: matched unrelated donor, HCT-CI: haematopoietic stem cell transplantation comorbidity index, AML: acute myeloid leukaemia, ALL: acute lymphatic leukaemia, MDS: myelodysplastic syndrome, MPN: myeloproliferative neoplasia, NHL: non-Hodgkin's lymphoma, HL: Hodgkin's lymphoma, CLL: chronic lymphatic leukaemia, Int: intermediate, CMV: cytomegalovirus, M/F: male recipient, female donor, MAC: myeloablative conditioning, Bu: busulphan, TBI: total body irradiation, RIC: reduced intensity conditioning, Flu: fludarabine, Cy: cyclophosphamide, NMA: non-myeloablative conditioning, PBSC: peripheral blood stem cells, BM: bone marrow.

Median time to follow-up of patients alive was 37.5 months for T cell replete haploidentical HSCT, 36.8 months for MRD HSCT and 46.0 months for MUD HSCT.

Outcomes of T cell replete haploidentical HSCT

Median time to neutrophil engraftment was 18 days (range 14 to 36) (**Figure 3.1**). Five patients (17%) did not have engraftment, most of these patients died before engraftment. These patients died of infectious complications. In univariate analysis, in this small group there was no relationship between the number of nucleated cells present in the graft and not having engraftment.

Figure 3.1 Neutrophil recovery.



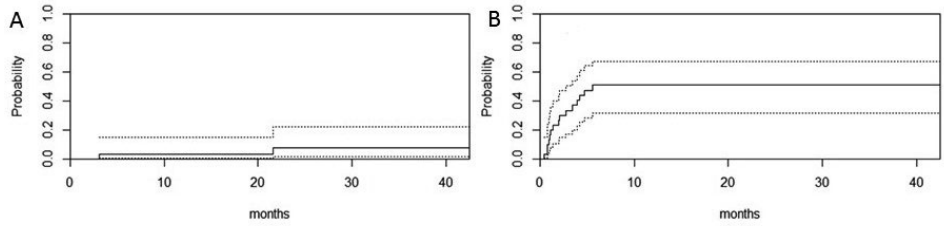
The cumulative incidence of neutrophil recovery after T cell replete haploidentical stem cell transplantation, day 0 is the day of transplant, n=30.

After a median follow-up of 37.5 months (range, 25.9 to 54.5), 13 out of 30 patients were alive and in remission. The 1-year OS and PFS was 47% (95% confidence interval (CI), 30-64), and 2-year OS and PFS 43% (95% CI, 26-60), with a 1-year relapse incidence of 3% (95% CI 0-9) (**Figure 3.2A**). NRM at 1 year was 50% (95% CI 31-67) (**Figure 3.2B**).

Causes of death in the first year are listed in **Table 3.2**. The main cause of death was infection (86%). Details about the type of infections can be found in **Table 3.2**.

In multivariate analysis (looking at age, gender, disease risk index, HCT-CI, recipient-donor sex match, recipient-donor CMV status, donor age), patient age was the only factor that correlated with OS (hazard ratio (HR) 2.4). GRFS at 1 and 2 year was 40% (95% CI 22-58) with no new events after 1 year.

Figure 3.2 Cumulative incidence of non-relapse mortality (NRM) and relapse in T cell replete haploidentical HSCT.



A. Relapse after stem cell transplantation (with 95% CI)
B. NRM after stem cell transplantation (with 95% CI)
HSCT: haematopoietic stem cell transplantation, NRM: non-relapse mortality, CI: confidence interval.

Table 3.2 Causes of death after T cell replete haploidentical HSCT.

Cause of death	Number of patients
Sepsis	7
Pneumocystis jirovecii pneumonia	3
Viral respiratory infection	3 (1 corona NL63, 2 parainfluenza type 3)
Aspergillus infection	1
Graft failure	1
Relapse	1

HSCT: haematopoietic stem cell transplantation

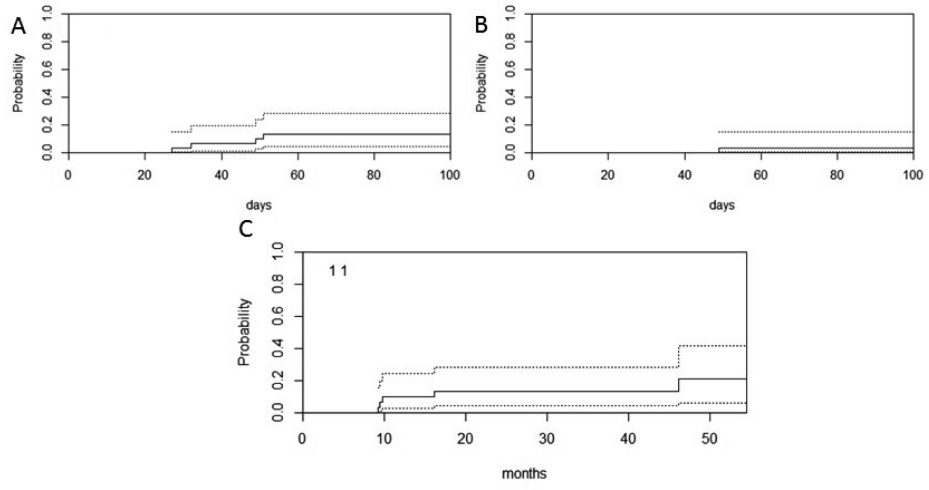
The cumulative incidences of grade II to IV and grade III to IV aGVHD at 100 days post-transplant were 13% (95% CI 4-28) and 3% (95% CI 0-15), respectively (**Figure 3.3A-B**). The cumulative incidence of cGVHD at 1- and 2-years post-transplant were 10% (95% CI 2-24) and 13% (95% CI 4-28), respectively (**Figure 3.3C**).

Comparison with outcomes of MRD and MUD HSCT

There were no significant differences between the outcomes of HSCT with a T cell replete donor, MRD or MUD. One year OS rates were 47% (95% CI 30-65), 53% (95% CI 37-69) and 48% (95% CI 35-61) for T cell replete haploidentical, MRD and MUD (p=0.63) (**Figure 3.4A**). One year relapse incidence was also similar in T cell replete haploidentical HSCT to MRD and MUD (respectively 3% (95% CI 0-9), 17% (95%CI 5-29) and 21% (95% CI 11-21) (p=0.08). NRM at 1 year was also not significantly different between these groups, respectively 50% (95% CI 31-67) for T cell replete haplo, 33% (95% CI 17-49) for MRD, and 36% (95%CI 24-48) for MUD, p=0.43). The cumulative incidences of grade II to IV and grade III to IV aGVHD at 100 days

post-transplant were not significantly different as well, but there was a trend towards a higher probably of aGVHD in MUD HSCT with a grade II to IV of 23% (95% CI 13-34) and grade III-IV 16% (95% CI 8-27) versus 13% (95% CI 4-28) and 3% (95% CI 0-15) in T cell replete haploidentical HSCT and 8% (95% CI 2-20) and 6% (95% CI 0-17) in MRD HSCT, respectively (p=0.08 and p=0.09). The cumulative incidence of cGVHD at 1- and 2-years post-transplant were 10% (95% CI 2-24) and 13% (95% CI 4-28) for T cell replete haploidentical HSCT, 11% (95% CI 4-25) and 19% (95% CI 8-34) for MRD HSCT and 23% (95% CI 13-34) and 25 (95%CI 16-36) for the MUD HSCT, respectively (p=0.32).

Figure 3.3 Cumulative incidence of GVHD in T cell replete haploidentical HSCT.



A. Grades 2-4 acute GVHD (with 95% CI)
B. Grades 3-4 acute GVHD (with 95% CI)
C. All chronic GVHD (with 95% CI)
GVHD: graft-versus-host disease, HSCT: haematopoietic stem cell transplantation, CI: confidence interval.

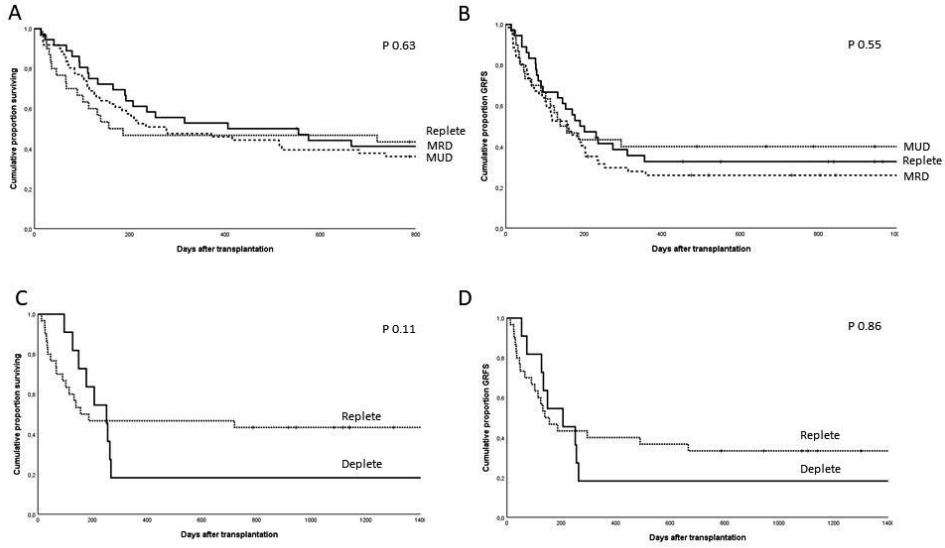
One- and 2-year GRFS rates were also similar (40% (95% CI 22-58), 33% (95% CI 17-49) and 26% (95% CI 14-38) for T cell replete haploidentical, MRD and MUD, respectively (p=0.55) (**Figure 3.4B**)).

Comparison with outcomes of TCD haploidentical HSCT

One year OS and GFRS was better in T cell replete haploidentical HSCT than in the historical TCD haploidentical HSCT cohort (though not significant due to

low numbers); OS was 47% (95% CI 30-65) and 18% (95% CI 0-41)(p=0.11) respectively, and GRFS was 40% (95% CI 22-58) and 18% (95% CI 0-41) respectively (**Figure 3.4C-D**) (p=0.86).

Figure 3.4 Adjusted estimated probabilities of overall survival (OS) and GVHD-free relapse-free survival (GRFS) in T cell replete haploidentical HSCT and all donor types.



A. OS T cell replete haploidentical vs. MRD vs. MUD HSCT
B. GRFS T cell replete haploidentical vs. MRD vs. MUD HSCT
C. OS T cell replete haploidentical vs. historical T cell deplete haploidentical HSCT
D. GRFS T cell replete haploidentical vs. historical T cell deplete haploidentical HSCT
HSCT: haematopoietic stem cell transplantation, MRD: matched related donor, MUD: matched unrelated donor.

Discussion

T cell replete haploidentical HSCT with PTCy is increasingly used worldwide, but data shown in literature are mostly from large multicentre analyses and generally from centres with high volumes. Here, we report the introduction of this transplantation method in our relatively small transplantation centre, which is the smallest in the Netherlands[26]. On average, we perform 40 to 50 allogeneic HSCT per year, with a staff of only 9 haematologists.

In 2016, we introduced haploidentical HSCT combined with PTCy. In this retrospective analysis for quality control, we find OS and GRFS of PTCy haploidentical HSCT to be similar to those of our MRD and MUD HSCT.

Remarkably, NRM rate was much higher than expected from literature. In large retrospective registry studies, NRM is 10 to 25%, while in our patients this was 50%[27-30]. The main cause of death was an infectious complication after transplantation. This could be partly due to an unfortunate and serious outbreak of respiratory viral infections at our ward during this period. Out of the 15 patients that received a haploidentical HSCT during these outbreaks, 7 patients were diagnosed with a respiratory viral infection and 3 died due to this infection. After this experience, in our second year of T cell replete haploidentical HSCT, we adapted our policy to prevent viral infections on the ward (for example by constant wear of mouth masks, limitation of visitors, early extensive testing, and stricter isolation). After all these measurements, we noticed a decrease in incidence of infections (only 1 out of 15 patients was diagnosed with a respiratory viral infection) and a decreased mortality (no patient died of respiratory viral infections in this period).

Another reason for the high NRM rate could be the high incidence of pneumocystis jirovecii pneumonia (PJP) (5 out of 15 patients in the first year). After 1 year of performing haploidentical T cell replete HSCT, we started to use prophylactic cotrimoxazole, and noticed a decrease in PJP incidence (1 out of 15 patients).

After all these adaptations, NRM decreased from 60% to 40%. This is still quite high but could partly be explained by the fact that most of the patients were of older age (median 60.3 years) and were all still receiving myeloablative conditioning. Recently after this analysis, we adapted our policy and decided to use a reduced intensity conditioning regimen in older patients to see if this could lower the mortality rate. However, this might be at the cost of a higher risk of relapse.

In other retrospective studies, a prominent cause of death was disease relapse (with 25 to 45% of patients experiencing relapse within 2 years)[9,27-30]. Only 1 of our patients (3%) experienced relapse in the first year in the T cell replete haploidentical group, so our relapse rate was much lower than expected. This could be partly explained by the high NRM that is a competing

risk for relapse and could also be linked to the myeloablative conditioning regimen.

Cumulative incidences of acute and chronic GVHD were low in our haploidentical T cell replete HSCT, and comparable to those in literature when BM is used as stem cell source[27-30].

Due to the low relapse and GVHD rate, OS, PFS and GRFS were comparable to outcomes found in literature. However, direct comparisons are difficult to be made since our numbers are limited and because these outcomes are dependent of different risk factors like age, co-morbidity, and disease status.

In our centre, HLA identical HSCT at time of the analysis was still performed without PTCy. The use of PTCy in HLA identical HSCT can potentially improve the outcome. In a recent randomized trial a 1-year GFRS in HLA-identical transplantations (MRD and MUD) of 45% was showed, but this is in the same range as our data with haploidentical HSCT[31]. Another option to improve GFRS in HLA identical HSCT could be the addition of ATG, as we did not use this in most of our patients. However, in most studies ATG only lowers GVHD and does not influence OS[32,33] and gives similar results to PTCy[34].

In the past, we performed several TCD haploidentical HSCT using the same method as described by the Perugia group. In contrast to their results, we had a very high NRM rate that was mainly due to infections, and we noticed that less than a fifth of patients survived. We hypothesize that the difference in climate in the Netherlands versus Italy, and perhaps also of living conditions, might be the reason for that. In addition, other centres could not reproduce the good outcomes of the Perugia group so these cannot be the only factors responsible for the worse outcome. Perhaps, these serious infectious problems could have been partly avoided with a different type of TCD. In the CD34 positive cell selection not only all T cells, but also natural killer cells and B cells are removed, while in other types of in vitro TCD, specific subsets are removed and thereby the immune system remains more intact. However, in our hands and in those of others, this type of TCD haploidentical HSCT was not seen as a suitable alternative for patients lacking an HLA-identical donor. We therefore stopped to perform haploidentical HSCT for several years. For the T cell replete haploidentical transplantations were performed more recently, we see better outcomes than in our historical patient group.

In light of this analysis and the logistical problems we experienced last year due to the COVID-19 pandemic, we recently adapted our search strategy and prefer a haploidentical donor above a MUD 10/10 when there are no DSA.

Conclusion

In summary, this comparison shows that outcomes after T cell replete haploidentical HSCT are comparable to those of HLA-identical HSCT in our hands. They are better than the outcomes of TCD haploidentical HSCT in our centre in the past. We performed this study as a quality control for our institute to see if our transplant data are comparable to those published. At the start, we experienced a learning curve but could decrease our initially high TRM due to infectious complications. Even though this study has its limitations due to the small number of patients, the heterogeneity between them and the retrospective nature, we conclude that the use of a haploidentical donor is a valid real-world choice for patients in need for an allogeneic HSCT. A haploidentical donor is usually quickly available for almost all patients, and the choice for this type of donor is logistically easier to arrange than a MUD.

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Part I

Haploidentical stem cell transplantation as alternative donor strategy

4

The impact of donor type on resource utilization and costs in allogeneic haematopoietic stem cell transplantation in the Netherlands

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Abstract

Background
Allogeneic haematopoietic stem cell transplantation (HSCT) is increasingly used, but this treatment is complex and costly. As clinical outcomes of HSCT with matched unrelated donor (MUD) and haploidentical donors are similar, costs could influence donor choice.

Method
We retrospectively compared resource utilization and costs of HSCT using the three different donor types (matched related donor (MRD) (n=32), haploidentical related (n=30) and MUD (n=60)) within the first year after transplantation. Costs were analysed through a bottom-up method. Non-parametric bootstrapping was applied to test for statistical differences in costs. Subgroup analyses were performed to identify predictors for costs.

Results
Cost pre-transplant for search and acquisition of the graft were significantly higher in MUD HSCT (€35,222) versus MRD and haploidentical HSCT (€15,356 and €16,097, respectively). The costs of haploidentical HSCT were the highest in the transplant phase. Main cost factors were inpatient days and medication. Overall, the costs for haploidentical and MUD HSCT were similar (€115,724 for MUD, €113,312 for haploidentical).

Conclusion
Our study suggests no difference in total transplantation costs between allogeneic HSCT using a MUD or a haploidentical donor. Since clinical outcomes seem similar as well, the choice of donor type might be based on availability, speed, and logistics.

Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is an important curative treatment option for various, especially malignant, haematological disorders. Over the last decades, procedures have been changed to make HSCT possible in a larger group of patients and the incidence of HSCT is rising exponentially[1]. However, the treatment is complex, resource intense and thereby costly[2-4]. The most ideal donor is a human leukocyte antigen (HLA)-identical family donor, as it is often easily obtained and clinical results of HSCT with this type of donor are best[5]. Since families in our community are getting smaller, the availability of this type of donor is decreasing. In the last years, there is an increase in the use of alternative donors[6], like HLA-identical matched unrelated donors (MUD), unrelated umbilical cord blood (UCB), or related haploidentical donors.

When looking for an alternative donor, in only 50 to 60% of patients worldwide a HLA-identical donor can be found in the extensive unrelated donor registries. For patients from non-European descent, this percentage is even much lower[7].

A haploidentical donor is a family donor that shares one haplotype with the patient and differs with a variable number of HLA-genes in the other, unshared haplotype. This means that every parent or child can be a suitable donor, and 50% of the siblings. Even second-degree family members still have 25% change to be a haploidentical match. This means that for almost all patients a haploidentical family donor can be found[8]. This donor type is increasingly used worldwide since the introduction of post-transplantation cyclophosphamide (PTCy)[9,10]. Traditionally, when using a haploidentical donor there was higher incidence of graft-versus-host disease (GVHD) and infections and thereby poor outcomes. However, studies have shown that PTCy has overcome this problem and even though some studies give contradicting results and prospective studies on this subject are lacking, outcomes (especially the composite end-point GVHD-free relapse-free survival (GRFS)) of haploidentical HSCT + PTCy seem quite similar to the outcomes in MUD HSCT[11-13].

Since outcomes are comparable, the choice between a MUD or haploidentical family donor is difficult and mostly based on local guidelines or logistical

aspects. Perhaps in this case, resource utilization and thereby costs might be an important aspect in donor choice. Larger studies on costs of allogeneic HSCT are conducted based on insurance claims with limited clinical information and show that allogeneic HSCT in general is a very costly treatment with high health care resource utilisation, but provide no information about different donor types[2,14]. Smaller studies from hospital perspective are limited and do not compare costs between MUD and haploidentical donors[3,15].

As clinical outcomes of HSCT with MUD and haploidentical donors are similar and no other studies have been performed on this subject, we investigated if there was a difference in intervention and hospital care costs between different donor types.

Therefore, the study aims to gain insight into the differences in resource utilization and costs between matched related donor (MRD), MUD and haploidentical donor HSCT from a hospital perspective looking in the time-period around the HSCT (pre, during, and after transplant) and identify the main cost-drivers.

Materials and methods

Design

We conducted a retrospective cohort study in a contemporary group of adult patients that received an allogeneic HSCT to describe the health resource utilization and costs of transplantation from a hospital-based perspective.

Study population

This retrospective cohort study included consecutive haematological patients treated with an allogeneic stem cell transplantation between January 2016 and September 2018 in the Maastricht University Medical Centre in the Netherlands. The clinical outcome has been published and shows comparable outcome on graft-versus-host free progression free survival in the different groups[16]. Patient characteristics such as age, sex, and diagnosis were obtained. All patients signed consent forms allowing analysis and dissemination of their outcome data.

Three phases of transplantation

We divided the transplantation into three phases: pre-transplantation, transplantation, and post-transplantation. The pre-transplantation phase included the donor search and selection and the harvesting of stem cells. The transplantation phase began on the first hospital admission day for the stem cell transplantation procedure including conditioning, until discharge. The post-transplantation phase was defined as the period from discharge to 1 year after the stem cell transplantation or death if it occurred earlier.

Perspective, costs and resource utilization

We took a hospital perspective and for the identification, all treatment-related hospital activities were incorporated. Data included all relevant treatment activities. In the pre-transplantation phase, only activities for the donor selection (including HLA typing of patient and potential donors, medical examination donors, and all costs related to the MUD search and acquisition) and harvesting of stem cells (both peripheral blood as well as bone marrow) were seen as relevant. In the transplantation and post-transplantation phase, the activities were subcategorised into six cost groups: inpatient days, outpatient visits (including day care), intensive care admission, medication, blood products, laboratory, and other activities (e.g., endoscopy and radiation).

For the resource use measurement, data were obtained from the hospital information system provided by System Applications and Products (SAP) that included detailed information about all relevant inpatient treatment activities. In addition, we collected information from electronic patient documents (EPD). For the pre-transplant phase, we only collected data on the number of potential donors that we assessed for HLA type for every patient to find a suitable match. We divided them in potential family donors and unrelated donors. For the transplant phase, we examined inpatient days, intensive care unit (ICU) admission and blood product use for all different donor types since these are activities are costly and well registered in the information systems.

For the valuation, costs were calculated by multiplying recorded units of healthcare resources used with corresponding unit prices[17]. Most of the unit costs were taken from the Dutch Manual for Healthcare Costing Research[17,18]. For costs of medical procedures that were not available in this manual, the tariffs from the Dutch health care authority (NZa) were used. Medication costs

were calculated per unit, based on the price per dosage of the drug in the Netherlands (medicijnkosten.nl) and multiplied by the total dosage given.

The index year for the study is 2020, and all cost-prices are updated to this year using consumer prices indices[19].

Analysis

For baseline characteristics, categorical variables were expressed as number and proportion, and continuous variables as median and range. Differences between baseline characteristics of the different groups were calculated using a Pearson's chi-squared test for categorical variables and a one-way ANOVA for continuous variables. Mean transplantation costs per patient were calculated for each phase and per cost category. The 95% confidence interval (CI) was determined using non –parametric bootstrapping with 5,000 iterations (resampling with replacement) and was used to test for statistical differences in costs between different donors[20]. If costs were not overlapping, they were considered significantly different. The main cost drivers for each type of HSCT were identified by calculating the proportion of total costs of each cost group. Differences in resource utilisation between groups were calculated with one-way ANOVA. Multiple linear regression was used to investigate the association between baseline characteristics (disease risk index (low/intermediate versus high/very high), age (<40, 40-60, >60 years), stem cell source (peripheral blood versus bone marrow), myeloablative conditioning (MAC) (yes or no), the occurrence clinical events (early death (within 100 days after transplantation), death 100 to 1 year after transplantation, relapse, all grades of GVHD, acute GVHD grade III/IV, chronic GVHD, the occurrence of cytomegalovirus reactivation/disease and intensive care unit (ICU) admittance)) and total costs. For all analyses, a p-value under 0.05 was considered significant. The analyses were performed using Microsoft Excel 2016 and SPSS, version 25.

Results

A total of 122 patients receiving HSCT were included in the analysis: 32 with MRD, 60 with MUD and 30 haploidentical family donors. Patient characteristics are listed in **Table 4.1** and are similar in all three groups.

Table 4.1 Patient and transplantation characteristics.

Parameter	MRD (n=32) N (%)	MUD (n=60) N (%)	Haplo (n=30) N (%)	p value
Median age (range)(years)	60.0 (23-71)	58.5 (21-76)	60.3 (19-74)	0.87
Sex				0.45
Male	22 (69)	34 (57)	20 (67)	
Female	10 (31)	26 (43)	10 (33)	
Diagnosis				0.33
AML	14 (44)	19 (31)	16 (53)	
ALL	3 (9)	5 (8)	6 (20)	
MDS/MPN	5 (16)	16 (26)	6 (20)	
NHL/HL	6 (19)	16 (26)	2 (7)	
Other	4 (13)	4 (7)	0	
Disease risk index				0.27
Low	4 (13)	4 (7)	0 (0)	
Intermediate	18 (56)	37 (62)	17 (57)	
High/very high	10 (31)	19 (32)	13 (43)	
Stem cell source				<0.0001
PBSC	31 (97)	57 (95)	3 (10)	
BM	1 (3)	3 (5)	27 (90)	
Conditioning intensity				<0.0001
MAC	4 (13)	14 (23)	28 (93)	
NMA/RIC	28 (87)	46 (77)	2 (7)	
TBI-based conditioning	25 (78)	48 (80)	3 (10)	<0.0001
1 year OS	18 (44)	29 (48)	14 (47)	0.71
1 year relapse	4 (13)	13 (22)	1 (3)	0.06
Acute GVHD, all grades	7 (22)	23 (38)	6 (20)	0.11
Acute GVHD, grade III/IV	2 (6)	10 (17)	1 (3)	0.10
1-year chronic GVHD	4 (13)	11 (18)	2 (7)	0.31
1-year CMV reactivation/disease	5 (16)	19 (32)	6 (20)	0.19

Abbreviations: MRD: matched related donor, MUD: matched unrelated donor, haplo: haploidentical, AML: acute myeloid leukaemia, ALL: acute lymphatic leukaemia, MDS: myelodysplastic syndrome, MPN: myeloproliferative neoplasia, NHL: non-Hodgkin's lymphoma, HL: Hodgkin's lymphoma, PBSC: peripheral blood stem cells, BM: bone marrow, NMA: non-myeloablative, RIC: reduced intensity conditioning, TBI: total body irradiation, OS: overall survival, GVHD: graft-versus-host disease, CMV: cytomegalovirus.

The median age was around 60 years, and there was a slight male predominance. Acute myeloid leukaemia was the most common diagnosis in all groups; disease risk index (DRI) was most frequently intermediate. Besides donor type, there were more differences in transplantation strategy between the groups. All patients receiving a haploidentical stem cell transplantation received post-transplantation cyclophosphamide (PTCy) as part of the GVHD prophylactic therapy, and none of the patients in the other groups did. The main stem cell source in patients with a haploidentical donor was bone marrow (90%), in both other groups it was peripheral blood (97

and 95%, respectively) (p<0.0001). A variety of conditioning regimens was used during the study period based on donor, patient, and disease type, but 93% of patients in the haploidentical cohort had myeloablative conditioning, versus 13% in MRD and 23% in MUD (p<0.0001). Furthermore, significantly more patients with a MRD and a MUD donor received TBI as part of the conditioning regimen (MRD 78%, MUD 80%, haploidentical 10%; p<0.0001).

Costs and health care resource utilization

Pre-transplantation phase

Table 4.2 presents the mean costs and health resource utilization for the pre-transplantation phase for all donor types. The mean costs for a MUD were significantly higher than for the other types of donors (€35,222, versus €15,356 for MRD and €16,097 for haploidentical donor). The mean costs for graft acquisition in MUD were €19,050. To identify an appropriate haploidentical donor, HLA typing was done on average in only one extra family member. However, the total amount of potential donors that were HLA-typed was similar in each group.

Table 4.2 Costs and resource utilization in pre-transplantation phase.

	MRD (n=32)	MUD (n=60)	Haplo (n=30)	p
Pre-transplantation costs, mean (95% CI)	€15,356 (13,581-17,511)	€35,222 (32,993-37,656)	€16,097 (14,141-18,324)	
Family members HLA typed, mean (range)	2.94 (1-7)	1.53 (0-4)	3.93 (1-13)	<0.0001
Unrelated donors HLA typed, mean (range)	0.25 (0-3)	2.57 (1-6)	0.57 (0-4)	<0.0001
Total potential donors HLA-typed, mean (range)	3.19 (1-8)	4.10 (1-8)	4.50 (1-14)	0.06

Abbreviations: MRD: matched related donor, MUD: matched unrelated donor, haplo: haploidentical, CI: confidence interval, HLA: human leukocyte antigen.

Transplantation phase

Table 4.3 presents the mean costs for the transplantation phase for all donor types. The mean costs for a haploidentical HSCT were significantly higher than for a MRD and MUD HSCT (€59,568, €35,874, and €42,154, respectively). Inpatient days are the largest cost category in all groups. In haploidentical HSCT, the second cost category was medication, in MRD and MUD HSCT this was the third cost category. The costs of medication, blood products,

laboratory tests and other costs were significantly different between donor types. The difference in other costs was almost fully explained by the difference in costs of radiation as part of the conditioning regimen.

Table 4.3 Costs during transplantation phase.

	MRD (n=32)	MUD (n=60)	Haplo (n=30)
Transplantation costs, mean (95% CI)	€35,874 (27,944-45,370)	€42,154 (33,237-52,270)	€59.568 (52,843-66,806)
Medication costs, mean (95% CI)	€6,514 (2,942-12,629)	€5,320 (3,034-7,970)	€14,870 (12,780-16,988)
% of total costs	11.9	9.7	25.0
Costs inpatient days, mean (95% CI)	€16,122 (13,047-19,923)	€15,432 (13,502-17,527)	€23,274 (20,820-25,760)
% of total costs	49.0	45.4	41.6
Blood product costs, mean (95% CI)	€2,936 (1,779-4,407)	€3,348 (2,544-5,079)	€8,456 (6,735-10,298)
% of total costs	6.8	7.2	13.6
ICU costs, mean (95% CI)	€817 (0-2,802)	€5,231 (888-11,658)	€6,175 (1,482-11,648)
% of total costs	0.6	4.1	7.6
Laboratory costs, mean (95% CI)	€2,916 (2,248-3,680)	€3,069 (2,491-3,715)	€5,814 (5,066-6,619)
% of total costs	8.2	7.7	10.1
Other costs, mean (95% CI)	€6,569 (4,627-9,631)	€9,354 (6,282-13,271)	€980 (337-1,751)
% of total costs	23.5	26.0	2.1

Abbreviations: MRD: matched related donor, MUD: matched unrelated donor, haplo: haploidentical, CI: confidence interval, ICU: intensive care unit.

Table 4.4 represents the resource utilization of all donor types. The total length of stay of patients receiving a haploidentical donor HSCT was significantly longer (mean 36.67 days) compared to MRD (23.81 days) and MUD (24.77 days), $p<0.0004$. There was no significant difference in the number of patients that needed to be admitted at the intensive care unit (ICU) and in the mean length of stay on ICU. However, there was a trend for an increased ICU admission in haploidentical HSCT compared to MRD that probably failed to have significance due to low numbers. In addition, the use of blood products in the haploidentical HSCT was more than double compared to MRD and MUD HSCT.

Table 4.4 Resource utilization transplantation phase.

	MRD (n=32)	MUD (n=60)	Haplo (n=30)	p
Length of stay on transplantation ward, mean (days)(range)	23.44 (9-85)	22.37 (6-53)	33.83 (15-57)	<0.0001
Number of patients with ICU admission (percentage)	3 (9.4)	6 (10.0)	8 (26.7)	0.07
ICU admission, mean (days)(range)	0.38 (0-12)	2.4 (0-73)	2.83 (0-24)	0.41
Number of transfusions, mean (range)	8.09 (0-43)	9.95 (0-50)	20.23 (4-50)	<0.0001

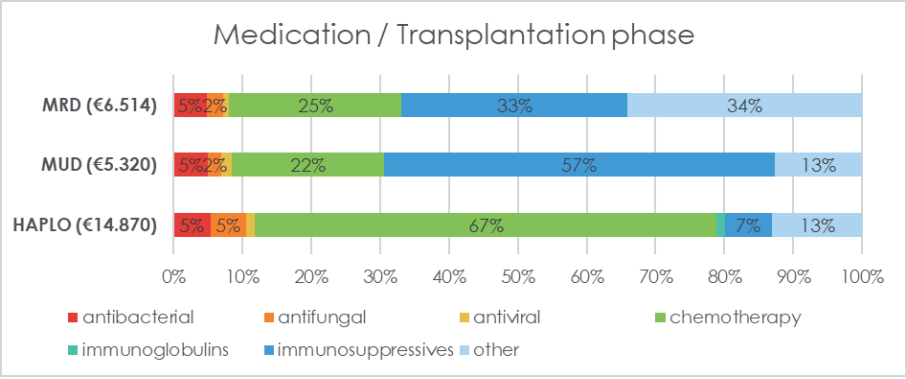
Abbreviations: MRD: matched related donor, MUD: matched unrelated donor, haplo: haploidentical, ICU: intensive care unit.

Since there was a major difference in medication costs and they were a significant cost driver in haploidentical HSCT, we divided the medication in 7 categories: antibacterial agents, antifungal agents, antiviral medication, chemotherapy, immunoglobulins and all other medication. In **Figure 4.1**, percentages of costs in the different medication categories are presented.
Most striking is the difference in the use of chemotherapy between haploidentical HSCT versus MRD and MUD HSCT. The explanation for this is that in the haploidentical group almost all conditioning was chemotherapy-based with thiotepa combined with fludarabine and busulphan. Thiotepa is expensive and mainly responsible for the difference medication costs in the transplant phase of the haploidentical group (€6,233). Most patients in the MRD and MUD groups had a conditioning regimen with a combination of fludarabine, which is much cheaper than thiotepa, and total body irradiation (included in the other costs category, €6,200). The difference in costs of immunosuppressive therapy between HLA-identical and haploidentical HSCT is caused by the use of high dosage of anti-thymocyte globulin in myelofibrosis, in this disease category there is a strong preference for a MRD or MUD according to local protocol.

Post-transplantation phase

Table 4.5 presents the mean costs for the post-transplantation phase for all donor types. Like in the transplant phase, inpatient days were still the largest cost category in all groups, followed by medication and laboratory costs. All costs and the division of costs between subcategories were similar in the three groups.

Figure 4.1 Mean percentage of medication costs per category for every donor type in the transplant phase.



Abbreviations: MRD: matched related donor, MUD: matched unrelated donor, haplo: haploidentical.

Table 4.5 Costs in the post-transplantation phase.

	MRD (n=29)	MUD (n=56)	Haplo (n=24)
One-year post-transplantation costs, mean (95% CI)	€45,626 (28,794-65,007)	€41,927 (33,409-51,221)	€42,695 (27,825-58,280)
Medication costs, mean (95% CI % of total costs	€10,530 (6,660-15,204) 24.2	€11,720 (7,287-15,420) 20.8	€6,337 (4,117-8,828) 22.4
Costs inpatient days, mean (95% CI) % of total costs	€19,119 (10,983-29,273) 29.9	€17,111 (12,467-22,274) 34.4	€13,127 (6,687-21,048) 24.2
Blood product costs, mean (95% CI) % of total costs	€4,034 (1,742-7,027) 7.4	€3,236 (2,278-4,276) 7.9	€5,510 (2,189-9,567) 9.1
ICU costs, mean (95% CI) % of total costs	€2,104 (363-4,474) 2.4	€3,386 (1,055-6,209) 4.7	€8,172 (2,076-15,801) 12.6
Laboratory costs, mean (95% CI) % of total costs	€7,075 (5,043-9,495) 22.9	€7,105 (5,741-8,626) 20.0	€7,158 (4,870-9,740) 20.8
Outpatient visit costs, mean (95% CI) % of total costs	€1,691 (1,343-2,052) 9.3	€1,701 (1,421-1,988) 7.9	€1,458 (960-2,056) 7.8
Other costs, mean (95% CI) % of total costs	€1,073 (675-1,537) 3.9	€1,580 (940-2,523) 4.4	€932 (535-1,429) 3.2

Abbreviations: MRD: matched related donor, MUD: matched unrelated donor, haplo: haploidentical, CI: confidence interval, ICU: intensive care unit.

Table 4.6 represents the resource utilization in the post-transplant phase. There were no significant differences in resource utilization (length of stay, ICU admission, number of re-hospitalisations and the use of blood products) for the different donor types.

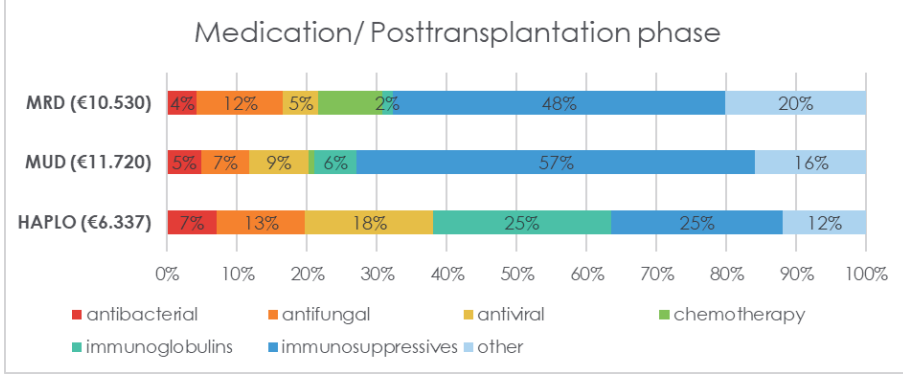
Table 4.6 Resource utilization in the post-transplantation phase.

	MRD (n=29)	MUD (n=56)	Haplo (n=24)	p
Length of stay on transplantation ward, mean (days)(range)	28.21 (0-168)	24.89 (0-140)	19 (0-103)	0.53
ICU admission (days)(range)	0.97 (0-10)	1.55 (0-23)	3.75 (0-27)	0.13
Number of re-hospitalizations during first year, mean (range)	1.83 (0-7)	1.86 (0-6)	1.29 (0-4)	0.27
Number of transfusions, mean (range)	11.55 (0-74)	10.14 (0-48)	15.08 (0-87)	0.49

Abbreviations: MRD: matched related donor, MUD: matched unrelated donor, haplo: haploidentical, ICU: intensive care unit.

In **Figure 4.2**, percentages of costs in the different medication categories are presented. The most striking difference here is the increased costs for immunoglobulins in the haploidentical HSCT. This high usage was caused by the standard use of immunoglobulins on several time point in our initial protocol for haploidentical HSCT. Another difference is the use of immunosuppressive medication between haploidentical HSCT versus MRD and MUD HSCT, which was caused by the use of ruxolitinib and anti-thymocyte globulin for GVHD in the last two groups.

Figure 4.2 Mean percentage of medication costs per category for every donor type in the post-transplant phase.



Abbreviations: MRD: matched related donor, MUD: matched unrelated donor, haplo: haploidentical.

Total transplant costs

The total transplant costs for the patients that reached all transplant phases were similar for MUD (n=56) and haploidentical donor (n=24) HSCT (€115,724 (95%CI 103,858-128,836) for MUD and €113,312 (95% CI 95,910- 131,583) for haploidentical donor HSCT). However, MRD (n=29) HSCT seems the cheapest option (€92,331 (95% CI 76,384-111,447). In the pre-transplantation phase, the total costs for the patients that reached all transplant phases were similar to the patients that died during the transplantation phase. In the transplantation phase, dying patients on average cost €5,000 more than the surviving patients. There were no significant differences in the mean observation time after HSCT between groups (MRD: 275 (94-365) days, MUD: 236 days (46-365), haplo: 245 days (17-365), p=0.37).

Multivariate analysis

The model estimating the costs of complications included both baseline data and clinical events and was calculated with the total transplant costs over all phases. Among all the transplant recipients, significant increased costs were associated with CMV reactivation/disease (€31,366, p=0.001) and death 100 days to 1 year after transplant (€42,407, p<0.0001). Lower costs were seen with relapse (-€42,047, p=0.0002) and early death (within 100 days after transplantation) (-€27,974, p=0.008). We did not find an association between costs and the occurrence of all grades of acute GVHD, acute GVHD grade III/IV, chronic GVHD and ICU admittance. However, especially for severe acute GVHD and ICU admittance numbers might just have been too low to reach significance. We also did not find an association between any of the baseline characteristics of patients and transplant analysed (donor type, disease risk index, age, stem cell source, myeloablative conditioning). The r^2 of the model was 0.316. Analysis of only transplant and post-transplant phase together led to comparable results.

Discussion

This single centre study aimed to gain insight into the differences in costs between MRD, MUD and haploidentical donor HSCT from a hospital perspective (pre-, during and post-transplantation) and identify the main cost-drivers.

In the pre-transplant phase, the mean costs for MUD were much higher than for MRD and haploidentical donors due to the costs of graft acquisition (mean €19,050). The number of potential donors that were HLA-typed was similar in each group.

In the transplantation phase, using a haploidentical donor was most expensive since these patients were hospitalized longer, and with a haploidentical HSCT, more resources like medication and blood products were used. This difference in resource utilization in the transplant phase is most likely caused by the increased use of myeloablative conditioning regimens in haploidentical HSCT, the use of bone marrow as graft source and the use of PTCy as GVHD prophylaxis, which all lead to longer and deeper cytopenia. During the time of the study, we did not use PTCy as GVHD prophylaxis in MRD and MUD allogeneic HSCT which might now been seen as standard treatment[21]. This might have an influence on the costs of MRD and MUD HSCT. In the last 10 months, we performed 14 HLA-identical HSCT (11 MRD, 3 MUD) using PTCy as GVHD prophylaxis. In those patients, the mean duration of hospitalisation in the transplant phase was 34.42 days, which is 10 days more than before and comparable with the hospitalisation in haploidentical HSCT. Those ten days of hospitalisation will cost only €6,880, but the longer cytopenia will probably also lead to an increased use of blood products, medication and laboratory test as that has been seen by the haploidentical transplantations.

Noteworthy is also the effect of the conditioning regimen on costs. Haploidentical HSCT were mostly conditioned with thiotepa, busulphan and fludarabine, MRD and MUD mostly with fludarabine and total body irradiation (TBI). The use of thiotepa is the most important cost-driver of the conditioning in haploidentical HSCT, but these costs are similar to the costs of TBI in MUD and MRD HSCT (€6.200).

In the post-transplant phase, costs and resource use were equal between all three groups. In total, no significant difference in cost was seen between HSCT from MUD or haploidentical donors. None of the baseline characteristics of patient or transplant was predictive for higher costs. In addition, the use of bone marrow instead of peripheral blood in haploidentical HSCT did not have an effect on the costs. It is intuitive that occurrence of major complications would increase costs of transplantation. However, in this population we only could see a significant raise in costs with CMV disease/reactivation and death between 100 and 365 days after transplantation. The costs of these complications are foremost related to long hospitalisation, ICU admittance and the use of expensive medication when having these complications. In these patients, we did not use the new antiviral drug letermovir as CMV prophylaxis in high-risk groups yet. It would be interesting to investigate the effect letermovir on costs, since this treatment is very expensive (€32,700 for 100 days in the Netherlands) but could lead to a significant decrease of CMV disease/reactivation, which is also one of the major cost drivers in HSCT[22].

No significant effect on costs was seen with acute GVHD. This is surprising in the light of findings of another investigator that did see increased costs with acute GVHD, but in this study almost double the number of patients were included and it was performed in the US where health care costs might be different than in the Netherlands[23]. Furthermore, we showed that in our situation patients with early death within 100 days had lower costs, and so did patients with relapsed disease.

At the time of the study, there was a preference for a 10/10 MUD over a haploidentical family donor in our centre, and tests were done to find a potential MUD donor even though there was already a haploidentical family donor present. If there is no preference between MUD or haploidentical donors, it could reduce the number of potential donors to analyse, and thereby reduce costs. Recently, because of logistical problems with MUD due to COVID-19, we decided to change standard policy now choose a haploidentical family donor over a MUD if possible. Since that change, we had an increase in haploidentical donor HSCT at the cost of MUD HSCT (in the last months 16 haploidentical donors versus 10 MUD, before 30 haploidentical donors versus 61 MUD, unpublished observation). In addition, by this strategy it could be possible to save costs for HLA typing a MUD donor if a haploidentical family donor is found.

There are a number of limitations to this study. One of them is the limited number of patients per group and the heterogeneity within and between groups. Furthermore, as in any single centre study, conclusions might be specific to our centre and reflect our specific patients and practice. Costs are also very sensitive to country rules and regulations and might only reflect our local Dutch situation. That is why we presented the data also with number of events, so that local cost can be calculated from these parameters as they might differ from ours. We obtained our real-world data from the hospital registration system, medical patient files, and electronic information systems. Our results depend on the completeness of registration, and unregistered hospital activities were not included in our study. No costs for home-care services and other providers were included in the study, since they were thought to only make a very small contribution to the total costs of transplantation. However, all these factors might have caused an underestimation of actual costs. Even more, in the pre-transplantation phase, we decided only to gather data on search, selection and acquisition of the graft. This excludes various other activities that are done in preparation for the stem cell transplantation, like consultations, laboratory tests, and pulmonary and cardiac function tests. However, these activities are not very costly, and it is difficult to differentiate between the use of the health resources in light of transplantation and other treatments, like remission-induction therapy. Most other activities in this phase were more likely to be connected with disease characteristics and not transplantation and are thought to be not different between the donor groups as the availability of a certain type of donor is not connected to the type of treatment before transplantation and can be considered biological randomisation. However, this could explain that the total costs we calculated are lower than in the study by Blommestein et al. that also took place in the Netherlands[3]. Additionally, we only regarded costs from a hospital perspective, and not from a societal perspective. Furthermore, since we performed hardly any UCB HSCT, we were not able to compare the costs of this donor type. Noteworthy is a recent retrospective study on outcomes between MUD HSCT with PTCy versus haploidentical HSCT that showed an OS advantage of MUD in the RIC setting[24]. In that case, outcome is more important than costs. However, in the only prospective study on the effect of PTCy compared to traditional GVHD prophylaxis (with 69% MUD HSCT and 99% RIC), this survival advantage was not seen[21].

Even though there are all these limitations, we believe our results can be informative for centres that want to determine best donor choice also with respect to costs. These data can also be useful for other centres to determine opportunities to adapt protocols to decrease costs and health resource utilisation in HSCT, for instance by replacing more expensive conditioning regimens or to use outpatient care in eligible HSCT candidates.

In summary, our study suggests that there is no substantial difference in total transplantation costs between allogeneic HSCT using a MUD or a haploidentical donor using the procedure described. Since clinical outcomes are similar as well in our procedure, the choice of donor type might be based on availability, speed, and logistics. However, costs will only be comparable in the future, if the choice of donor and protocol has no influence on cost drivers, like post-transplant cyclophosphamide. A substantial cost driver in MUD HSCT that will not change by changing the clinical procedure is the graft acquisition (15% of the total costs). However, if the adaptation of protocols leads to a difference in GRFS between different donor types, the cost factor might be less relevant.

Conclusion

In summary, this comparison shows that outcomes after T cell replete haploidentical HSCT are comparable to those of HLA-identical HSCT in our hands. They are better than the outcomes of TCD haploidentical HSCT in our centre in the past. We performed this study a quality control for our institute to see if our transplant data are comparable to those published. At the start, we experienced a learning curve but could decrease our initially high TRM due to infectious complications. Even though this study has its limitations due to the small number of patients, the heterogeneity between them and the retrospective nature, we conclude that the use of a haploidentical donor is a valid real-world choice for patients in need for an allogeneic HSCT. A haploidentical donor is usually quickly available for almost all patients, and the choice for this type of donor is logistically easier to arrange than a MUD.

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Part I

Haploidentical stem cell transplantation as alternative donor strategy

5

Haploidentical transplantation in patients with multiple myeloma making use of natural killer cell alloreactive donors

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Abstract

Disease relapse is an important problem after allogeneic stem cell transplantations in multiple myeloma (MM). To test the hypothesis that natural killer (NK) cell alloreactivity in the setting of a haploidentical haematopoietic stem cell transplantation (HSCT) can reduce the risk of myeloma relapse, we performed a small prospective phase 2 study in which we transplanted poor risk MM patients using a killer-cell Immunoglobulin-like receptor (KIR)-ligand mismatched haploidentical donor. Patients received bone marrow grafts after reduced-intensity conditioning, with post-transplantation cyclophosphamide (PTCy) graft-versus-host-disease (GVHD) prophylaxis. The primary endpoint was 1.5-year progression-free survival (PFS), stopping rules were installed in case interim results made a benefit of 50% PFS at 1.5 years unlikely. After inclusion of 12 patients, of which 9 were evaluable for the primary endpoint, all patients relapsed within a median time of 90 days. All except 1 patient showed engraftment, with a median time to neutrophil recovery of 18 (12-30) days. The study was prematurely terminated based on the predefined stopping rules after the inclusion of 12 patients. With this small study we show that in chemo-resistant myeloma patients NK cell KIR- mismatch is not superior to conventional allogeneic HSCT. This strategy, however, can serve as a platform for new treatment concepts.

Introduction

In recent years, the use of haploidentical donor cells for treatment of haematological malignancies has been increasing and has proven effective and safe, especially with the use of PTCy[1,2]. There is limited documentation about haploidentical HSCT in MM, but small retrospective studies show this is feasible with relatively low non-relapse mortality (NRM). Results in terms of PFS are however similar to HLA-identical HSCT[3-5]. Haploidentical HSCT offers the attractive opportunity to introduce natural killer (NK) cell alloreactivity based on a KIR-ligand mismatch. In myeloid malignancies this NK alloreactivity, in the context of a T cell deplete haploidentical HSCT, leads to a decreased relapse rate and improved survival without causing GVHD[6-8]. Unlike in T cell depleted haploidentical HSCT, the benefit of NK cell alloreactivity in T cell replete haploidentical HSCT is less clear and the limited number of small sized studies concerning this effect are not conclusive[9-12].

There is ample evidence to support a role of NK cells in fighting MM[13-16]. It has been proven that therapeutic interventions like lenalidomide and elotuzumab result in increased NK cell mediated anti-myeloma responses[17,18]. In preclinical studies performed by our research group, we show that NK cells are able to kill MM cells, and this killing is improved in the presence of NK alloreactivity[19,20]. This was shown in vitro as well as in a humanized mouse model. Two small clinical studies also implicated a beneficial role for KIR-ligand mismatch in MM. In one study, the role of administration of alloreactive NK cells before autologous HSCT was examined. High remission rates were observed, although they were short-lived[21]. In another study, the impact of KIR-ligand mismatch in a HLA-identical and mismatched HSCT setting was investigated and showed that KIR-ligand mismatch in the graft-versus-host direction was protective for relapse[22].

The aim of this phase 2 study was to prospectively evaluate if KIR-ligand mismatched haploidentical bone marrow transplantation (haploBMT) with PTCy improves PFS in poor risk MM patients.

Methods

Patients

In this prospective, single-arm, multicentre trial, we recruited poor risk MM patients aged below 66 years with good clinical performance from hospitals in the Netherlands. Poor risk was defined as high-risk cytogenetics, or relapse within a year after autologous HSCT, or relapse after three or more previous lines of therapy. Furthermore, patients had to be responsive to their last line of therapy, defined as at least partial response according to the International Myeloma Working Group consensus criteria[23]. Another prerequisite of enrolment was the permissiveness to NK alloreactivity and availability of a KIR-ligand mismatched haploidentical family donor. Patients were excluded if donor-specific HLA-antibodies were present.

Donor selection

All patients were transplanted with a KIR-ligand mismatched haploidentical family donor. The opportunity for KIR-ligand mismatched haploBMT was determined by Luminex Sequence Specific Oligonucleotide Hybridization (SSO) typing for the three possible inhibitory KIR ligands: HLA-C group 1 (ligands for KIR2DL2/3), HLA-C group 2 (ligands for KIR2DL1), HLA-Bw4 including HLA-A harbouring Bw4 motifs as ligands for KIR3DL1 (A*23, A*24, A*32).

In case of an opportunity for KIR-ligand mismatched haploBMT, a KIR-ligand mismatched haploidentical family donor was searched in the wide family tree of the patients. In case a probable KIR-ligand mismatched donor was identified by low resolution, a second blood sample was drawn from this potential donor for confirmation in high resolution, for KIR typing, by low resolution Luminex SSO assay. Protein expression of the mismatched KIR was confirmed by immune phenotyping of the peripheral blood NK cells for KIR expression as described below.

Immune phenotyping for KIRs during donor selection and NK cell reconstitution

Peripheral blood mononuclear cells were isolated by gradient density centrifugation and stained with monoclonal antibodies with specificity for CD3 (SK7, BD), CD56 (B159, BD), NKG2A (Z199, Beckman Coulter), KIR2DL1

(143211, R&D), KIR2DL2/3/S2 (DX27, Miltenyi Biotech) or KIR3DL1 (DX9, Miltenyi Biotech) followed by acquisition of the samples on a BD FACS Canto II machine. Acquired data were analysed using Diva software by gating on CD3- CD56+ lymphocytes followed by analysis of the percentage of positive cell for the individual KIRs.

Conditioning and transplant procedure

Conditioning regimen consisted of cyclophosphamide 14.5 mg/kg on day -6 and -5, fludarabine 30 mg/m^2 from day -6 to -2 and 200 cGY total body irradiation at day -1 in all but one patient that received busulphan instead of cyclophosphamide pre-transplant. Donor bone marrow cells were infused on day 0. Bone marrow cells used in all patients since they are preferred over peripheral blood stem cells because of a lower risk of GVHD[24,25].

GVHD prophylaxis and supportive care

GVHD prophylaxis consisted of cyclophosphamide 50 mg/kg at day +3 and +4. Mycophenolate mofetil was used from day +5 to day +35. Tacrolimus 0.1 mg/kg was added to this combination day +5 to day +180.
To prevent infections, patients received immunoglobulins 0.2 g/kg once every 4 weeks from 1 week before conditioning until the immunosuppressive drugs were stopped. Anti-microbial prophylaxis furthermore consisted of cotrimoxazole and valaciclovir, and during neutropenia, ciprofloxacin and fluconazole were given as selective digestive decontamination.

Study endpoints and statistical analysis

Primary endpoint was PFS at 1.5 years. Since haploBMT is a demanding and costly treatment for the patients we considered the effect that has to be realized by this procedure needed to be substantial and chose for the PFS goal of 50% in 1.5 years compared to around 25% with conventional allogeneic HSCT according to historical data.

To test this hypothesis, a Simon's Two-Stage design was used. We hypothesized that the 1,5-year PFS will be 50% after haploBMT, while this is maximum 25% in the hypothetical standard treatment group (conventional allogeneic HSCT according to historical data). To demonstrate this difference with a power of 80% and a type 1 error rate, alpha (one-sided), of 0.05%, 24 patients were needed. If 1 or less of the first 9 patients experienced

1.5-year PFS, the relevant predefined positive effect was considered very unlikely, and the study would be stopped.
Secondary endpoints were engraftment, bone marrow reconstitution, NK cell reconstitution and repertoire, GVHD, infections and NRM.

To ensure safety, we build in decision rules to prematurely terminate the study if the NRM at 100 days exceeded a certain percentage that was calculated beforehand based on a modification of the standard 3+3 scheme.

Analyses were performed as of February 2020. Pre-transplantation patients' characteristics were given as median and range for continuous variables and as frequency and proportion for categorical variables. PFS was analysed using a Kaplan Meier estimate. For NRM, relapse and GVHD a competing risk framework was used. The analysis was performed with R software.

Results

In total, 12 poor risk patients were included from 3 hospitals in the Netherlands from April 2016 to April 2018. Pre-transplantation patient characteristics are described in **Table 5.1**. They were all heavily pre-treated with both proteasome inhibition and immunomodulatory drugs. One patient had high-risk cytogenetics. We excluded one patient for further analysis even though he was transplanted due to disease progression just before BMT since this was a predefined exclusion criterion.

Table 5.1 Patient characteristics.

Patient characteristics	
Gender - n (%)	
Male	10 (91)
Female	1 (9)
Age – Median in years (range)	61 (40-66)
Response to last therapy - n (%)	
PR	5 (45)
VGPR	5 (45)
CR	1 (9)
Previous lines of treatment- median (range)	3 (2-7)
Previous HSCT - n (%)	
1x Autologous	8 (73)
2x Autologous	3 (27)
Allogeneic	1 (9)

Abbreviations: PR: partial response, VGPR: very good partial response, CR: complete response.

At this moment (April 2020), median time to follow-up is 30.2 months (range 11.8-44.9 months).

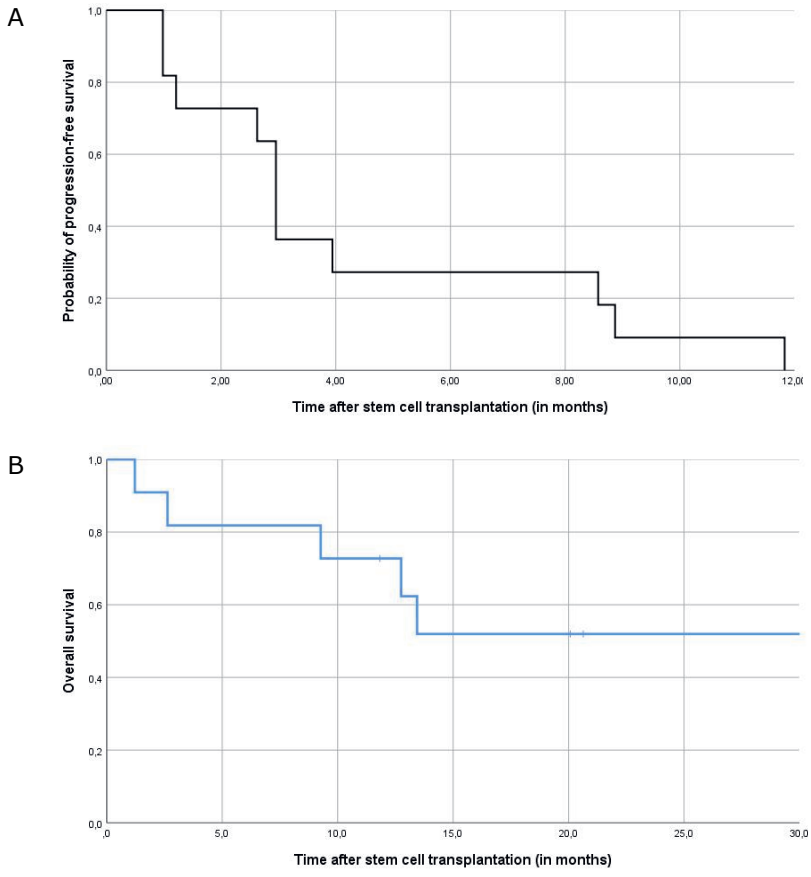
Clinical endpoints

Of the 11 evaluable patients, 10 achieved primary engraftment (91%), with a median time to neutrophil and platelet engraftment of 18 (12-30 days) and 30 (20-49 days) days, respectively. Grade 2-4 acute GVHD occurred in 2 of 11 patients (none grade 3-4) and chronic GVHD occurred in 4 of 11 patients. Two of the 11 patients died of treatment-related mortality (18%) within the first year.

Of the 9 for the primary endpoint evaluable patients, all patients relapsed within 1 year (**Figure 5.1A**). The median time to relapse was 90 days (range 30-360 days). Though all relapsed, 8 of 9 patients had to start anti-myeloma treatment. The median time for next treatment was 186 days (range 40-330 days); two third of the patients at first only biochemically relapsed without a need for treatment. Overall survival was 73% after 1 year and 52% after 2 years (**Figure 5.1B**).

Noteworthy is one patient, who relapsed quickly after HSCT with an increase of her involved free light chain, displayed a spontaneous decrease (except for stopping immune suppressive therapy as planned) 60 days after haploBMT to pretransplant levels (**Figure 5.2**). She did not require any treatment until 400 days after the HSCT. Interesting is also a second, heavily pre-treated patient that was already progressive at day 40 after HSCT but had a complete remission on daratumumab and has continuous bone marrow proven remission more than 2 years after stopping this treatment.

Figure 5.1 Clinical outcomes after HaploBMT.

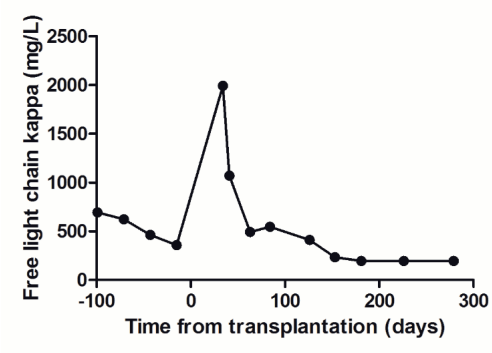


A. Probability of progression-free survival.
B. Overall survival.
HaploBMT: haploidentical bone marrow transplantation.

NK cell reconstitution

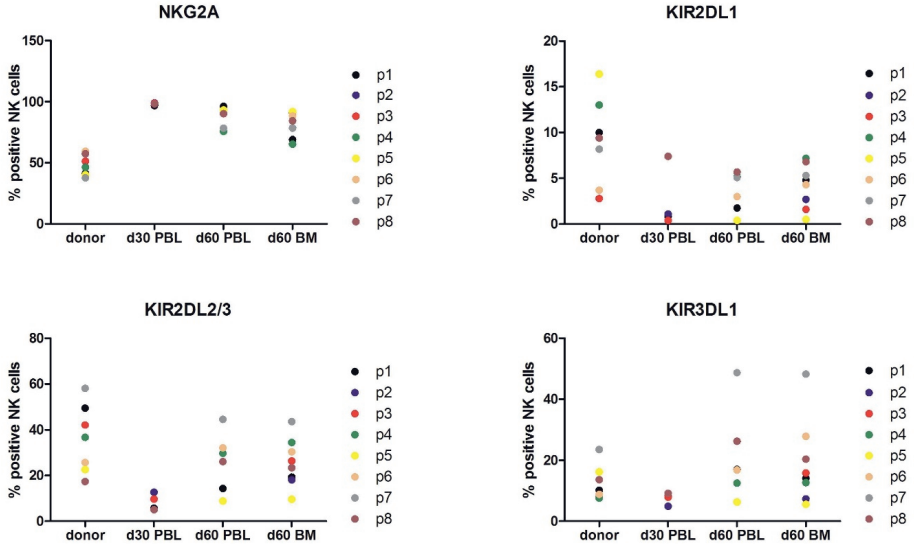
At day 30, all of the 8 analysed patients showed NK cell recovery, though with an immature phenotype ($NKG2A^+$, KIR^-). At day 60 in both the peripheral blood as well as bone marrow, mature NK cells (KIR^+) could be identified (**Figure 5.3A-D**). We have no data on the functionality of these cells.

Figure 5.2 Serum free light chain response after haploidentical bone marrow transplantation in one individual patient.



Free light chain kappa over time for one individual patient. Progression 30 days after transplantation and decreasing thereafter without treatment.

Figure 5.3 A-D Assessment of natural killer cell phenotype after stem cell transplantation.



Mononuclear cells were isolated from peripheral blood (PBL) or bone marrow (BM). To analyse KIR expression in the donor, PBL were harvested before from the donor before transplantation. To determine NK reconstitution upon transplantation, PBL or BM cells were isolated from the patient at 30 (d30) or 60 (d60) after transplantation. Isolated cells were stained and the percentage of CD3-CD56+ NK cells expressing KIR2DL1, KIR2DL2/3, KIR3DL1 or NKG2A was determined by flow cytometry. Patients and their donors are depicted as P1 – P8 in the legend and every dot represents one data point of cells analysed at day 30 (d30) or day 60 (d60) after transplantation.

Discussion

With this study, we evaluated the safety and the efficacy of a KIR mismatched haploBMT in high-risk MM patients. We observed that the treatment is safe since there was a high engraftment rate, low NRM (18% at 1 year) and no unexpected adverse events. In the only two other retrospective studies concerning haploidentical HSCT with PTCy in MM where there was no selection on KIR-mismatch, comparable results were seen with respect to NRM (10-21% in 1.5 to 2 years), however they had a slightly higher PFS (17-33% in 1.5 to 2 years). In our small study, unfortunately, there is a 1.5-year PFS of 0%. This 1,5 years PFS seems lower than in conventional allogeneic HSCT studies. However, the study is underpowered to draw this conclusion, since the study was prematurely terminated due to the defined stopping rules based on a 1.5 years PFS of 50%. Furthermore, this low PFS was also not unexpected since these were heavily pre-treated patients with very high-risk chemo-resistant myelomas. Though these patients showed a biochemical relapse, many of them did not require treatment for a long time after which is very unlikely for this fast progressive patient population. Even though it is difficult to draw conclusions in such a small group of patients, our results show that KIR-ligand mismatch in this patient category is not harmful, but also not more effective than non-matched haploidentical HSCT or conventional allogeneic HSCT in curing MM[3,4].

We hypothesize that the late reconstitution of functional mature NK cells is responsible for the lack of response. This has already been described in other patient groups after PTCy: within days after HSCT, mature graft-derived NK cells appear, but they are rapidly eliminated by PTCy. Full reconstitution of a mature and functional NK cell population took 6 to 12 months[12]. The median time point of relapse in our patients occurred shortly after transplantation (around 3 months). We detected that 30 days after haploBMT most NK cells still had an immature phenotype without KIR expression and were not able to stop disease progression at this early stage after transplantation when myeloma cell load was still low. We hypothesize that the delayed NK cell reconstitution may be overruled by infusion of mature, functional donor NK cells shortly after PTCy administration. In myeloid malignancies, this concept has already been used and seems successful in a small group of high-risk patients[26]. In this context, it may also be helpful to bridge the time to full NK cell restoration after the HSCT with additional anti-MM therapy like

elotuzumab or daratumumab, or to combine these two modalities. This concept is supported by the patient that showed a long-lasting complete response after temporary treatment with daratumumab. Another option would be to improve NK cell function and thereby increase the alloreactive effect by blocking antibodies, such as monalizumab, a novel checkpoint inhibitor against NKG2a[27].

In conclusion, in this study we show that haploBMT in MM patients is safe and feasible in terms of engraftment and late NK cell reconstitution. HaploBMT in MM forms a possible platform for future immunotherapeutic strategies in which the KIR-ligand mismatch might be beneficial. In the setting of haploBMT in combination with PTCy however, a KIR mismatch probably is not clinically relevant.

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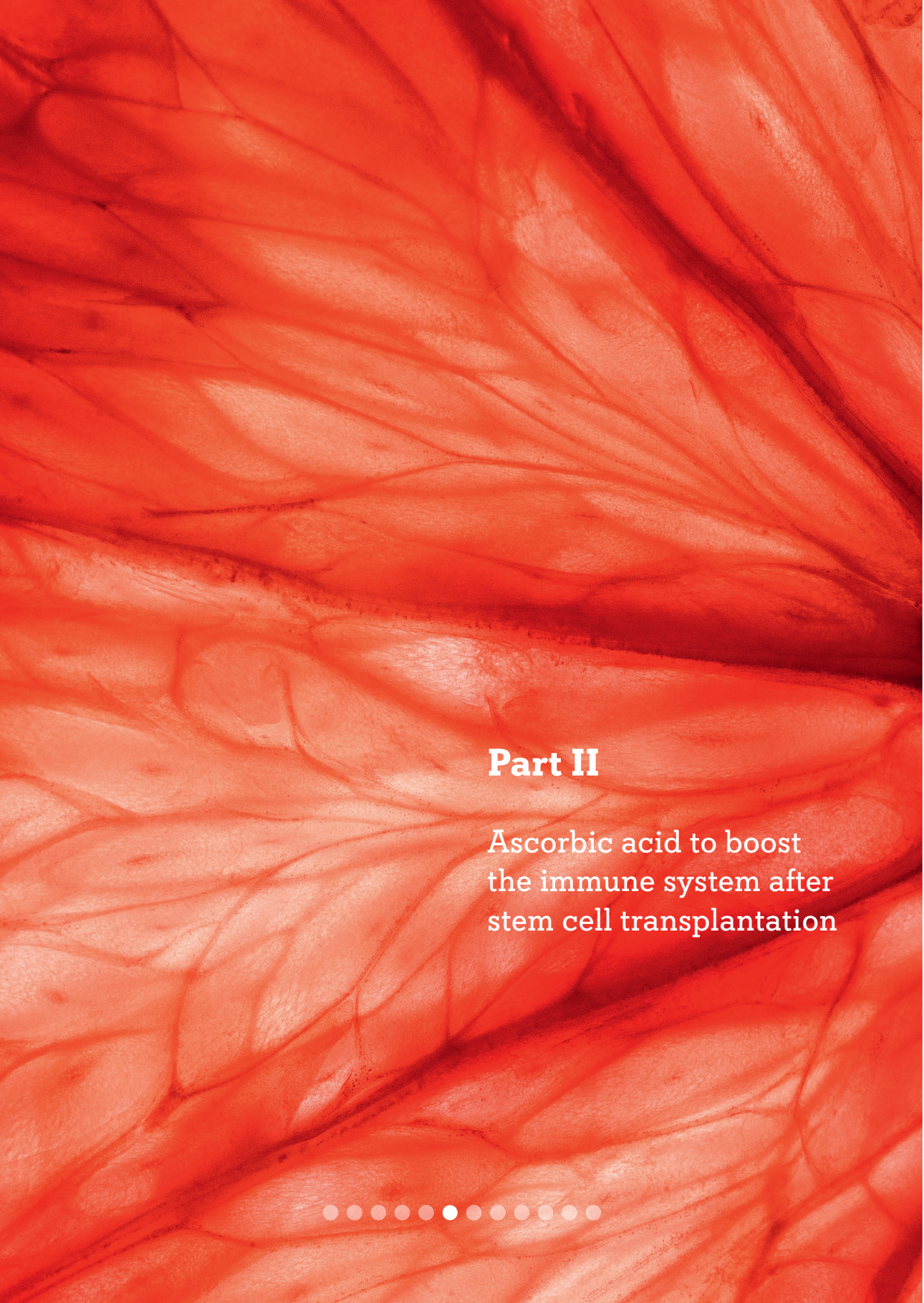

Part II

Ascorbic acid to boost the immune system after stem cell transplantation

6

Easy-to-use HPLC method to measure ascorbic acid levels intracellular in human peripheral blood mononuclear cells as well as in plasma

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Abstract

Given the growing interest in ascorbic acid (AA), there is a need for a reliable and reproducible method to measure AA status in the human body. Serum AA concentrations do not correlate well with tissue levels, but AA levels in leukocytes do. However, a standard method for clinical application is lacking. This present study describes a method to measure AA in the peripheral blood mononuclear cells (PBMCs) with hydrophilic interaction liquid chromatography (HILIC). The method can also be used in plasma and other leukocyte subsets. The measurements of AA in PBMCs and plasma were performed with HPLC with HILIC separation and UV detection. The sample preparation involved the isolation of PBMCs and lysis and precipitation with acetonitrile. European Medicine Agency guidelines for bioanalytic method validation were followed for the evaluation. A highly precise execution of the method was found with intra- and inter-assay variations at a maximum of 7.8%. In 40 healthy donors, a mean intracellular AA concentration of 7.9 microgram/10^8 cells was found in PBMCs. A correlation between plasma and PBMC AA concentration was not present (r=0.22). In conclusion, we developed a convenient, reliable, and reproducible method for the quantitative determination of AA within PBMCs and plasma from human blood.

Introduction

There is an increasing interest in the potential health benefits of vitamin C, or ascorbic acid (AA). AA is an essential vitamin that, unlike most other mammals, the human body cannot produce by itself, and has to be obtained from the diet[1]. Inside the human body, AA functions as an essential cofactor in numerous enzymatic reactions and as a potent antioxidant, due to its strong reducing potential[2]. For clinical purposes, AA concentration in serum or plasma is routinely determined by high-performance liquid chromatography (HPLC) in many laboratories globally and is an accepted golden standard for this measurement[3,4]. However, plasma and serum AA levels are very low compared with the rest of the human body and are highly affected by dietary intake, age, gender, and circadian rhythm. After oral intake of AA, the plasma AA increases rapidly, but this effect is only short-lived. These levels are thought to reflect the metabolic turnover rate of the vitamin[5,6]. Leukocytes can concentrate AA intracellular 80 times more than in plasma[2,5]. The leukocyte AA level is more stable than serum AA level because it is less affected by dietary changes and is also thought to be a more accurate assessment for AA storage[5]. Furthermore, AA is important for the development, proliferation, and function for different types of leukocytes[7,8]. Nevertheless, the measurement of intracellular leukocyte AA is hardly carried out because there is no well-established, standard, clinical method available.

For clinical investigators, the relationship between immune function and the AA concentration in leukocytes and plasma is highly relevant. Several methods for direct quantification of AA intracellular in leukocytes have been described with the use of the spectrophotometer (dinitrophenylhydrazine method) and reversed phase (RP) HPLC with UV or coulometric electrochemical detection[5,9,10]. With the described methods, however, estimates of the concentration of AA intracellular are quite variable, as AA degrades fast, and it is not possible to use the same procedure for the measurement of AA in plasma. Therefore, we tried to adapt a standardized reverse phase (RP) HPLC method with UV detection for AA serum and plasma measurements in our routine clinical hospital laboratory to make it suitable for intracellular AA measurements. Sample preparation was optimized using several published methods of cell lysis, but they were either insufficient or interfered with the HPLC measurement. We left the standard method to measure AA in serum and developed a new method, which, in comparison to

previous published methods for PBMC AA detection, can measure AA both in PBMCs and in plasma by hydrophilic interaction liquid chromatography (HILIC). In this article, we show that this method is effective, reliable, and reproducible. AA concentrations in separate cell fractions can also be measured with the same methodology, and only small amounts of cells are necessary. Furthermore, we show a very effective lysis process, which does not interfere with the measurement.

Materials and methods

HPLC and conditions

The HPLC system used for the AA detection was as follows: HPLC liquid chromatograph pump, UV/VIS detector, column oven, auto sampler, degassing unit, and communications bus module (all Shimadzu) with HPLC column XBridge Amide 3.5 µm, 4.6 × 150 mm (Waters) and Guard column XBridge Amide 3.5 µm, 4.6 × 20 mm (Waters, Milford, United States (U.S.)). The UV detection was at 255 nm wavelength. The mobile phase contained 85% acetonitrile and 15% water with 10 mM NH_4Ac pH 7. The flow rate was 1 mL/min, the pressure 63 bar, the temperature of column oven 25°C, the temperature of the auto sampler was 19°C, and the injection volume was 20 µL. The concentration of vitamin C in the sample was specified using a calibration curve prepared beforehand using known concentrations of AA (Sigma Aldrich, Saint Louis, U.S.) and D-(-) Isoascorbic acid (Sigma Aldrich, Saint Louis, U.S.).

Sample preparation

Lymphoprep density centrifugation was used to separate peripheral blood mononuclear cells (PBMCs) and plasma from heparin blood (600 g, 30 min). For enrichment of the different PBMC subsets (NK cells, T cells, B cells, and monocytes), MACs cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany) with CD56, CD3, CD19, and CD14 microbeads was used (positive selection), according to the manufacturer's protocol. In addition, cell sorting was performed on a BD FACS Melody to separate all different PBMC fractions.

Isolated cells were washed twice with PBS (centrifuged 283 *g*, 8 min) and erythrocytes were lysed when necessary, using lysis buffer. Lysis buffer was made with 200 mL Milli Q water plus 155 mM Ammonium chloride (NH_4Cl,

Merck), 10 mM potassium hydrogen carbonate ($KHCO_3$, Merck) and 1 mM ethylene diamine tetra acetic acid (EDTA, Sigma Aldrich).

After cell count, 2×10^6 cells were re-suspended in 100 µL 50 mM ammonium acetate followed by the addition of 400 µL precipitation agent. Precipitation reagent was made with 200 mL acetonitrile (Sigma Aldrich) plus 40 µL internal standard (D-iso ascorbic acid (Sigma Aldrich) stock solution 50 mg/mL in 0.1% metaphosphoric acid (Sigma Aldrich)).

After mixing thoroughly, the sample was incubated for 10 min in the dark at room temperature. To eliminate aggregated cell proteins, the procedure was followed by a last step of centrifugation (18.620 g, 10 min). Supernatant, containing the extracted AA, was then stored in brown vials at −80°C until further analysis.

Method validation

The validation was performed according to the European Medicine Agency's (EMA) bioanalytical method validation guideline[11].

Linearity and quantification limits

Linearity and quantitation limits were determined for ascorbic acid in plasma and in PBMCs. The limit of blank (LoB) was calculated using the formula: LoB = mean of blank + (1.645 × standard deviation (SD) of blank). Limit of detection (LoD) was calculated using the formula: LoD = LoB + (1.645 × SD of low concentration of AA). The lower limit of quantification (LLOQ) was determined by measuring serially diluted samples and determined to be the lowest value in which the coefficient of variation (CV) was under 10%.

Precision

The intra-assay variation of the HPLC was investigated by measuring 4 quality control (QC) samples with different AA concentrations covering the range of the calibration curve (11 µM, 20 µM, 35 µM, and 50 µM) 5 times in the same run and the inter-assay variation was calculated by measuring those 4 different concentrations 6 times on 2 different days.

The inter-assay variation of the isolation and extraction of intracellular AA from the PBMCs was investigated by performing the isolation, extraction, and analysis of different blood samples from a single individual 10 times.

Accuracy

The results of plasma AA levels with our method were compared with those measured by a standardized method by Chromsystems, which is used in our hospital for clinical diagnostic purposes and that is validated with external controls provided by Instand every 3 months. Furthermore, accuracy for different concentrations was calculated by comparing the obtained concentration with the target concentration of the QC samples.

Furthermore, the accuracy was assessed by comparing the results of QC samples of the intra-assay variation with the target concentration of AA.

Sample stability

Samples were measured after different storage conditions. The first was isolated, lysed and measured immediately after the blood collection and served as reference point; the other blood samples were stored at room temperature and at 4°C for 4 h and 72 h. Extracted samples were also measured fresh, frozen at −80°C for a day, a week, and for 88 days.

Carryover

Carryover was estimated by alternating injections of 10 samples with high (71 µM) and 11 with low (3 µM) concentrations of AA in a specified order according to an EP Evaluator® (Data Innovations®, Colchester, U.S.). Carryover results were compared to the error limit, which was defined as 3 times the standard deviation of the low after the low sample results.

Clinical application

All blood specimens for validation were taken from healthy volunteers. The medical ethical committee, which is in agreement with the Declaration of Helsinki, approved the sampling and the volunteers signed informed consent.
In order to gather local reference values, PBMC and plasma AA were determined in 40 healthy volunteers. None of these donors had any chronic diseases or was physically impaired.

Calculations and statistics

The concentration of AA in every sample was calculated in µmol/l with a formula that was based on the calibration curve. The plasma AA level was identical to the concentration that was measured. From the concentration in the sample (100 µL), the intracellular amount of AA in µg/10^8 cells was

calculated as intracellular AA is most often displayed in these units (intracellular AA (in μg/10^8 cells) = measured concentration (in μmol/l) × 1.76). The concentration within the PBMCs was estimated based on the known average mean corporal volume of PBMCs (282.9 fL)[12].

Statistical analysis was performed with SPSS version 25. Results were expressed as mean, maximum, and minimum. A confidence interval of 95% was considered to indicate statistical significance. We used a Pearson's correlation coefficient to investigate the association between PBMC AA level and serum AA level, and between serum and plasma AA level. For comparison of PBMC AA concentration in different genders and between leukocyte subgroups, an independent sample t-test was performed.

Results

Sample preparation

Purity of the cells was established by flow cytometry and was found to be greater than 95%. After re-suspending the PBMCs in ammonium acetate, adding the precipitation reagent, and 10 min incubation, the lysis efficacy was evaluated by microscopy using Trypan blue. In a viable cell, Trypan blue is not absorbed; however, it traverses the membrane in dead cells. There were no intact lymphocytes visible, and thereby it was determined that the lysis method was efficient.

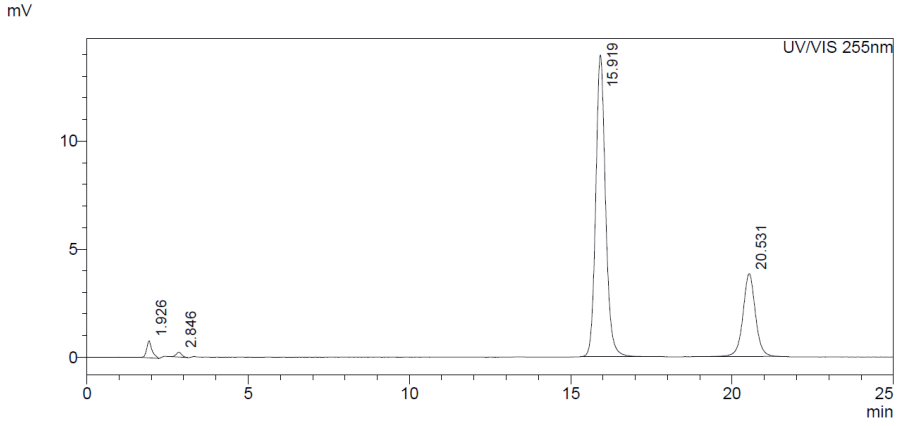
Method validation

Chromatography

The maximum AA peak was determined by performing a wavelength scan with AA in the mobile phase solution and showed a maximum peak at 255 nm. Using a flow rate of 1 mL/min, the AA and the internal standard peak were completely separated from other peaks and arrived at the detector after approximately 20.5 and 15.9 min, respectively (**Figure 6.1**). The peak authenticity was established by sample spiking. By running a sample with and without internal standard, it was shown that there were no underlying peaks at this timeframe in the sample. D-iso ascorbic acid was chosen as internal standard since it has almost the same properties as AA with regard to loss and decay, so even if the AA concentration in the sample is decreasing (for

example while staying in the auto sampler of the HPLC), the ratio between internal standard and AA stayed equal after a maximum of 6 hours.

Figure 6.1 Chromatogram.

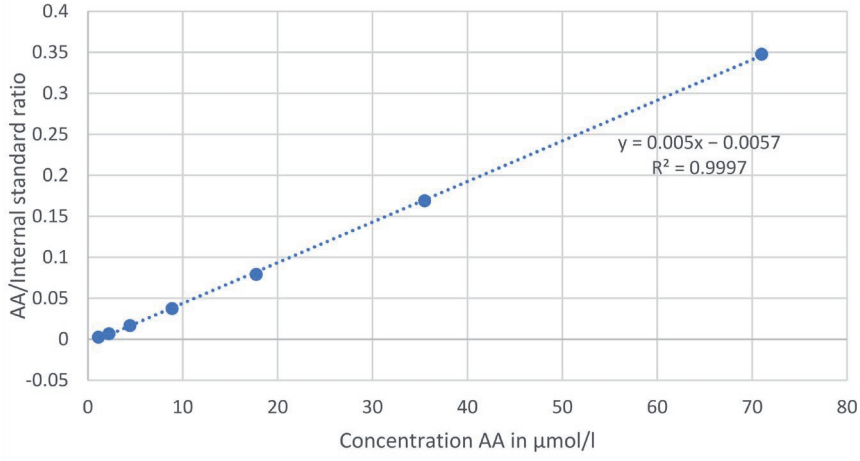


Chromatogram of a PBMC sample, in particular the ascorbic acid peak (at 20.5 min) and isoascorbic peak (internal standard) (at 15.9 min), spectrum taken as mass absorbance units.

Linearity

The 7-point calibration graphs for AA standards (0–71 µmol/l) were plotted as the ratio of AA and internal standard showed linearity, passing though the origin (**Figure 6.2**): y=0.005x − 0.0057, R2=0.9997.

Figure 6.2 Example of a calibration curve.



AA: ascorbic acid.

Quantification limits

For the determination of the LoB, 9 blank samples were measured. LoB was calculated to be 1.65 µM (SD 0,26) and subsequently the calculated LoD was 1.93 µM (SD 0.17), when using repetitive measurements of a known sample of 1,1 µM from our standard curve. The lower limit of quantification (LLOQ) was determined by measuring serially diluted samples, with a concentration around the LoD. The deviation was >10% if there was less than 3.8 µM AA in the sample; thereby, the measurement is unreliable under this limit, according to our limit of 10%. From this, the calculated lower limit of quantification in the cells is 3.3 µg/10^8 cells.

Precision

The intra-assay variation of the HPLC measurement was investigated by measuring 4 QC samples with different AA concentrations (11 µM, 20 µM, 35 µM, and 50 µM) 5 times, and was 0.6–7.7% (**Table 6.1**). The inter-assay variation of the HPLC measurement was calculated by measuring the samples with 4 different concentrations 6 times on 2 different days and was 0.9–2.5% (**Table 6.1**). Thereby, the total imprecision was 1.1–7.8%. Results gathered by this approach are therefore considered as reliable.

Table 6.1 Precision test with intra- and inter-assay variation for AA measurement with HPLC.

Ascorbic Acid	QC1		QC2		QC3		QC4	
	Intra-Run	Inter-Run	Intra-Run	Inter-Run	Intra-Run	Inter-Run	Intra-Run	Inter-Run
Target (µM)	11		20		35		50	
Number	5	6	5	6	5	6	5	6
Mean (µM)	9.5	9.0	17.1	17.0	30.0	29.9	43.1	43.4
SD	0.73	0.1	0.19	0.2	0.27	0.7	0.25	0.7
CV (%)	7.7	1.1	1.1	1.0	0.9	2.5	0.6	1.6
TI (%)	7.8		1.1		2.7		1.7	

Target (µM) is the required concentration of the quality control samples (QC1, QC2, QC3, QC4). SD is the standard deviation. CV is the ratio of the standard deviation to the mean. TI is the total imprecision.

The inter-assay variation of the extraction of intracellular AA from the PBMCs was investigated by performing the isolation, extraction, and analysis of different blood samples from a single individual 10 times. The variation coefficient (CV) of the inter-assay variation of the isolation and extraction AA in the PBMCs was determined by performing the isolation, extraction, and

analysis of separate blood samples of the same volunteer 10 times on the same day and was 7.7% (**Table 6.2**).

Table 6.2 Inter-assay variation of the intracellular AA measurement of the PBMCs.

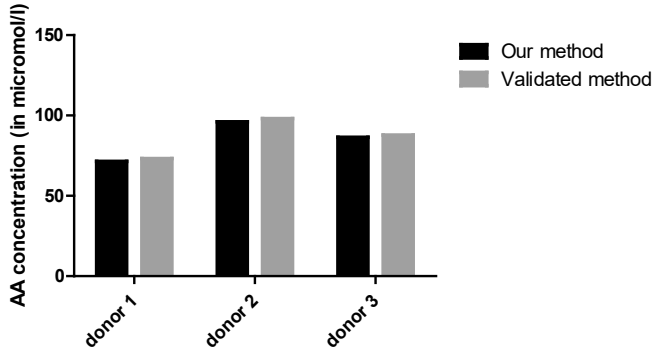
Extraction Number	PBMC AA (in µg/10^8 Cells)
1	11.31
2	11.79
3	9.80
4	11.16
5	9.31
6	10.75
7	10.61
8	10.97
9	9.71
10	9.89
MEAN	10.53
SD	0.81
CV (%)	7.72

Abbreviations: AA: ascorbic acid, PBMC: peripheral blood mononuclear cells, SD: standard deviation, CV: variation coefficient.

Accuracy

The plasma AA levels determined with this method were compared with a validated method from Chromsystems used in our clinical diagnostic laboratory, and the results were similar (**Figure 6.3**).

Figure 6.3 Comparison between plasma AA concentration with our method compared to the validated method.



Plasma concentration of AA in 3 healthy volunteers measured at the same time with the 2 methods show almost identical values. AA: ascorbic acid.

Furthermore, the obtained values of the QC samples of the intra- and inter-assay variation were compared with the nominal concentration (82–87% of the target value) of AA and the deviation was under 15% for all concentrations, except for the lowest concentration, which is acceptable, since it is within 20% of the nominal value (**Table 6.3**).

Table 6.3 Accuracy for AA measurement with HPLC.

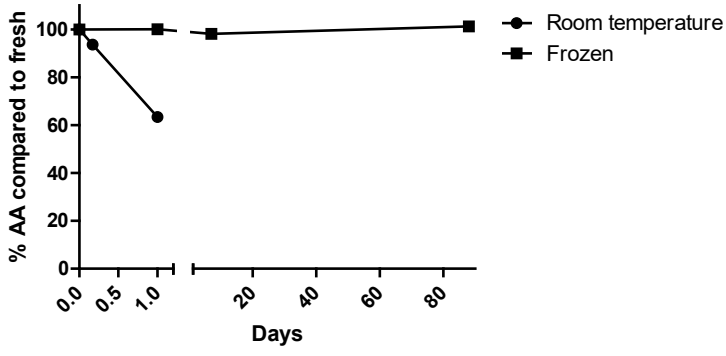
Ascorbic Acid	QC1		QC2		QC3		QC4	
	Intra-Run	Inter-Run	Intra-Run	Inter-Run	Intra-Run	Inter-Run	Intra-Run	Inter-Run
Target (µM)	11		20		35		50	
Number	5	6	5	6	5	6	5	6
Mean (µM)	9.5	9.0	17.1	17.0	30.0	29.9	43.1	43.4
Accuracy (%)	86	82	86	85	86	85	86	87
Deviation (%)	14	18	14	15	14	15	14	13

Target concentration (in µM) is the required concentration of the quality control samples (QC1, QC2, QC3, QC4).

Stability

The AA concentration decreased by only 11% after the blood samples were stored for 4 h at room temperature in the dark, so we consider this as acceptable (**Figure 6.4**). After 24 h storage at room temperature, the AA levels decreased on average 38%. Storage at 4°C was even less stable. The freezing process of prepared samples did not influence AA levels, and after 1, 7, and 88 days of storage at −80°C, there was less than 5% change in repeated measurements (**Figure 6.4**).

Figure 6.4 Stability of AA.



Stability of AA in blood sample stored at room temperature (mean of 3 samples) and of extracted AA in frozen at −80°C (mean of 5 samples). AA: ascorbic acid.

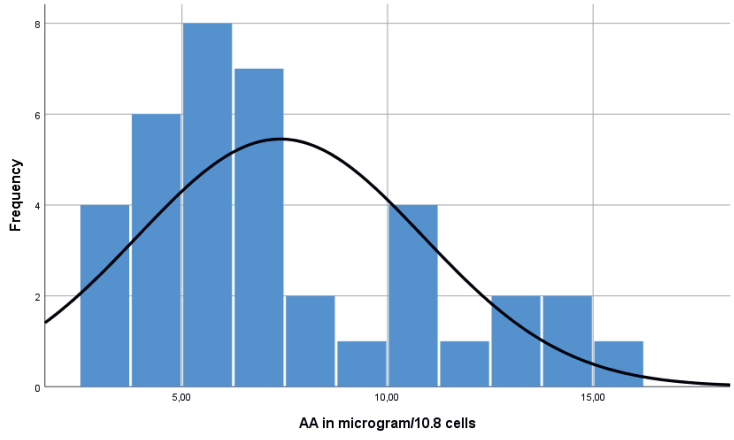
Carryover

Carryover was measured by subtracting the mean of low–low results (low values that follow a low value, 3.03 µM) from high–low results (low values that follow a high value, 3.17 µM) of 10 replicates of a sample with high (71 µM) AA concentration, and 11 replicates of a low (3 µM) AA concentration (Supplementary Table S6.1). There was no significant carryover since the measured carryover (0.13) was lower than the error limit (0.40).

Reference values

In order to gather local reference values, PBMC and plasma AA were determined in 40 healthy volunteers. These consisted of 11 males and 29 females with a mean age of 35 years (range 18 to 62). The mean of the PBMC AA concentration was 7.9 µg/10^8 cells (range 3.17 to 27.47). There was no Gaussian distribution of the intracellular AA concentrations with a skewness of 2.16 and a kurtosis of 6.76 (**Figure 6.5**). On average, the PBMCs contained 24 times higher AA concentrations than plasma (1586 µM vs. 67 µM). No differences based on age or gender were found in this population.

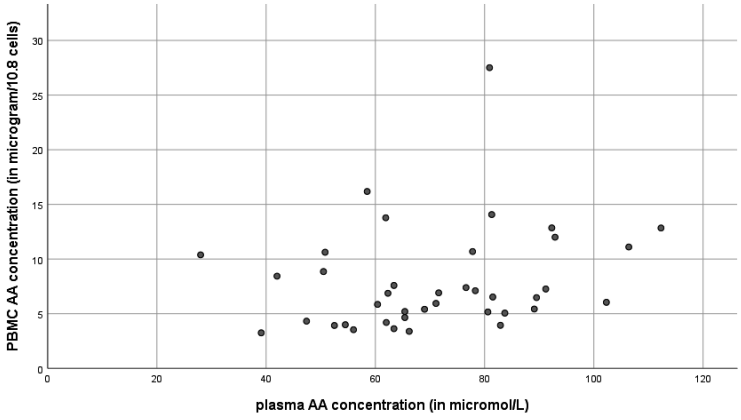
Figure 6.5 Distribution of PBMC AA concentration in healthy volunteers.



Histogram with a distribution fit of the intracellular AA (in µg/10^8 cells) in PBMCs in healthy volunteers, n=40. PBMC: peripheral blood mononuclear cells. AA: ascorbic acid.

Data on plasma and PBMC AA concentrations are represented in **Figure 6.6**. There was no correlation (r=0.22) between plasma and PBMC AA levels. Even though the values were not always equal, plasma AA levels had a strong positive correlation with serum AA levels, which are normally used in clinical applications (r=0.70, p<0.01) (data not shown).

Figure 6.6 Correlation between the plasma and PBMC AA.

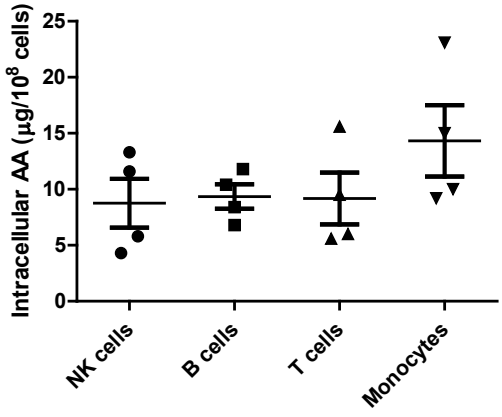


Correlation between the plasma AA concentration and the PBMC AA concentration in healthy volunteers, n=40, r=0.22.
PBMC: peripheral blood mononuclear cell, AA: ascorbic acid.

PBMC subsets

To determine if there was a difference in AA content in different PBMC subtypes, we measured AA levels in PBMC subsets (T cells, B cells, NK cells, and monocytes) in 4 healthy volunteers. In 3, we isolated the cells using MACS separation, and in 1 we used cell sorting by flow cytometry. Results are given in **Figure 6.7**. The AA levels in different subgroups were not significantly different (p=0.32). They were also similar with the different separation methods.

Figure 6.7 Amount of AA in PBMC subsets.



Amount of AA in µg/10^8 cells for each PBMC subset (n=4)
AA: ascorbic acid, PBMC: peripheral blood mononuclear cell, NK: natural killer.

Discussion

In this article, we describe an optimized method for quantitative determination of AA in human PBMCs and in plasma using HPLC with HILIC mode. HPLC is already considered as a golden standard for the measurement of AA in serum, but a standard clinical method to measure it intracellular in PBMCs was not available. At first, we tried to measure intracellular AA using a standardized method for serum AA determination, but we did not succeed to use this HPLC protocol due to interfering signals after lysis. After that, we chose HILIC mode, as it works well for small, very polar molecules, such as AA, and it is well studied for the detection of AA in other circumstances[13].

In most articles about intracellular AA measurements in leukocytes, the method is not validated according to the European Medicine Agency guidelines for bioanalytic method validation. In one article, in which the validation is clearly described, RP HPLC was used and information about lysis efficacy was lacking[10]. We tried to use sonication for lysis based on this article, but the efficacy was very low in our hands (30–40%). If the lysis is that inconsistent, AA concentrations intracellularly will be underestimated. Our lysis procedure was very efficient. Furthermore, plasma AA levels measured with our method

were almost identical to plasma AA levels determined with the standardized method used for clinical applications.

We analysed 40 blood samples of healthy adult volunteers. These results showed a mean AA concentration of 7.9 µg/10^8 cells in PBMCs. In other studies, there is a large variety in these results (5.3 to 122 µg/10^8 cells)[5,10,14-16]. The values we found are in the same range as earlier findings. One study, in which AA levels of 20 healthy volunteers were measured by HLPC with coulometric electrochemical detection, shows identical results (5.3 to 10.6 µg/10^8 cells)[14]. In other studies, the detected amount of AA in PBMCs was higher, but in these studies the measured samples were possibly contaminated with platelets, which are known to have intracellular AA as well[5,17,18]. In one of the more recent papers, AA is measured in lymphocytes using RP HPLC with UV detection and a mean AA of 23.5 µg/10^8 cells was found[10]. However, these measurements were done in blood samples of children that already needed blood examination; therefore, these measurements might not represent healthy control values, especially in adults[10]. In our reference population, the calculated intracellular concentration of the PBMCs was around 1.6 mM. This is also in line with earlier findings of Levine et al. who describe the effects of AA supplementation and deprivation on intracellular values of lymphocytes and monocytes in 7 healthy volunteers[6].

In the literature, more is known about the amount of AA that can be found in leukocytes and in most articles and textbooks reference ranges of 20 to 53 µg/10^8 cells are given[9,18–20]. However, in most of these analyses, the buffy layer is used, containing not only leukocytes but also platelets and, thereby, again, overestimating the AA found in the leukocytes[18]. The relationship between buffy layer and leukocyte AA levels was researched by Gibson et al. and it was determined that with a normal number of platelets, the buffy layer concentration shall be divided by 2.0, but this conversion factor shifts if the number of platelets is abnormal[17]. We checked the number of platelets that were left in our cell samples by flow cytometry and found that the percentages of thrombocytes in the samples were quite low (median 1% of total cell count, whilst in the buffy layer with normal cell counts the leukocyte:platelet ratio is around 1:40). These small amounts of platelets will have an irrelevant influence on the total AA concentration measured with this method as it is thought that platelets only carry 0.25–0.55 µg AA/10^8 cells[5,17].

Our reference values have limitations, for instance, underrepresentation of higher ages. They are probably regional and are influenced by lifestyle and diet. Samples were taken in summer; values could be different in other seasons. For clinical use of this method, it is thereby advised to create local reference values. Similar to data from other studies, AA concentration in lymphocytes was not well correlated with plasma concentration in healthy volunteers.

We also used the same method to investigate AA levels in different leukocyte subsets and noticed no significant differences. This shows that determination in even small subsets of the peripheral blood leukocytes is possible since only 2×10^6 cells are necessary for a reliable result with this method.

In our clinical setting, in an earlier investigation, we observed low serum AA levels in patients after stem cell transplantation, a patient group in which the immune system functioned less, which is very vulnerable to infections[21]. This is interesting, as we discovered before that in vitro lymphocytes and NK cells are dependent on AA for proper development and/or proliferation[22,23]. For an accurate estimation of the relevance of AA in relation to the function of the PBMCs in these kinds of clinical situations, it is interesting to measure intracellular AA levels in the PBMCs, or even in subgroups. Moreover, our method can be used for in vitro studies on the function of leukocytes in correlation to intracellular AA levels where we have little knowledge on how optimal intracellular concentrations should be in relation to the function of these cells.

Conclusions

We developed an effective and reliable method for the quantitative determination of ascorbic acid in human PBMCs by HPLC in HILIC mode, for which you only need a small number of cells and that can also be used for measurement in plasma and in different leukocyte subgroups. Intracellular AA values found with this method are in line with earlier results in the literature. The presented methodology can be of use in a large variety of patients, as it can possibly better determine if there is a true deficiency in the total body AA. Therefore, this measurement could be added to AA measurements in plasma in many laboratories. Furthermore, AA seems relevant in many diseases, but

a clinical effect of supplementation is not always present in intervention studies. However, in these studies, the effect of AA supplementation is measured in plasma, which is not a proper reflection of total AA. This measurement can be useful in these intervention studies to determine the optimal dose and administration route of AA supplementation and to identify patients with a true AA deficiency.

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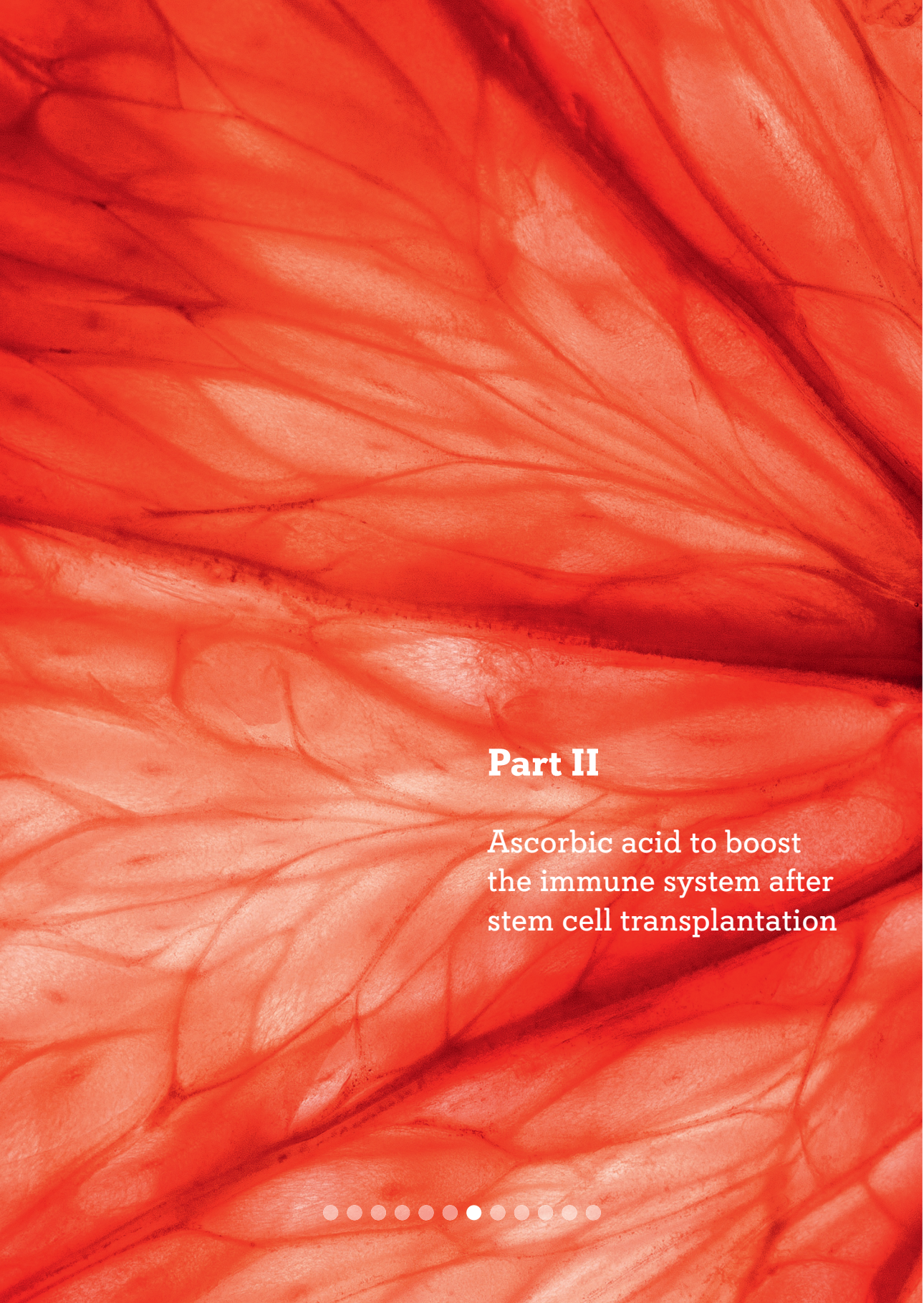
Supplementary material

Table S6.1 Carry over determination. Low-Low: Low concentration sample measured following a low concentration.

Sample	Results (µM)
Concentration low	3.0
Concentration high	71.0
High-Low mean	3.17
Low-Low mean	3.03
Low-Low SD	0.13
Error limit	0.40

High-Low: Low concentration sample measured following a high concentration sample. SD: Standard deviation.



Part II

Ascorbic acid to boost the immune system after stem cell transplantation

7

The Effect of Vitamin C (Ascorbic Acid) in the Treatment of Patients with Cancer: A Systematic Review

Gwendolyn N.Y. van Gorkom, Eline L. Lookermans, Catharina H.M.J. Van Elssen, Gerard M.J. Bos

Nutrients. 2019 Apr 28;11(5).

Abstract

Many cancer patients on intensive chemotherapy lack vitamin C. Vitamin C stimulates the production and activation of immune cells, so perhaps supplementation could be used to improve the immunity in those patients. This review assesses the effectiveness and safety of vitamin C administration in cancer. The PubMed and EMBASE databases were searched and all study designs except for phase I studies, and case reports were included in this review. A total of 19 trials were included. In only 4 trials randomization was used to determine if patients received vitamin C or a placebo. The result of this review does not prove that there is a clinically relevant positive effect of vitamin C supplementation in cancer patients in general on the overall survival, clinical status, quality of life (QOL) and performance status (PS) since the quality of the studies published is low. Interventions and patient groups are very diverse; hence, an effect in some patient groups is possible. There seems to be a better effect with intravenous than oral administration. Nevertheless, treatment with vitamin C is safe with minimal side effects. Thereby, we think it is safe to examine the effects of vitamin C on specific groups of patients in a randomized controlled setting.

Introduction

Vitamin C is an essential micronutrient that plays a vital role in numerous physiological processes in the human body. Unlike most mammals, humans lack the ability to generate endogenous vitamin C due to a mutation in the *GULO* gene and are thereby completely dependent on dietary intake. The biological efficacy of vitamin C depends on its redox abilities, and it functions as a cofactor in many enzymatic reactions. In physiological concentrations, it also functions as an antioxidant.

By the 1970s, Nobel Prize winner Linus Pauling had already developed a strategy to use intravenous (IV) vitamin C in cancer patients[1,2]. He treated patients with advanced cancer with high doses of vitamin C and reported a positive effect on survival. However, these studies have been methodologically criticized on several aspects such as data collection and data analysis. This resulted in a limited use of vitamin C in cancer patients. Other studies performed afterwards could also not reproduce these results; however, opposed to the intravenous use of vitamin C by Pauling et al, in most of these studies, oral vitamin C supplementation was used[3]. Pharmacokinetic studies show that the way of administration makes a big difference as peak plasma vitamin C concentrations after intravenous administration are much higher (up to 70-fold) than after oral intake[4]. Peak plasma concentrations also continue to increase when the intravenous dose of vitamin C is increased, while peak plasma concentrations plateau around 220 µM even though oral doses are increased.

There are multiple hypotheses about the way vitamin C has anti-tumour effects. An important possible mechanism of action is that in pharmacological concentrations (especially after intravenous use) vitamin C functions as a pro-oxidant and stimulates the formation of hydrogen peroxide. This hydrogen peroxide can create reactive oxygen species (ROS) that directly have cytotoxic activity on cancer cells[5]. Another important hypothesis is that vitamin C can create important epigenetic changes due to its effect on 2-oxoglutarate-dependent dioxygenases, like histone and DNA demethylases[6]. In preclinical studies, investigators also show that vitamin C can have a synergetic effect with some types of chemo- and immunotherapy[7–11].

Additionally, we showed in pre-clinical studies that vitamin C has a significant role in the immune system, as it stimulates the production and/or activation of immune cells, like T-lymphocytes and natural killer cells, which have a function in fighting against pathogens and cancer cells[12–14].

In our previous research on vitamin C we noticed that many of our patients receiving intensive chemotherapy and/or stem cell transplantations for haematological malignancies have low vitamin C plasma concentrations[15]. This could be the result of low dietary intake of these patients, or of an increased need for vitamin C in tumour cells or immune cells. In extension of our results, other researchers observed that low vitamin C plasma levels in patients with various types of advanced cancer were associated with worse survival[16].

Patients that receive intensive chemotherapy and/or stem cell transplantations are prone for infectious complications. Boosting their immune system with vitamin C to hasten immune recovery and thereby prevent infectious complications is attractive, since vitamin C is cheap and generally available. However, since some vitamins have been shown to promote cancer development, we were interested in the effects of vitamin C on cancer progression and its safety. To this end, we conducted a systematic review of the literature on vitamin C administration in cancer patients. We focused on administration route, efficacy and on the side effects in combination with or without other cancer treatment.

Materials and methods

Objectives

The aim of this review is to assess the effectiveness of vitamin C in the treatment of cancer, with or without adjuvant standard anti-cancer treatment like chemotherapy and radiotherapy.
We researched the literature on the following hypotheses:

- Vitamin C administration is more effective in the treatment of cancer than placebo or no treatment in susceptible populations.
- Different routes of vitamin C administration (intravenous/oral) may differ in effectiveness in treating cancer.

The reached serum and/or tissue vitamin C concentrations with supplementation were also of interest and were noted when given.
An attempt was also made to quantify toxicity and side effects of vitamin C and the findings were considered in the discussion to determine the risk–benefit ratio of the treatment.

Protocol and registration

This systematic review was written conform the PRISMA statement for reporting systematic reviews of studies that evaluate health care interventions[17]. It is registered with the University of York Centre for Reviews and Dissemination International Prospective Register of Systematic Reviews.

Eligibility criteria

Types of studies

Studies on the effect of vitamin C administration in cancer patients after diagnosis were included. All study designs were allowed except for Phase I trials and case reports, since there was a lack of extensive randomized controlled trials (RCTs), but the quality of the studies was weighted during analysis and discussion. Language was restricted to English.

Types of participants

Inclusion criteria

Studies with patients of all ages and both genders with all types of diagnosed cancer.

Exclusion criteria

- Studies investigating the effect of nutritional supplements.
- Studies on the effect of vitamin C administration in the prevention of cancer.

Types of intervention

Studies on the effect of clinical vitamin C administration, as mono-therapy or in combination with other standard cancer treatment regimes. The dose and mode of delivery were considered in subgroup analyses.

Types of outcome measures

Primary outcome measure was overall survival. Secondary outcome measures were progression-free survival, tumour response, response rate, disease-free survival, adverse effects, quality of life (QOL), clinical response and performance status (PS).

Literature search

Studies were identified by searching the PubMed and EMBASE databases and snowballing from review articles and relevant studies. The last search was run on the 11th of March 2019.

The following search terms were used to conduct the search: Neoplasms; Cancer; Malignancy; Leukemia; Lymphoma; Ascorbic acid; Vitamin C; Ascorbate; Dehydroascorbic acid; Randomized controlled trial; RCT; Randomized; Controlled clinical trial; Prospective study; Clinical trial; Case-control; Cohort; Phase 2; Observational study; Reduced infection; Overall survival; Progression-free survival; Toxicity; Quality of life; Tumor response; Response rate; Disease-free survival. Bibliographies of identified articles were also reviewed and searched manually for additional references.

Data collection and analysis

Assessment of eligibility of the articles for inclusion in this review was performed and peer reviewed by two of the authors. The identified articles were screened on title and abstract in agreement with the inclusion and exclusion criteria by E. Lookermans (E.L.), who discarded studies that were clearly ineligible but aimed to be overtly inclusive rather than risk losing relevant studies. Subsequent full text assessment resulted in the final study selection.

Data were collected by E. L. and peer reviewed by G. van Gorkom (G.G.) with use of a data extraction sheet based on the Cochrane Consumers and Communication Review Group's data extraction template[18]. For each included study, information was extracted regarding the methods of the study (aim, study design, number of groups), the participants (number of patients, patient description, geographic location, methods of recruitment, inclusion criteria for participation, exclusion criteria for participation, age, gender) the intervention (vitamin C treatment, dose, schedule, mode of delivery, additional treatment, previous cancer treatments received, setting) and the

outcomes and comparison groups (primary outcome measures, secondary outcome measures, method of assessing outcome measures, method of follow-up for non-respondents, outcome assessment, length of follow-up, frequency, relevant adverse events). Discrepancies in the data extraction were resolved through discussion.

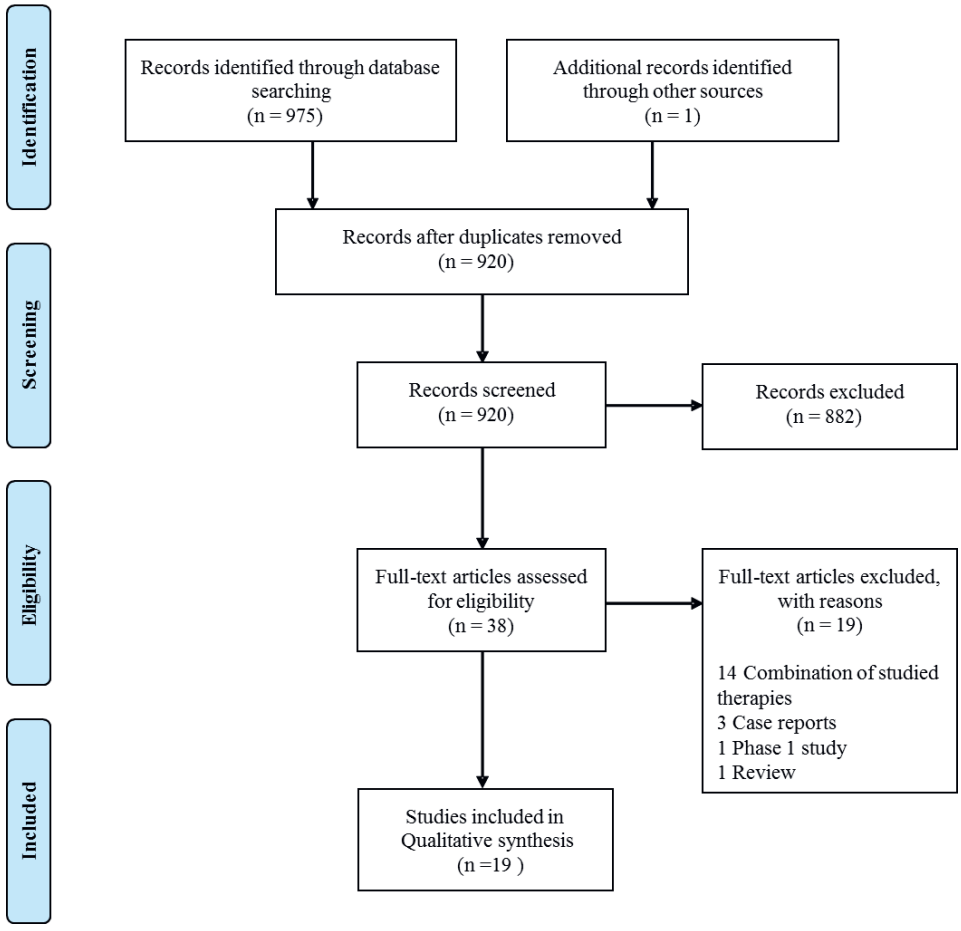
The quality of all eligible studies was assessed independently by two authors (E.L. and G.G.) with use of predefined risk of bias criteria, with discrepancies resolved by discussion and a third author (G.B.) when necessary. For randomized-controlled trials "The Cochrane Collaboration's tool for assessing risk of bias in randomized trials" was used[19], for non-randomized comparison studies (studies were there is a group of patients included without vitamin C) the ROBINS-1 tool was used, a Cochrane risk of bias assessment tool for non-randomized studies of interventions[20] and the Effective Public Health Practice Project (EPHPP) Quality Assessment Instrument was used for non-comparison studies[21]. These tools were used to make judgments about the extent of bias that may be present in each of the studies and to rate the information in each component of the paper.

Results

Study selection

A total of 975 articles was retrieved by the PubMed and EMBASE databases search. An additional article was found through article references, bringing the total number of records suitable for further evaluation to 976. After removal of duplicates there were 920 articles left for investigation. By scanning the title and abstract of these records, 882 records were excluded because they clearly did not match the inclusion criteria (mostly they were preclinical studies). Thirty-eight articles were evaluated on their full text. Of these articles, 19 records were excluded based on the inclusion criteria. In most of these articles, vitamin C supplementation was not the primary intervention, but combined with other experimental treatments, like other vitamins or arsenic trioxide. This resulted in 19 records being included for qualitative synthesis (**Figure 7.1**).

Figure 7.1 Flow diagram of the article selection.



Study characteristics

The study characteristics of the 19 articles selected for this review are described in **Table 7.1**.

Table 7.1 Characteristics and outcome of the included studies in alphabetical order.

Study	N (Vitamin C/ controls)	Type	Participants	Intervention	Concomitant therapy	Main endpoints	Adverse events
Bazzan 2018[22]	86 (86/0)	Retrospective cohort	All types of cancer in different settings	50-150 g IV, at least 5 times (total 3034 doses)	32 patients none, 54 patients chemotherapy	20 of 40 patients improvement of fatigue, 15 of 86 patients improvement of appetite.	Mostly mild AEs, like nausea, vomiting and discomfort at injection side (<3% of infusions). Self-limiting to time of infusions. No SAEs related to vitamin C
Cameron 1974[2]	50 (50/0)	Retrospective cohort	Advanced stage cancer patients	10 g a day IV for 10 days + oral vitamin C	None	10 minimal response, 11 growth retardation, 3 stable disease, 5 tumour regression. Less pain, reduction in ascites/pleural effusions.	Fluid retention, oedema, dyspeptic symptoms, tumour haemorrhage/necrosis.
Cameron 1976[1]	1100 (100/1000)	Case-control	Incurable cancer patients	10 g a day IV for 10 days + oral vitamin C	Conventional anti-cancer treatment	Mean OS 210 days vs. 50 days in controls (4.2x more)	NR
Cameron 1978[23]	1100 (100/1000)	Case-control	Terminal cancer patients	10 g a day IV for 10 days + oral vitamin C	None	Recalculation of Cameron 1976. Average OS (7.7x more =288 days)	NR
Cameron 1991[24]	1826 (294/1532)	Case-control	Terminal cancer patients	10 g a day IV for 10 days + oral vitamin C	None	OS 343 days vs. 180 days in controls	NR
Creagan 1979[3]	123 (60/63)	RCT	Advanced stage cancer patients	10 g a day orally	NR	Identical survival. PS identical. 58% vs. 63% some improvement in symptoms	Nausea, vomiting
Hoffer 2015[25]	14 (14/0)	Uncontrolled phase II	Advanced stage cancer patients	1.5 g/kg IV 2-3 times per week.	Chemotherapy	6 transient, partly long-lasting stable disease	Oedema, thirst, nausea, vomiting, headache, chills
Günes-Bayir 2015[26]	39 (15/24)	Case-control	Bone metastases from various types of cancer	2.5 g IV a day	NR	OS 10 months vs. 2 months in controls. Decrease in pain in 9/15 vs. 5/24 in controls. PS improvement in 4/15 vs. 1/24 in controls.	40% mild diarrhoea, 30% mild oliguria

Table 7.1 (continued)

Study	N (Vitamin C/ controls)	Type	Participants	Intervention	Concomitant therapy	Main endpoints	Adverse events
Ma 2014[27]	25 (13/12)	RCT	Newly diagnosed stage III and IV ovarian cancer after debulking	IV two times per week using a dose escalating protocol (final dose either 75 or 100g) for 12 months.	Paclitaxel and carboplatin chemotherapy	Trend in improvement OS, 25.5 months vs. 16.75 months in controls, (not significant).	Fewer chemotherapy related side effects with vitamin C, no relevant AEs of vitamin C.
Mikirova 2012[28]	45 (45/0)	Retrospective cohort	Various types of cancer, mostly metastatic	Escalate to 50 g IV 3 times per week for a median of 9 times	NR	76% reduction in C–reactive protein, 75% reduction in PSA	NR
Moertel 1985[29]	100 (51/49)	RCT	Advanced colorectal cancer	10 g a day orally	None	Median OS 2.9 months vs. 4.1 months in controls. 7/11 symptom relief vs. 11/17 in controls	Low incidence of AEs, mild.
Murata 1982[30]	130 (111/19)	Nonrandomized clinical trial	Terminal cancer patients	Site 1: 6-30 g a day orally and 10-20g IV. Site 2: 0.5-3 g or 5-30 g per day orally.	NR	Site 1: average OS with high dose vitamin C 246 days vs. 43 days with low dose. Site 2: average OS with high dose vitamin C 115 days vs. 48 days in controls. Less use of narcotic drugs in vitamin C treated patients: 17% in high dose vs. 50% in low dose vs. 79% in controls. Improved state of wellbeing, improved appetite, increased mental alertness	No SAEs
Nielsen 2017[31]	23 (23/0)	Uncontrolled phase II	Chemotherapy-naïve metastatic castration-resistant prostate cancer	Weekly infusions for 12 weeks. Week 1: 5 g, week2: 30 g, week 3-12: 60 g + oral 500 mg/day.	None	75% of patients PSA increase at 12 weeks, one PSA decrease of 27%. 80% unchanged PS at week 12, 2 improved, 2 worse score. QOL identical baseline to week 12.	53 AEs, mostly mild and not related to vitamin C. 11 SAEs, explained by progression of prostate cancer. 2 pulmonary embolisms.

Table 7.1 (continued)

Study	N (Vitamin C/ controls)	Type	Participants	Intervention	Concomitant therapy	Main endpoints	Adverse events
Poulter 1984[32]	66 (27/25)	Nonrandomized clinical trial	Newly diagnosed breast cancer	3 g a day orally	NR	No change in survival rates	NR
Riordan 2005[33]	24 (24/0)	Uncontrolled phase II	Late-stage terminal cancer, mostly colorectal (19)	150 to 710 mg/kg/ day IV for 8 weeks	None	1 patient stable disease, all others progressive disease.	Mild: nausea (46%), oedema (29%), dry mouth or skin (29%), fatigue (25%). SAEs: 1 kidney stones, 1 hypokalaemia.
Takaha-shi 2012[34]	60 (60/0)	Prospective cohort	Newly diagnosed cancer of various types	IV dose of 12.5-15 g, 25 g and 50 g + vitamin C orally 2-4 g a day.	Chemotherapy (n=33), radiation therapy (n=1), none (n=2)	Improvement in QOL: score 44.6 before treatment vs. 53.2 at 2 weeks and 61.4 at 4 weeks.	Mild (grade 1), most often headache (8.3%), nausea (8.3%)
Voll-bracht 2011[35]	125 (53/72)	Retrospective cohort	Breast cancer stages IIa–IIIb.	IV 7.5 g once a week for at least 4 weeks	Primary surgical treatment +/- adjuvant chemotherapy +/- adjuvant radiotherapy	Reduced QOL related side effects, slight increase PS during adjuvant treatment (80% vs. 71%) and aftercare (87% vs. 78%).	None
Yeom 2007[36]	39 (39/0)	Uncontrolled phase II	Terminal cancer of various types, stage IV.	10 g IV twice + 4 g oral vitamin C daily for a week.	NR	Health score improved from 3637 to 5537 after vitamin C.	No vitamin C supplementation stopped because of side effects.
Zhao 2017[37]	73 (39/34)	RCT	Newly diagnosis elderly with acute myeloid leukaemia	50-80 mg/kg IV for 10 days/ month, at most 10 months	Decitabine, cytarabine and aclarubicin chemotherapy	Median OS 15.3 months vs. 9.3 months in controls (p=0.039). Complete remission rate higher with Vitamin C: 84.6% vs. 70.6% after 2 courses.	Identical number of AEs and SAEs in both groups.

Abbreviations: IV: intravenous, AEs: adverse events, SAEs: serious adverse events, NR: not reported, OS: overall survival, PS: performance status, PSA: prostate-specific antigen, QOL: quality of life, RCT: randomised controlled trial.

Participants

The number of participants in these studies ranges from 14 to 1826.
All studies described different individual patients except the 2 studies by the same authors. In both of these studies, 100 patients with terminal cancer, which were treated with IVC, were compared to 1000 similar control patients. The second study 10 of the original studied patients were replaced since there were not enough suitable control patients, but 90 patients and most of the controls were identical to the first study[1,23].

Most trials included patients with a variety of cancer types, and most of the time patients were in a terminal or advanced stage. In only 6 studies, a specific cancer type was treated: 2 times breast cancer, 1 ovarian cancer, 1 colorectal cancer, 1 prostate cancer and 1 acute myeloid leukaemia.

All trials included both sexes, except for the studies on breast cancer and ovarian cancer that logically treated only women and the study on prostate cancer that logically treated only men. The average age of the participants was approximately 60 years.

Intervention

In 8 studies, vitamin C was given intravenously (IV), all in different doses and time intervals[22,25–28,33,35,37]. In 8 studies intravenous vitamin C (IVC) was given followed by or in combination with oral vitamin C supplementation[1,2,23,24,30,31,34,36]. In 3 studies vitamin C supplementation was only prescribed orally[3,29,32].

Other treatments

In 7 studies, conventional anti-cancer treatment was given in addition to the administration of vitamin C[22,24,25,27,34,35,37]. In 6 studies, this was not specifically documented, but it seems unlikely that patients had concomitant treatments[3,26,28,30,32,36] and in 6 articles it was written that no additional treatment was given at the time of the intervention with vitamin C[1,2,23,29,31,33]. In 4 articles, the researchers described that patients received conventional cancer therapy prior to participation[2,31,32,35], 13 articles report no information of previous treatments[1,3,22–28,30,33,34,36] and in 2 studies patients received no prior treatment[29,37].

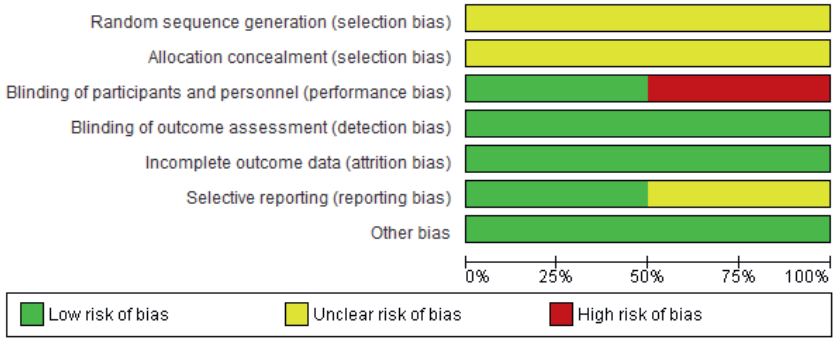
Outcome measures

Ten articles discuss the effect of vitamin C on overall survival[1,3,23,24,26,27,29,30,32,37], 9 articles the effect on clinical response in general[2,25,26,28–31,33,37], 7 on QOL and PS[3,26,30,31,34–36] and 14 articles report on the safety and toxicity of vitamin C treatment[2,3,22,25–27,29–31,33–37].

Risk of bias in included studies

Figure 7.2 presents the bias risk assessment as percentages across all RCTs. Bias judgment was based on "The Cochrane Collaboration's tool for assessing risk of bias in randomized trials".

Figure 7.2 Itemized Judgments for risk of bias based on "The Cochrane Collaboration's tool for assessing risk of bias in randomized trials".



Risk is presented percentages across all included RCTs.

In all RCTs, the selection process and the randomization process were not clearly described. It was therefore impossible to make a statement about the selection bias in the studies. Two of the 4 studies were blinded. There was a low risk of detection bias since the main outcome in the RCTs was overall survival. There did not seem to be other forms of bias in the selected RCTs.

The ROBINS-I tool, used for the non-randomized comparative studies, showed moderate to high risk of bias for the majority of the comparative studies as seen in **Table 7.2**. The Effective Public Health Practice Project tool, used for the non-randomized non-comparative studies, showed moderate to weak quality of the majority of non-comparative studies as seen in **Table 2.3**. Although certain studies used broad selection criteria, selection bias is hard to avoid without randomization.

Table 7.2 **Risk-of –bias assessment of comparative studies. Judgment for risk of bias based on the ROBINS-I tool for each included comparative study (high, moderate, low).**

Study	Evidence of selection bias/prognostic imbalance	Bias due to confounding factors	Bias in measurement of outcomes	Follow-up of participants sufficiently complete	Bias due to selection of reported results or due to missing data	Comparability of cohorts on important confounding factors
Cameron 1976[1]	High risk	High risk	Moderate risk	Moderate risk	Low risk	Moderate risk
Cameron 1978[23]	High risk	High risk	Moderate risk	Moderate risk	Low risk	Moderate risk
Cameron 1991[24]	Moderate risk	High risk	Low risk	Moderate risk	Low risk	Moderate risk
Günes-Bayir 2015[26]	Moderate risk	Moderate risk	Moderate risk	Moderate risk	Low risk	Moderate risk
Murata 1982[30]	High risk	High risk	Moderate risk	Moderate risk	Moderate risk	Moderate risk
Poulter 1984[32]	High risk	Moderate risk	Moderate risk	High risk	Moderate risk	Moderate risk
Vollbracht 2011[35]	High risk	Moderate risk	Moderate risk	Low risk	Low risk	High risk

Table 7.3 **Quality assessment of non-comparative studies. Judgment for quality of the studies based on the Effective Public Health Practice Project tool for each included non-comparative study (strong, moderate, weak).**

Study	Selection	Study design	Confounders	Blinding	Data collection cethods	Withdrawals and drop-outs
Bazzan 2018[22]	Weak	Weak	Weak	Weak	Weak	Not applicable
Cameron 1974[2]	Moderate	Weak	Weak	Weak	Moderate	Not applicable
Hoffer 2015[25]	Moderate	Weak	Weak	Weak	Strong	Strong
Mikirova 2012[28]	Weak	Weak	Weak	Weak	Moderate	Strong
Nielsen 2017[31]	Moderate	Weak	Moderate	Weak	Strong	Strong
Riordan 2005[33]	Moderate	Weak	Weak	Weak	Strong	Strong
Takahashi 2012[34]	Moderate	Weak	Weak	Weak	Moderate	Strong
Yeom 2007[36]	Moderate	Weak	Weak	Weak	Moderate	Strong

Results of individual studies

Results of the individual studies are summarized in **Table 7.1**.

Overall survival

Ten of the included studies measured overall survival in vitamin C treated patients[1,3,23,24,26,27,29,30,32,37] (**Table 7.1**). All studies compared their results with those obtained with a control group. In three studies, no effect of vitamin C on survival time was observed[3,29,32]. Two of those studies were RCTs. In 7 studies, the researchers found a positive effect of vitamin C on survival time[1,23,24,26,27,30,37], two of those studies were RCTs. One of these RCTs was done in acute myeloid leukaemia in a small group of patients treated with a hypomethylating agent (decitabine) that in vitro has a synergistic effect on vitamin C on TET2 expression, apoptosis, and proliferation of tumour cells. Patients received a relatively low dose of vitamin C intravenous and median overall survival increased with 6 months[37]. In the other RCT, the overall survival also trended toward improvement with vitamin C addition to standard chemotherapy for ovarian cancer, but since the patient groups were very small, the increase in median overall survival was not significant[27].

Intravenous versus Oral Vitamin C

All studies in which researchers suggested a positive effect of vitamin C on survival time, supplementation was administered intravenously, with[1,23,24,30] or without[26,27,37] oral supplementation. In the 3 studies, in which no effect of vitamin C on survival was observed, the supplementation was administered only orally[3,29,32].

Clinical response

The effect of vitamin C on clinical response was assessed in 9 studies[2,25,26,28-31,33,37], of which six showed at least some clinical improvement[2,25,26,28,30,37]. In the first study in patients with advanced stage various types of cancer, in which some positive effects were described, 10% of the patients experienced tumour regression[2]. This regression, however, was mostly measured based on clinical findings (and not imaging) and earlier tumour progression or metastasis was not always histologically proven. Other clinical improvements observed in that study were also highly subjective.

When IVC was given in 14 patients with various types of advanced cancer in combination with cytotoxic chemotherapy, 43% of the patients experienced a transient, but sometimes long-lasting stable disease; the investigators thought this was the effect of the vitamin C since it was highly unlikely to be due to chemotherapy alone[25]. However, no tumour regression was seen. In 15 patients with bone metastasis IVC had a positive effect on relief of pain[26]: with IVC 53% of patients had an significant improvement of the pain versus 13% with chemotherapy and 0% in controls without treatment. Another research group also observed a decrease in pain and decreased use of narcotic drugs[30]. They also saw some subjective other clinical improvements possibly related to the treatment with vitamin C.

In 75% of 20 prostatic cancer patients, the prostate-specific antigen (PSA) decreased after the administration of IVC[28]. This effect, however, was not confirmed in another trial were none out of 23 patients with prostate cancer experienced a reduction of the PSA[31].

In 2 other studies, there was also no objective clinical improvement[29,33]. In one of these (an RCT) 64% of vitamin C patients claimed relief of pre-treatment symptoms, but this was similar in the placebo group (65%)[29].

The only objective improvement of clinical response was seen in patients with acute leukaemia; patients with vitamin C had significantly better complete response rates after chemotherapy than without[37].

Intravenous versus Oral Vitamin C

All investigators that reported positive effects of vitamin C on clinical response administered vitamin C IV, with[2] or without[25,26,28,37] oral vitamin C.

Quality of life and performance status

QOL and/or PS after vitamin C treatment were assessed in 7 of the included studies[3,26,30,31,34–36]. In 5 studies, investigators showed a beneficial effect of vitamin C[26,30,34–36] while in 2 studies no benefit of vitamin C could be demonstrated[3,31].

Remarkable is one RCT in which 63% of the 60 patients with various types of advanced stage cancer on vitamin C claimed some improvement in symptoms but also 58% of the patients on placebo[3].

Intravenous versus Oral Vitamin C

Vitamin C was given IV (+/- orally) in the studies in which the investigators demonstrated a beneficial effect of vitamin C on QOL and PS[26,30,34–36]. In 1 study in which no effect was seen vitamin C was given intravenously in combination with a low dose of vitamin C orally, in the other study without benefit only oral supplementation was used[3,31].

Safety and toxicity

Fourteen articles report on the safety and toxicity of vitamin C treatment[2,3,22,25–27,29–31,33–37]. In all studies, no vitamin C related toxicity was observed. In 10 studies, possible side effects of vitamin C were seen but these were generally mild, and in general not more than in a control group. In one of these studies in which vitamin C was given through continuous infusions, the investigators described 2 serious adverse events (SAEs) that were possibly related to vitamin C treatment: kidney stones and hypokalaemia[33]. In 2 studies, no vitamin C related side effects were observed[27,35]. Investigators of 2 studies only described that there were no SAEs[30,36].

Vitamin C concentrations after supplementation

In 8 of the studies, plasma or serum concentrations of vitamin C were measured[24,25,27,28,31–34]. In 4 of those studies, baseline values were mentioned by the authors[25,31–33]. In 3 of these studies, the plasma vitamin C concentrations before supplementation were normal to high, ranging between 45 to 66 µM on average. In 14 of 22 patients with late-stage terminal cancer, the investigators describe low mean baseline values, but write down that the mean plasma value was 100 µM before supplementation[33].

All 8 studies mentioned plasma or serum vitamin C concentrations after the start of supplementation. Two studies dosed vitamin C in participants based on peak plasma concentrations after IVC with a target range of 20–23 mM. In 13 patients with ovarian cancer, it is not mentioned if this goal was always reached; in 60 patients with various types of newly diagnosed cancer only 54% of patients reached the required level at the end of the treatment period. Three other articles also describe peak plasma levels directly after IVC between 14 to 19.3 mM[25,28,31].

In 3 studies, it is not explained at what moment in time plasma vitamin C concentrations were measured after supplementation. In the study in which mostly vitamin C deficient patients were treated, mean plasma levels during IVC were 1.1 mM (range 0.38–3.0)[33]. In terminal cancer patients, the majority of the 1532 control patients had plasma concentrations of less than 85 μM, while the majority of the 294 vitamin C supplemented patients had concentrations higher than 114 μM[24]. In newly diagnosed breast cancer patients, researchers used oral supplementation in patients with normal baseline plasma vitamin C concentrations and measured values between 111 to 124 μM after supplementation[32].

In 2 studies it is mentioned that leukocyte vitamin C levels were measured[24,32], but in only 1 study the results are given. In that study, the investigators did not see a significant difference in leukocyte vitamin C concentrations in 27 patients with newly diagnosed breast cancer before and after the start of oral vitamin C supplementation.

Discussion

Summary of the main results

This systematic review presents the results and quality assessment of 19 studies to evaluate the effect of vitamin C treatment in cancer patients. It is difficult to draw any conclusions, since these studies have a large variety of outcome measures and the included studies also differ in study population (from newly diagnosed to advanced cancer), co-interventions (none or various adjuvant anti-cancer treatments), and vitamin C treatment (different doses, schedules, and modes of delivery). The results of some studies do suggest that vitamin C might have a positive effect on overall survival, clinical response, QOL and PS. However, this effect cannot be generalized to all patient groups with cancer. The best indication of a positive effect of vitamin C is seen in the RCT where it was used intravenously in elderly acute myeloid leukaemia patients who were also treated with decitabine. This effect is most likely due to direct regulation of ten-eleven-translocation (TET) activity by vitamin C in synergy with decitabine. TET enzymes are dioxygenases that are important for DNA methylation and are often less functional in patients with AML. One of the potential working mechanisms of decitabine already is the

upregulation of the TET proteins; it is thought that vitamin C enhances this effect[37]. In all other studies, especially those that included patients with various types of cancer, the results were less clear. Therefore, it is not proven and seems quite unlikely that the pro-oxidant capacity of vitamin C in high dosage creates a positive effect on overall survival, clinical response, QOL or PS in cancer patients in general.

More than half of the studies that researched QOL or PS saw a beneficial effect. However, since these studies were not blinded, patients may have experienced the well-known 'placebo effect'. In this respect, the observation of Creagan et al. is important since he demonstrates a positive effect in nearly 60% of the patients on placebo. These factors might have favoured a positive outcome of vitamin C treatment in all studies, especially those in which outcome was assessed through self-administered questionnaires that are highly subjective.

The mode of delivery seems to be an important factor in the effectiveness of the vitamin C treatment. In the studies with positive effects, intravenous delivery was used, while the absence of effect was mostly after oral administration. This suggests that the vitamin C levels that can be reached by oral supplementation might not be high enough for a possible effect, or that vitamin C is not absorbed from the gastrointestinal tract.
In the 14 studies in which investigators reported on side effects of vitamin C, these side effects were mostly mild or none at all and could have been related to the cancer itself or to the concomitant therapy patients received. There was no obvious difference in side effects of oral vitamin C compared to intravenous supplementation.

If measured at baseline, only 1 of the studied patient groups was vitamin C deficient. Perhaps supplementation would be more efficient and useful in patient groups that indeed are deficient. Unfortunately, also not many vitamin C plasma concentrations after supplementation were documented, but with oral supplementation plasma concentrations were much lower than with IVC.

Quality of the evidence

The quality of the evidence is poor due to the lack of extensive double-blinded RCTs. There are only 4 small RCTs that have been undertaken on this subject,

and in only 2 of these RCTs intravenous supplementation of vitamin C was used, which seems to be the most optimal to increase vitamin C concentrations in the patients.

All articles showed one or more forms of bias in the study procedures. Since only 4 studies were randomized, there is a high risk for selection bias. Especially the absence of blinding procedures has resulted in performance and detection bias. Outcome assessors might have overestimated the effects of vitamin C in patients, based on knowledge of their characteristics and clinical status.

In general, most studies examined in this review show some positive effects of vitamin C supplementation on cancer. However, this could also be due to a publication bias of studies with a good result and are at best indicative that it is unlikely that vitamin C supplementation in any form is harmful in patients with cancer.

Potential biases in the review process

Article selection and data extraction was peer reviewed by a second author. Assessment of the quality of included studies was performed independently by two authors, with discrepancies resolved by discussion and a third author when necessary. The authors therefore believe the review process was unbiased. The only limitation is the quality of the included studies.

Conclusions

The results of this review do not prove that there is a clinically relevant positive effect of vitamin C supplementation in most cancer patients on the overall survival, clinical status, QOL and PS. The quality of the studies published is low and the interventions and patient groups are very diverse. The best indication of a positive effect is seen in acute myeloid leukaemia patients in combination with decitabine, and in vitro data also show a synergistic efficacy of both treatments. An effect in some other patient groups might still be possible and might have been overlooked. In addition, clear pharmacological data might be needed to optimize treatment plans.

Treatment with vitamin C is likely to be safe, with almost no serious adverse events and minimal mild side effects, even with high doses of intravenous supplementation. There are also no indications that cancer progresses faster under vitamin C supplementation.

We see the results of this review as an indication that it is safe to examine vitamin C supplementation further in a randomized controlled setting. Therefore, we are planning to investigate the effect of vitamin C supplementation on immune recovery in patients that receive intensive chemotherapy and/or stem cell transplantation.

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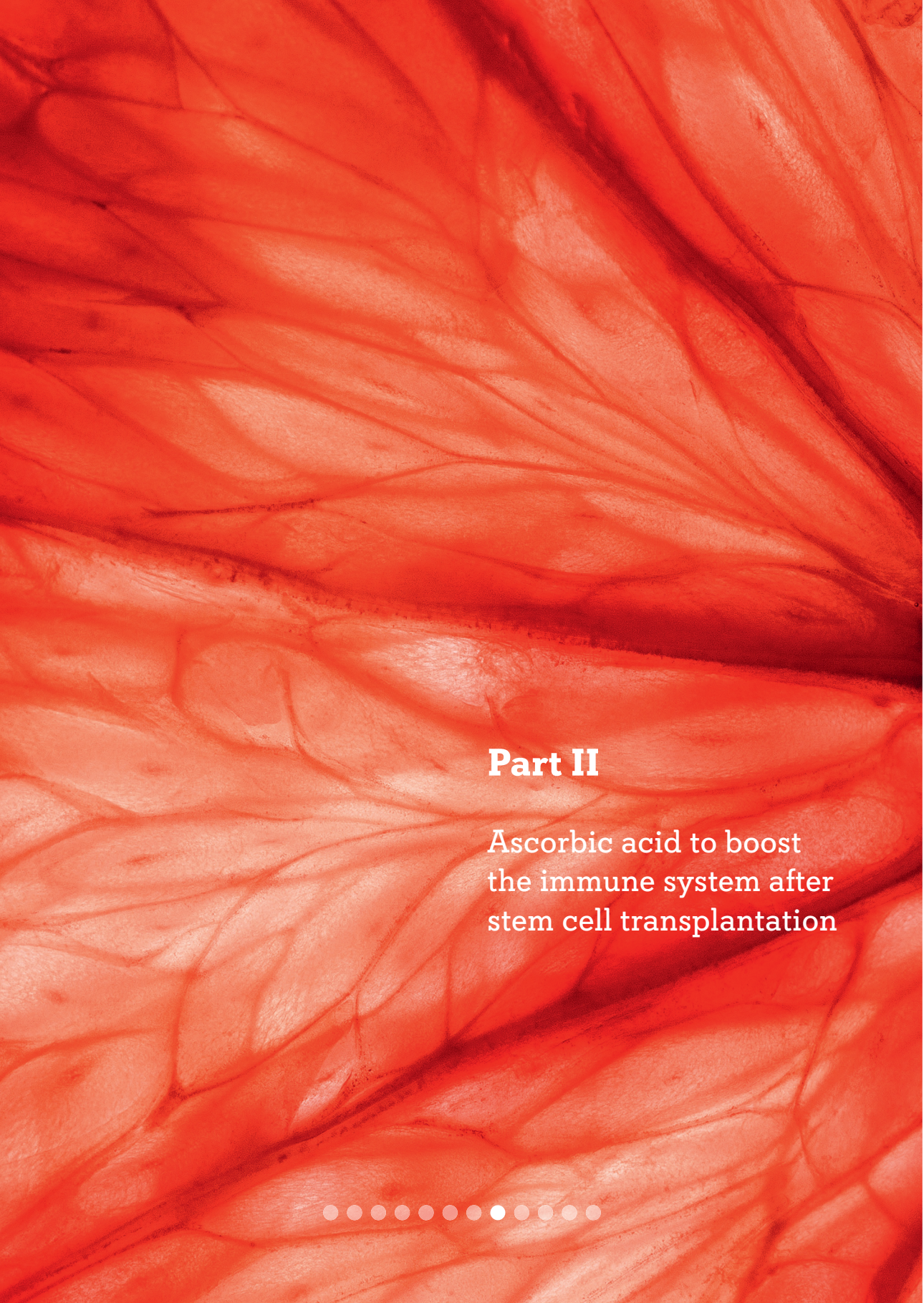

Part II

Ascorbic acid to boost the immune system after stem cell transplantation

8

Effect of vitamin C supplementation in autologous stem cell transplantations (ViCAST study protocol)

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Summary

Rationale
Recent studies showed that ascorbic acid (AA) stimulates proliferation and maturation of T lymphocytes and NK cells. Chemotherapy results in depletion of those cells and thereby an increased infection rate. A pilot study showed low levels of AA in the plasma of several patients after chemotherapy followed by autologous stem cell transplantation for haematological malignancies. AA supplementation could be beneficial to the recovery of the immune system in these patients.

Objective
The aim of this study was to examine the effect of AA supplementation on immune recovery in patients with autologous stem cell transplantation.

Study design
double-blinded randomized controlled trial

Study population
All participants are adults (minimally 18 years old) that received an autologous stem cell transplantation for multiple myeloma or lymphoma and were recruited at the MUMC+. In total there are 44 participants included in this study. The inclusion took place between 11-2019 and 11-2021.

Main study parameters/endpoints
Primary endpoints is the day of neutrophil recovery after stem cell transplantation. Secondary endpoints are AA lymphocyte levels, infection rate, duration of hospital stay, side effects of chemotherapy, overall survival, amount of T and B cells, maturation of NK cells, coagulation parameters, platelet reactivity, fibrinolysis, and quality of life.

Nature and extent of the burden and risks associated with participation, benefit, and group relatedness
AA supplementation could be beneficial for the immune recovery in the participants of this study. The risks associated with participation in this study are low. Vitamin C supplementation is safe and hardly has any documented side effects.

Introduction and rationale

Introduction

Cancer patients receiving stem cell toxic chemotherapy and haematopoietic stem cell transplantation (HSCT) have low immunity during a longer period due to slow immune reconstitution. The dietary intake of these patients is often diminished and we measured significantly lower serum AA levels in these patients[1]. Perhaps supplementation of AA to these patients could lead to faster immune reconstitution by stimulating proliferation of T and NK cells, the differentiation of T cells and improving the function of phagocytic cells.

AA supplementation is easy, cheap, and safe up to 2 gram daily in healthy volunteers and equal to the dosage in earlier clinical studies in critically ill patients[2,3]. In this study, we investigate the role of AA supplementation in patients that received high dose chemotherapy plus an autologous stem cell transplantation (SCT) to rescue the effect of the high dose treatment.

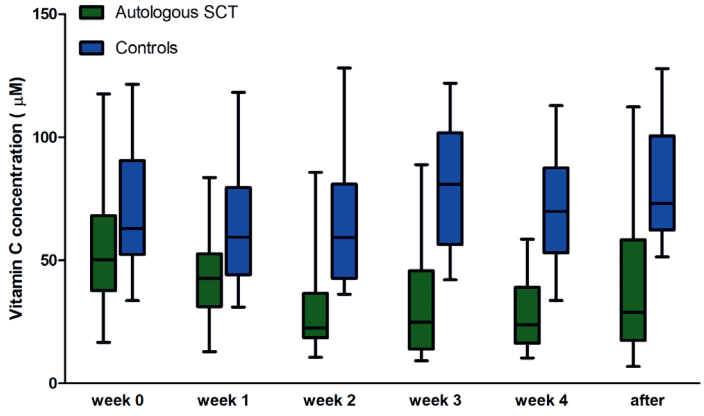
In an observational study done in our hospital that focussed on patients receiving an autologous stem cell transplantation, we discovered that more than 90% of those patients had a significant decrease in plasma AA level during therapy (n=31). The plasma AA values were also significantly lower than those of healthy family members followed over the same time period to observe season influence (n=19) after the start of the chemotherapy continuing for at least 3 months (**Figure 8.1**).

Supplementation of AA during the treatment of these patients might lead to a faster immune recovery, reduce infections, and shorten hospital stay in these patients.

AA and platelets

Platelets can hold up to 4 mmol/L AA, and the intraplatelet concentration thereby is 20-80 fold higher than the plasma AA concentration[4,5]. Platelets can compensate for fluctuations in plasma AA levels by modulating the expression of the sodium dependent transporter SVCT2, allowing them to actively transport AA intracellularly[5]. Platelet AA contributes to the whole blood content (16%) and may also provide an estimate of tissue status. AA is consumed during platelet activation and aggregation[6].

Figure 8.1 AA concentration in plasma over time in autologous SCT patients vs. healthy controls.



AA concentrations in µM in plasma of patients receiving an autologous SCT and of their healthy family members as controls. Week 0 is at baseline before the start of the myeloablative chemotherapy, after that blood samples were taken weekly. After is after discharge in the outpatient clinic on different time periods between 5 to 12 weeks after baseline.
AA: ascorbic acid, SCT: stem cell transplantation.

Studies have shown that AA plays several roles in platelet function, including reduction of reactive oxygen species (ROS) in platelets[7], inhibition of the proinflammatory and prothrombotic trans membrane protein CD40L expression, which might lead to impaired platelet aggregation[8,9], decrease of platelet-derived nitric oxide production in smokers, inhibition of thromboxane B2 formation[10] and stimulation of prostaglandin E1 production[11]. Furthermore, AA modulated surface sulfhydryl groups on platelets. Thiols and disulphides on platelet surface play an important role in platelet responses, including aggregation, secretion and activation of integrins. Changes in intracellular AA concentrations changed the surface sulfhydryl content[5].

Previous studies show contradicting results of the effect of AA on platelet aggregation tests. One study shows inhibited platelet aggregation in 6 patients with diabetes after 1 week of oral AA supplementation[4]. Another study shows no effect on platelet aggregation in three groups receiving placebo, low dose AA or high dose AA[12].

AA and blood coagulation and fibrinolysis

Several small studies show some effect of AA on blood coagulation. Studies in septic mice show that AA infusion corrected endotoxin induced coagulopathy by restoring prothrombin time (PT) and activated partial thromboplastin time (APTT) times to near normal. Furthermore, AA restored haemostasis in septic mice as observed by normalisation of thromboelastography (TEG) viscoelastic properties and AA also significantly induced expression of tissue factor pathway inhibitor (TFPI) and tissue plasminogen activator (tPA) in the lungs[13]. Another study showed that short-term treatment with high doses of AA decreased plasma levels of tPA and von Willebrand factor (VWF) in patients with type 2 diabetes[14].

Also, the effect of AA on endothelial function was investigated in a few studies (next to coagulation markers). One study showed that combined administration of AA and vitamin E at high dosages, improved endothelial function and decreased plasma levels of plasminogen activator inhibitor-1 (PAI-1), VWF and PAI-1/tPA ratio in chronic smokers[15]. Another study showed that AA inhibited expression of P-selectin and the release of VWF form endothelial cells in an in vitro model of sepsis, thereby inhibiting platelet-endothelial adhesion[16]. Even more AA inhibited rise in plasma thrombomodulin (as a marker of endothelial injury) in patients with severe sepsis[17].

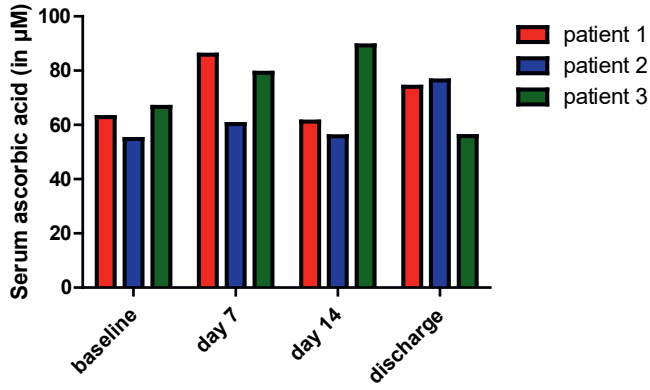
To unravel the possible effect of AA supplementation on thrombocyte function and coagulation parameters, we decided to perform a side-study on blood samples of all patients in the primary study if they did not use anticoagulants.

Determining the optimal dosage of intravenous AA

We started with a run-in phase to determine the magnitude of the effect of intravenous AA supplementation on serum levels of AA in patients with autologous stem cell transplantation order to verify that the prescribed AA dose was sufficient (in accordance with reference values in healthy people). To calculate sample size for this run-in period we used a Simon's two-stage design. In our historic reference group only 10% of patients had an optimal (>50 μM) AA concentration in serum on day 14. To see if the dosage of AA (70 mg/kg/day) was adequate, 3 patients were necessary. If all of the patients had a concentration of serum AA of >50 μM on day 14, we continued with the randomized part of the trial with this concentration.

The mean concentration of serum AA at day 14 in the 3 patients of the run-in phase was 69 µM and all of the values were >50 µM (**Figure 8.2**).
After the evaluation of the run-in phase, we thereby started with the randomised placebo-controlled trial with this AA dosage of 70 mg/kg/day.

Figure 8.2 AA serum levels over time for the 3 patients of the run-in phase.



AA serum levels of over time for the first 3 patients in the run-in phase. With AA supplementation all patients not only had an optimal AA concentration >50 µM at day 14 of the study, but at all timepoints.
AA: ascorbic acid.

Study objectives

Primary objective

The aim of this randomised-controlled trial was to investigate the effect of AA supplementation in the immune recovery – i.e. number of granulocytes - of patients after chemotherapy plus an autologous stem cell transplantation for multiple myeloma or lymphoma.

Secondary objectives

- To examine the effect of AA supplementation on serum and lymphocyte concentrations of AA
- To study the effect of AA supplementation on the recovery of subpopulations of lymphocytes, and on maturation of NK cells
- To study infection rate after autologous stem cell transplantation with and without AA supplementation

- To study duration of hospital stay after autologous stem cell transplantation with and without AA supplementation
- To study overall survival after autologous stem cell transplantation with and without AA supplementation
- To study relapse rates after autologous stem cell transplantation with and without AA supplementation
- To study side effects of chemotherapy after autologous stem cell transplantation with and without AA supplementation
- To study quality of life after autologous stem cell transplantation with and without AA supplementation
- To investigate the effect of AA supplementation on platelet reactivity, mitochondrial dysfunction, and ROS production
- To investigate the effect of AA supplementation on coagulation parameters and fibrinolysis

Study design

The study was a double-blind randomized placebo-controlled trial. For this study, subjects were individually randomized with a 1:1 ratio between the experimental group (Arm A: AA), and the control group (Arm B: placebo).

Duration

After enrolment, hospital admission of subjects was planned. Subjects were treated with intensive chemotherapy, followed by an autologous SCT, according to the institutional protocol. Two groups of patients were included: those with multiple myeloma and those with lymphoma, various subtypes, for which high dose chemotherapy and reinfusion of stem cells is standard of care. Patients with multiple myeloma received melphalan as chemotherapy. Patients with lymphoma received a standard combination of 4 different types of chemotherapy combined (BEAM: BNCU, etoposide, cytarabine, melphalan).

Subjects were treated with AA or a placebo. This was intravenous during hospitalization and oral after discharge of the hospital. For the individual subject, the study treatment ended 6 weeks after SCT. This endpoint for supplementation was chosen because most infectious complications are during these 6 weeks. After this period, there are hardly any infections anymore, so the immune recovery is usually adequate enough in this

treatment strategy at 6 weeks. Follow-up of these patients was until 3 months after stem cell transplantation; this will be in end of February 2022 for the last patient. When all subjects have completed the follow-up period and all the outcomes are assessed, we will de-blind the individual treatment (AA or placebo).

Setting

During the SCT and the episode the intravenous study drug was applied, subjects were hospitalized at our haematology ward. The last part of the study was performed at the outpatient clinic.
The study was conducted at the Maastricht University Medical Center.

Study population

Population (base)

Study participants were patients with a haematological malignancy (lymphoma or myeloma), that were in need of intensive chemotherapy plus an autologous stem cell transplantation as rescue for haematological cell recovery. They were all treated in the MUMC. In the last three years, the MUMC treated on average 72 patients per year with an autologous stem cell transplantation. At this moment due to changes in protocols, this number is even increasing. However, a part of the patients was treated in an outpatient setting after day one of the treatment (those who live close by and choose for this option) in a new protocol. These patients were excluded from this study since daily intravenous vitamin C could not be realized.

Inclusion criteria

In order to be eligible to participate in this study, a subject had to meet all of the following criteria:

- 18 years or older
- written informed consent
- diagnosis of malignant lymphoma or multiple myeloma
- chemotherapy plus autologous stem cell transplantation as standard of care for the disease at that stage
- central venous catheter in place or planned

Exclusion criteria

A potential subject who met any of the following criteria was excluded from participation in this study:

- inability to understand the nature and extent of the trial and the procedures required
- history of kidney stones
- kidney failure requiring dialysis or estimated glomerular filtration rate (eGFR) <30 mL/min
- history of glucose-6-phosphatedehydrogenase (G6PD) deficiency
- life expectancy < 1 month
- use of immunosuppressive medication other than chemotherapy and corticosteroids
- active AA supplementation other than normal daily multivitamin use
- any concurrent medical or psychiatric condition or disease that is likely to interfere with the study procedures or results, or that in the opinion of the investigator would constitute a hazard for participating in this study
- patients that are eligible after transplantation for a follow up in the outpatient setting and want to use this option

Sample size calculation

Our hypothesis was:

- Supplementation of AA will lead to a faster immune recovery after autologous stem cell transplantation of 1.5 days.

To calculate sample sizes, we first investigated the average duration of the immune recovery after stem cell transplantation of our patients treated with chemotherapy plus autologous stem cell transplantation in the last 3 years (2014-2016). There were 216 patients treated in this period with a mean duration from transplantation to neutrophil recovery (neutrophils ≥0.5) of 11.67 days (range 9-20, SD 1.357).

Based on these numbers, we used a two-tailed unpaired t-test to calculate the desired sample size to test our hypothesis and stated:

- α=0.05 (5% chance we determine there is a relevant effect of AA supplementation on immune recovery when such difference does not actually exist)
- power=0.9 (90% chance to be able to detect a relevant effect (=at least 1.5 days earlier) on neutrophil recovery of AA supplementation on immune recovery when a difference actually exists)

For this, the sample size should be 18 participants in each group.

This meant that to test of our hypothesis taking into account a withdraw of participants of 20% (z) an adjusted sample size (N) of 22 persons in each group would be adequate (N = n / (1-(z/100)).

Treatment of subjects

Investigational product

The investigational product used in this study is AA (vitamin C).
The intervention was the administration of a daily intravenous dose of AA, starting on the day of the first day of chemotherapy, until the end of hospitalization. Subjects in this part of the trial were treated with AA in a dose earlier described to be beneficial and safe in acute leukaemia patients and tested in the run-in phase of 70 mg/kg/day[18]. The comparator was administration of a daily intravenous dose of placebo, starting on the first day of chemotherapy, until the end of hospitalization. After termination of the hospitalization patients received oral AA supplementation daily or placebo. Subjects were randomized between the experimental and control group, with a 1:1 ratio.
Experimental group (arm A): AA intravenously once daily 70 mg/kg during hospitalization followed by oral AA 2 times daily 1 gram at home for in total six weeks.
Control group (arm B): placebo intravenously once daily during hospitalization followed by an oral placebo 2 times daily at home for a total of six weeks.

Use of co-interventions

Subjects have been treated with conditioning chemotherapy (melphalan or BEAM), followed by an autologous stem cell transplantation.

All subjects received the fluoroquinolone ciprofloxacin for prophylaxis against bacteraemia due to gram-negative bacilli, as well as fluconazole as fungal prophylaxis (except in case of allergies when a suitable alternative was provided). In case of fever, defined as a tympanic temperature ≥38.5°C, empirical therapy with piperacillin/tazobactam (or meropenem in case of allergy) was initiated during neutropenia according to local protocol.

Patients were treated with haematopoietic growth factors (filgrastim) until immune recovery according to our local stem cell transplantation protocol. Patients also received folic acid supplementation until immune recovery.

Aside from the above-mentioned drugs, subjects were allowed to use all treatments, which were prescribed by their treating haematologists.
Examples of allowed co-interventions:

- Haematological supportive care involved prophylactic platelet transfusions when counts were below 10 x 10^9/L as well as therapeutic transfusions when clinically indicated.
- Filtrated packed red blood cells were given on clinical indication, and when counts were below an individually specified level.
- Subjects received anti-emetics and pain medication

Medication or therapies that were not allowed during the study:

- Other vitamin supplementation than the prescribed AA or placebo and folic acid, including self-care and over-the-counter products

Methods

Main study endpoints

The main study endpoint was the day of repopulation (return of neutrophil to at least 0.5 × 10^9/l) after autologous stem cell transplantation.

Secondary study endpoints

Secondary endpoints were:

- AA serum levels
- AA peripheral blood mononuclear cell (PBMC) levels
- incidence of infections/ neutropenic fever
- days of hospitalization
- days with fever (≥ 38.5° C)
- incidence of bloodstream infections i.e. bacteraemia
- type of bloodstream infections
- quality of life according to the EORTC QLQ-C30
- overall survival (3 months)
- relapse rates (3 months)
- use of systemic antimicrobial agents (incidence and duration)
- recovery of lymphocyte subsets

- NK cell maturation
- platelet reactivity
- coagulation parameters
- tPA-ROTEM and fibrinolytic parameters
- number and severity of bleeding episodes during admission

Other study parameters

Other parameters that were documented (at baseline):

- sex
- age
- weight
- length
- ethnicity
- comorbidity
- performance status
- disease

Randomization, blinding and treatment allocation

Subjects have been randomized by block randomization stratified for disease (lymphoma or myeloma) using the randomization program ALEA through the clinical trial centre Maastricht. The sequence was concealed at all times throughout the study. Investigators, treating physicians and subjects were blinded to both the randomization schedule and subsequent treatment allocation. The pharmacy was responsible for providing the right medication for each subject.

Investigators and healthcare providers did not have access to the randomization code except in case of a serious adverse event (SAE) and/or suspected unexpected serious adverse reaction (SUSAR).

Study procedure

Venous blood sampling procedure

Participants that received an autologous stem cell transplantation already had a central venous catheter for standard care to take the blood samples. The first weeks they were certainly admitted in the hospital, and routine venous blood samples were taken daily this period already. After hospitalization, all patients were already seen frequently in the outpatient setting and blood

samples were always taken during those visits. We tried to combine these necessary blood evaluations with our research sampling. **Table 8.1** shows the timing of the required blood evaluations.

Table 8.1 Required blood evaluations.

	Baseline	First thrombocyte count of <50x10^9/l ± 3 days	Day 8 (± 3 days)	Repopulation (Leucocytes ≥1x10^9/l ± 3 days)	End of study medication (+/- day 42)
AA levels in serum/ PBMC	x		x	x	x
Flowcytometry on lymphocyte subsets	x			x	x
Flowcytometry for NK cell maturation					x
Thrombocyte and coagulation tests		x			

Abbreviations: AA: ascorbic acid, PBMC: peripheral blood mononuclear cell, NK: natural killer.

Laboratory tests performed on the venous blood samples

- AA level in serum
- AA level in PBMCs
- B, T and NK cell quantity (by flowcytometry)
- NK cell maturation (by flowcytometry)
- Thrombocyte and coagulation tests

Determining AA level in plasma and PBMCs

The determination of AA levels in serum is a standardized measurement performed in the clinical laboratory of the MUMC+ by HPLC.

The intracellular AA levels in PBMCs were determined in our research laboratory at the University of Maastricht. The method by which this was performed is described in chapter 6.

Thrombocyte and coagulation tests

To investigate the effect of AA on platelet reactivity, platelet reactivity was measured by flowcytometry and flow chamber.

To investigate the effect of AA on coagulation parameters and fibrinolysis coagulation parameters (PT, APTT, thrombin time, d-dimer, FII, FV, FVII,

FVIII, FIX, FX, FXI, and FXIII activity) and fibrinolytic parameters were measured (fibrinogen, plasminogen, α2-antiplasmin, tPA activity, PAI-1 activity and thrombin activatable fibrinolysis inhibitor). Furthermore, tPA-rotational thromboelastometry (ROTEM) was performed, a whole blood assay for fibrinolysis.

Quality of life questionnaire

The concept of health-related quality of life (HRQoL) has gained much interest during the last decades and is considered a second endpoint for evaluation following cancer treatment. Several instruments measuring HRQoL are applied in the field of oncology, with the 'European Organization for Research and Treatment of Cancer' (EORTC) Quality of Life Core Questionnaire QLQ-C30 being the instrument most frequently used. This questionnaire is most often implemented in clinical trials and is the current standard in measuring HRQoL in Europe.

The QLQ-C30 is a 30-item instrument, has been extensively tested for reliability and validity, and is translated in Dutch.

The QLQ-C30 addresses six multi-item scales of functioning (physical, role, social, emotional, and cognitive functioning, global health). Furthermore, three multi-item scales (fatigue, nausea and vomiting, pain) as well as six single items (dyspnoea, constipation, sleeping problems, appetite loss, diarrhoea, and financial problems) are related to symptoms or problems.

We asked participants to fill in this questionnaire 3 times: at baseline, at discharge from the hospital and at the end of the study period.

Withdrawal of individual subjects

Subjects were free to discontinue their participation in the study at any time, without prejudicing further treatment.

Statistical analysis

The baseline characteristics of the study population will be described by presenting categorical data as proportions and continuous variables as means

(+standard deviation) for normal distributed and as medians (with inter quartile range) for skewed data.

Primarily, we will perform an intension-to treat analysis. Secondary, we will do a per protocol analysis as a sensitivity analysis.

Primary study parameter(s)

To analyse the difference of duration of immune recovery (neutrophils ≥0.5) in days between AA supplemented patients and controls at first an unpaired two-tailed t-test will be performed. In a secondary analysis, we will do a linear regression to study the influence of covariates.

Secondary study parameter(s)

For all continuous variables (like AA levels, days of hospitalization, days with fever, coagulation parameters), we will use an unpaired two-tailed t-test for normal distributed data and the Mann-Whitney U test if there is no normal distribution.

For all categorical variables (like the occurrence of infection or bleeding), we will use a Chi-squared test and a Fisher's exact test if necessary.

Survival data will be analysed using Kaplan Meier with a log-rank test, and if necessary, using a Cox regression analysis.

For the majority of the QLQ-C30 items, a four-point Likert-type response scale is used. Exceptions are the items for the global quality-of-life scale (where a seven-point scale is used). Results will be reported in graphs per scale as mean, median and ranges.

The aspect of missing data will be solved through multiple imputations.

Ethical considerations

Regulation statement

The study was conducted according to the ethical principles of the Declaration of Helsinki 64th WMA General Assembly, Fortaleza, Brazil, October 2013 and

in accordance with the Medical Research Involving Human Subjects Act (WMO)[36].

Recruitment and consent

Written informed consent of patients was required before enrolment in the study and before any study related procedure took place. Patients were informed of the study by their treating physician. The physician followed ICH-GCP and other applicable regulations informing the patients. The research staff provided the patients with further information. Before informed consent was obtained, the research staff provided the patient ample time and opportunity to inquire about the details of the study and to decide whether to participate. All questions about the study were answered to the satisfaction of the patient and – if appropriate - their family members. The set time limit for the patient to make a decision was one week maximum, because it was not in the interest of the patient to postpone standard treatment. If patients already decided before, the period was shorter.

Benefits and risks assessment, group relatedness

Risk of specific measurements

AA is a well-known and safe medical substance and in the given dose dosage there are no serious adverse events to be expected. The risks of participants thereby were low. There were no extra investigations for the study except for peripheral blood withdraw, so the risk for complications was minimal.

Time and inconvenience

Since all participants were already admitted in the hospital and received intravenous medication through a central line, the extra time and inconvenience has been minimally if at all present.

Benefits

Patients that receive chemotherapy are prone to infections because of the negative impact on the immune status. AA supplementation could have a positive effect on this and decrease hospital stay and infection rate in the participants.

This study might demonstrate that improvement of the treatment of patients that receive an autologous stem cell transplantation is possible if it shows that

AA supplementation is beneficial for the immune recovery and lowers the risk of infection.

Since the risks of AA supplementation were minimal, and the time and inconvenience to participants was limited, we are convinced the possible benefits (participating in a scientific trial that possibly improves health care for this patient group) outweigh the risks. Those that randomised to the AA treatment arm and not placebo might already have profited from a positive outcome by participating in the study.

Administrative aspects, monitoring and publication

Handling and storage of data and documents

All studied subjects were assigned a study code. No patient specific data were included in this code. Only the principal investigators, the monitor and study coordinator had access to the key of this code to identify the patient. All regular diagnostic tests were coded on patient number and saved in the patient chart, available to their haematologist, the principal investigators, and the study coordinator.

Data were collected on electronic Case Report Forms (eCRF) in macro to document eligibility, safety and efficacy parameters, compliance to treatment schedules and parameters necessary to evaluate the study endpoints. Data collected on the eCRF were derived from the protocol and included:

- inclusion and exclusion criteria;
- baseline status of patient including medical history and stage of disease;
- baseline concomitant diseases and adverse events;
- parameters for response evaluation;
- survival status of patient;
- reason for end of protocol treatment.

All patient data, blood samples and details will be stored by MUMC+ for a period of fifteen[15] years after the onset of the study. For collection and storage of blood samples explicit informed consent was asked. For this

purpose, all subjects were requested to fill out a YES / NO answer, as stated in the informed consent letter.

Public disclosure and publication policy

The publication policy is based on the Central Committee on Research Involving Human Subjects statement (CCMO) of March 2002. Results of this study will be submitted for publication in peer-reviewed medical journals until accepted by one of these.

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9

General discussion

General discussion

Haematopoietic stem cell transplantation (HSCT) is an important treatment option for high-risk, malignant haematological diseases. The goal of a successful HSCT is to provide an effective treatment, with limited treatment related mortality and in case of an allogeneic HSCT limited graft-versus-host disease (GVHD)-associated morbidity and sufficient graft versus leukaemia/lymphoma (GVL) to prevent relapse. During the past decades, the use of HSCT has rapidly increased from 4,234 in 1990 to 48,512 in 2019 in Europe, due to substantial improvements in transplant procedures and supportive care[1,2]. This created more opportunities, but also more complexity. When considering a HSCT, there are multiple choices to make with regards to the type of transplant and conditioning regimen. In case of an allogeneic HSCT, donor selection, graft type and GVHD prophylaxis have to be considered. Choosing the best available combination for every patient is important and influences outcomes. However, further optimisation of HSCT procedures is still necessary, as there remains considerable mortality and morbidity related to it. Even if the procedure seems successful, many patients relapse afterwards. This makes HSCT an intense and dangerous treatment, and patients have to be fit in order to cope with these problems. Consequently, many patients are excluded from HSCT, since most haematological malignancies are diagnosed at older age.

This thesis focuses on strategies to improve the outcome of both autologous as well as allogeneic HSCT. At first, we discuss on the use of a haploidentical donor in allogeneic HSCT; secondly we explore the role of ascorbic acid (AA) in immune constitution in both autologous as well as allogeneic HSCT.

Haploidentical stem cell transplantation as alternative donor strategy

In the first part of this thesis, we investigate the role of haploidentical family donors as an alternative for the traditional HLA-identical donor. One of the major problems of allogeneic HSCT is finding a suitable donor. In the past, allogeneic HSCT was only performed using stem cells from an HLA-matched related (sibling) donor (MRD). Due to the reduction in family size in the Western world, there is a decreased availability of MRDs[3,4]. In case a MRD is not available, there are several alternative donor options including a matched unrelated donor (MUD), a haploidentical related donor and umbilical cord

blood. Traditionally, a MUD from the worldwide donor registries is considered the best alternative option, as MUD HSCT outcomes are similar to MRD HSCT with regard to overall survival (OS) and progression-free survival (PFS), even though there is an increased risk of GVHD[4-6]. However, finding a MUD donor is not always easy, and can be costly and time consuming[7,8]. Finding a MUD is especially challenging for the non-Caucasian population due to poor representation in donor registries as well as a high degree of genetic diversity within this population.

Haploidentical family donors are easy to find for almost every patient. However, the HLA disparity creates a higher risk of GVHD and graft rejection. To minimize these complications, extreme T cell depletion was used in the first haploidentical HSCT. This T cell depletion, however, made patients vulnerable to infectious complications. With the aim of reducing the chance of GVHD and graft rejection while maintaining the defence against infections a new regimen with high dose post-transplantation cyclophosphamide (PTCy) was developed to selectively deplete alloreactive T cells after HSCT. More and more data show that using this regimen a haploidentical donor is a very good alternative for an HLA-identical donor[9-12]. Due to these developments, the problem of finding a donor has faded to the background.

During the last decades, the use of a haploidentical donor for allogeneic HSCT has risen exponentially (Europe: none in 1990, 3,538 in 2019), as it entails many important advantages[2]. For instance, a haploidentical donor is widely and rapidly available, so it can facilitate faster HSCT for patients with urgent needs. It also creates easy access to the donor to collect additional cellular products to be used after transplant to prevent graft failure or disease relapse[13]. Furthermore, the use of PTCy instead of complex graft engineering approaches is clinically simple and relatively cheap. Nevertheless, there are some important disadvantages of this strategy. PTCy is a toxic regimen that can induce severe side effects, such as vulnerability to viral infections and haemorrhagic cystitis[14-16]. These can even be fatal or lead to longer hospitalisation, especially in older patients or patients with comorbidities[17]. Another problematic side effect is the occurrence of early cardiac events after PTCy that is seen in 19% of patients[18]. The most common cardiac toxicity is left ventricular systolic dysfunction, which is associated with older age, a history of cardiovascular problems, and prior exposure to cyclophosphamide[18]. Another prominent issue in haploidentical HSCT are

donor specific antibodies (DSA), which are induced by previous pregnancies or transfusions. They can negatively affect transplant outcome by reducing the chance of engraftment[19]. Another major problem for all donor types is the high amount of relapse that is seen after HSCT + PTCy, especially when reduced intensity conditioning regimens are used. A randomised controlled trial that provided a direct comparison between double unrelated cord blood HSCT versus haploidentical bone marrow HSCT as alternative donors in the absence of an HLA-identical donor showed superior OS in favour of the haploidentical HSCT due to lower NRM[20]. Relapse rates, though, were very high with a 2-year incidence of relapse of 48% in haploidentical HSCT in the reduced intensity setting. Even after myeloablative conditioning, relapse rates after haploidentical HSCT were still excessive (around 40%)[21-23]. This could potentially be explained in part by the suppression of NK cell proliferation and development by PTCy as it eliminates all dividing alloreactive NK cells. This reduces the important anti-tumour response of NK cells in the first several months after HSCT[24].

In **chapter 2**, we confirm that a haploidentical donor is a suitable alternative for patients with chronic lymphatic leukaemia (CLL) in need of an allogeneic HSCT. Prior to the availability of targeted novel agents, like the Bruton's tyrosin kinase inhibitors (ibrutinib and acalabrutinib), the phosphoinositide 3-kinase inhibitors (idelalisib and duvelisib), and the BCL2 inhibitor (venetoclax), allogeneic HSCT was advised for all fit poor risk CLL patients, especially those with early relapse, refractory disease or TP53 mutated disease. Allogeneic HSCT was a good option in these patients as it can create long-lasting responses, sometimes even curation. Its success is not influenced by the presence of high-risk molecular abnormalities, such as TP53 mutations[25-27]. However, the introduction of novel agents and thereby new treatment options changed the landscape of CLL. They dramatically improved OS and PFS, even in these high-risk patients[28,29]. Consequently, the number of allogeneic HSCT for CLL worldwide has steadily declined over the last years. Currently it is indicated as a last resort option for young and fit CLL patients who are refractory to one or more of these novel agents and in whom other agents are not expected to create long-term disease control[30,31]. The optimal timing of this allogeneic HSCT has to be considered, as we and others have shown that active disease at the time of transplants is one of the main risk factors for a poor outcome. HSCT in CLL should therefore only be performed in responsive disease[32]. We were the first to investigate the impact

of the use of a haploidentical family donor in CLL and showed that outcomes are similar to those with HLA-identical donors, as it is seen in other diseases[33-39]. Therefore, the lack of an HLA-identical donor should not be a reason to withhold allogeneic HSCT in these patients.

In **chapter 3**, we present the results of the introduction of the haploidentical HSCT with PTCy as GVHD prophylaxis in our centre. We performed a retrospective study as quality control and experienced first-hand that when adopting a new and complex treatment regimen from the literature in your centre, one can encounter unexpected problems. It is not always possible to generate similar outcomes, so implementation of new local treatment plans should not only depend on worldwide literature. Other aspects, such as geography, local habits and amenities, should be taken into account. For instance, in the T cell deplete (TCD) setting and later also in T cell replete settings, in our patients NRM was much higher due to infections than seen in the studies on which we based our regimens[11,40]. It is difficult to precisely identify the causes of this difference, but it could be related to our colder climate in which there is a higher incidence of viral infections in the general population[41,42]. The development of respiratory viral infections post-transplantation is related to inferior survival[43]. In our country, the distance between the hospital and the patients' hometown is generally small. Patients are discharged from the hospital quite fast after transplantation, where they are more exposed to domestic pathogens. This could have an impact on infection rates and thereby survival. Other aspects that can lead to inferior outcomes are certain less favourable baseline patient characteristics that are not always equally spread. These differences will be difficult to manage, so it is always important to evaluate the outcomes after changes in treatment regimens and to adapt local strategies accordingly. Benchmarking can be a useful tool to compare outcomes between centres but depends on complete and correct registration of all aspects that are important in relationship to outcome[44].

We did not see a difference in outcome of HSCT between matched unrelated donors (MUD) and haploidentical family donors in our investigated cohort. Therefore, we decided to investigate if costs would be a reason to choose for a specific alternative donor type if availability was not an issue. In **chapter 4**, we present the data and show that, overall, costs were similar for MUD and haploidentical HSCT. However, at the time of the study we did not yet use

PTCy as GVHD prophylaxis in HLA-identical donors. This could be of influence on the costs. There is likely an increase during the transplantation phase as patients that receive PTCy have a longer hospitalisation and cytopenic period. However, it could also lower the costs of the posttransplantation period, as it could decrease the incidence of costly complications like GVHD.

Nevertheless, calculations of costs are only relevant in this situation if clinical outcomes are similar. In many studies in which outcomes of haploidentical HSCT were compared with HLA-matched HSCT equivalent results were seen[12,35]. However, in these studies, GVHD prophylaxis in HLA-matched HSCT consisted of traditional immunosuppressive agents (calcineurin inhibitors, methotrexate, mycophenolate mofetil), and haploidentical HSCT even seemed to be beneficial as patients experienced less acute and chronic GVHD than patients undergoing MUD HSCT[35-37,45]. A recent retrospective study compared haploidentical with MUD HSCT, both followed by PTCy as GVHD prophylaxis. It showed an advantage in outcome when PTCy was used in the HLA-identical MUD setting. There was a significant reduction of both acute and chronic GVHD, and a reduction of NRM. This even translated in a better OS in MUD than in haploidentical HSCT[46]. However, this advantage was only seen in the reduced intensity setting and not in the myeloablative setting. Nevertheless, it was impressive with a 2-year OS of 54% in haploidentical vs. 67% in MUD HSCT. We must be cautious to make a definitive conclusion as these are retrospective data and there are no randomised controlled trials that address this subject. Furthermore, several studies contradict OS improvement with PTCy in HLA identical donors[47,48]. Even if a MUD HSCT with PTCy would be superior based on outcome, availability and speed may also guide donor choice.

In conclusion, the introduction of PTCy makes a haploidentical donor a suitable alternative if no MRD is present. This makes an allogeneic HSCT accessible for patients in need, for whom otherwise no donor would be available. However, there are still several important questions to be addressed. The introduction of T cell replete haploidentical HSCT does not improve HSCT outcomes, and many patients will relapse after it. Novel approaches will need to be developed to further optimize this platform to limit the risk of relapse, and thereby truly extent OS.

We asked ourselves if donor selection based on NK (natural killer) cell alloreactivity could be useful in haploidentical HSCT setting to induce NK cell killing of tumour cells and thereby reduce relapse rate. In **chapter 5**, we investigated this option in a small prospective study in high-risk multiple myeloma (MM) patients. Unfortunately, we had to stop this study prematurely as we did not see any improvement of PFS after including 12 patients. We had built very strict stopping rules since previous studies showed very high NRM in allogeneic HSCT for MM. We hypothesize that late reconstitution of functional mature NK cells due to PTCy is responsible for the lack of response, as most patients relapsed before these cells were present[24]. This thought is supported by the fact that one of the patients biochemically relapsed shortly after transplantation but had spontaneous improvement without any additional treatment. This was very remarkable, as her myeloma had a very aggressive course before the haploidentical HSCT but the improvement could also have been caused by the anti-tumour response of donor T cells. Nevertheless, it was clearly an effect of the HSCT. Furthermore, the fact that the patients already received several treatment lines (average 3, range 2 to 7) could also be of influence to the lack of response, since the best responses of allogeneic HSCT in MM in general are seen when it is used in an earlier course of the disease[49].

Several studies address the effect of a killer immunoglobulin like receptor (KIR)-ligand mismatch, but the results have not been consistent. This is probably related to the use of different transplant protocols with varying levels of T cell depletion *in vivo* or *in vitro*. One study in TCD haploidentical HSCT showed a dramatic decrease in relapse rates, and thereby improved survival compared to acute myeloid leukaemia (AML) patients without a KIR-ligand mismatch[50]. Until now, these results have not been equalled in unmanipulated haploidentical setting, and different studies show contradicting results from sometimes small beneficial effects, to sometimes no effects at all and sometimes even deterioration of outcomes[24,51-55]. The effects seem to be related to NK cell reconstitution, as one of these studies showed better outcomes in patients who had a faster NK cell reconstitution[24]. The lack of benefit of KIR-ligand mismatch in haploidentical HSCT with PTCy might have several causes[56]. For instance, early activated alloreactive donor-derived NK cells are eliminated by PTCy[24,53], and replaced by immature NK cells that are hypofunctional until at least 6 months after transplant. Another reason is the negative impact that graft-derived T cells can have on NK cell function and

KIR repertoire reconstitution as was shown in a study that compared NK cell reconstitution in T cell deplete and unmanipulated haploidentical HSCT[57]. The exact mechanism by which the T cells influence NK cell maturation is not yet exactly known. In this study, impaired KIR expression on day 100 after transplantation was an independent predictor of decreased OS, showing the importance of the development of a mature NK cell repertoire. Another possible mechanism behind the lack of effect of NK alloreactivity in the PTCy setting might be the increased amount of regulatory T cells (Tregs), that are naturally resistant to cyclophosphamide due to an overexpression of aldehyde dehydrogenease[58]. Tregs are known to interact with NK cells and have a negative impact on NK cell proliferation and functions[59,60].

Future perspectives of haploidentical HSCT

HSCT using a haploidentical donor has increasingly been performed and nowadays it is seen as a standard option in patients lacking an HLA identical donor. PTCy has been an important addition to haploidentical HSCT as it limits GVHD in this setting in an efficient, easy, and relatively cheap way. However, since NK cells play a key role in the GVL effect in HSCT and PTCy impairs them, it can be argued that this causes disease relapse. Future research shall focus on strategies to optimize the NK cell anti-tumour effect in haploidentical HSCT. There are multiple potential routes to overcome this limitation, and I will discuss three options more thoroughly.

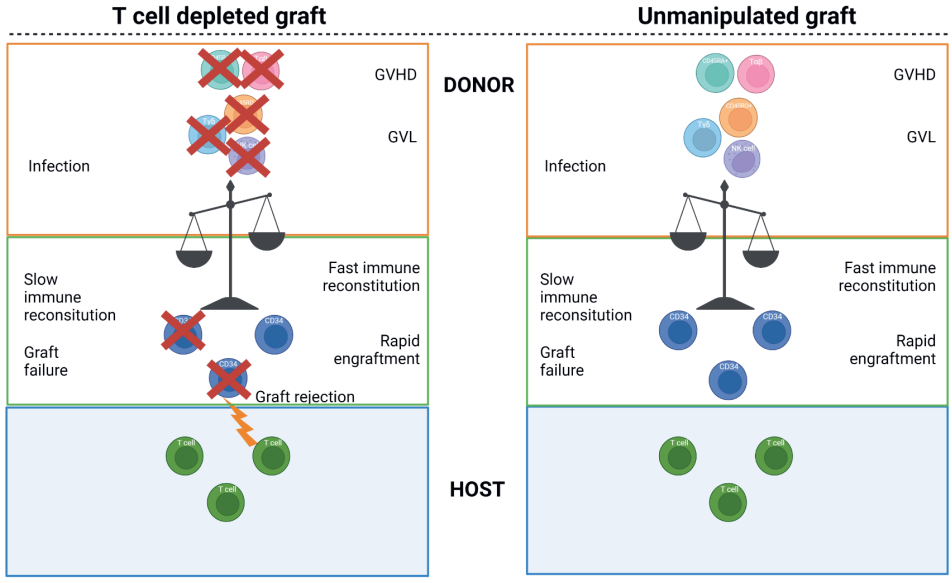
- The adaptation of anti-GVHD strategies to reduce the negative effect on GVL function of the graft
- The improvement of the NK cell presence and function
- Fastening immune reconstitution with ascorbic acid.

The adaptation of anti-GVHD strategies

In haploidentical HSCT, the immunological balance determines the outcome after transplantation (**Figure 9.1**). Extensive T cell depletion of a graft leads to an immunological imbalance that can cause slow immune reconstitution resulting in a high incidence of opportunistic infections and NRM. Furthermore, residual host T cells can cause graft rejection. On the other hand, without T cell depletion, a small percentage of donor T cells can create alloreactive reactions leading to GVHD. However, these donor T cells facilitate neutrophil

engraftment and immune reconstitution. This improved immunity prevents post-transplant infections and can improve the GVL effect.

Figure 9.1 The immunological balance of haploidentical HSCT.



Left: extensive T cell depletion (CD34 selection) from the graft results in an imbalance between residual host T cells and the graft that can lead to graft rejection. It also results in slow immune reconstitution and leads in that way to infections and poor disease control.
Right: Without T cell depletion, there is a faster engraftment and immune reconstitution that can lead to a faster GVL effect. However, donor T cells can also cause GVHD.
HSCT: haematopoietic stem cell transplantation, GVL: graft-versus-leukaemia, GVHD: graft-versus-host disease.

A transplant strategy to reduce relapse can be to selectively eliminate the subtype of T cells from the graft that are responsible for GVHD and leave the T cells that facilitate GVL unharmed. Another option is to deplete all T cells from the graft and administer special subgroups of donor lymphocytes (DLI) after HSCT to overcome post-transplant life-threatening infections and disease relapse.

Gamma/delta (γδ) T cells are a small T cell subset (3 to 10% of T cells in the peripheral blood of a healthy individual). In recent years, they are gaining interest of their special properties. These cells possess features from both innate and adaptive immune cells, and have a MHC-independent mode of

action. They show cytolytic activity through perforin/granzyme secretion and death receptor ligands. The dominant subset of γδ T cells in the human peripheral blood express a Vγ9Vδ2-encoded TCR, whereas Vδ1 γδ T cells are more present in the tissues[61]. Vγ9Vδ2 γδ T cells recognize microbial pyrophosphate molecules, which are secreted by many bacteria and some parasites. They can also be activated by high concentrations of isopentenyl pyrophosphate, a product that is synthesised by human cells in the mevalonate pathway of cholesterol synthesis, a pathway that is often dysregulated in tumour cells. This could lead to cytotoxicity against tumour cells, and thereby γδ T cells are interesting potential effector cells in cancer immunotherapy[61,62]. More importantly, these γδ T cells are not involved in GVHD. It is possible to perform a αβ/B cell depletion on the graft to limit the chances of developing GVHD and spare the γδ T and NK cells to optimise GVL effect. Mainly in paediatric patients, results of this method have been promising in the haploidentical setting[63-66]. However, in these studies relapse incidences were still high (24-42%). The lack of clinical benefit is possibly due to the large heterogeneity of polyclonal derived γδ cells with differences in TCR affinity and thereby anti-tumour activity[67]. Furthermore, HSCT with these TCRαβ/CD19+ depleted grafts were associated with high incidence of CMV and EBV viremias, but this was not linked to inferior survival[68].

Furthermore, αβ T cells can be divided into distinct subsets: naïve, stem cell memory, effector, effector memory and central memory T cells[69]. In mouse studies, the naïve T cells, which are CD45RA positive, mediate severe GVHD[70]. The memory T cells, which downregulate CD45RA and upregulate CD45RO, are more important to fight infections and exhibit GVL effect[71,72]. To deplete the graft of CD45RA positive cells could prevent GVHD while keeping the GVL effect. However, up to now, clinical results of studies using this graft selection method have been limited to children[66,73-77]. In these studies, high rates of engraftment, enhanced T cell reconstitution and low rates of acute GVHD were seen, and sometimes even a reduction of NRM. However, in one study the researchers observed an unexpected high rate of human herpes virus-6 (HHV-6) encephalitis (32%)[78]. HHV-6 is a member of the subfamily of human herpes viruses and is highly prevalent in the human population. Like other herpes viruses, HHV-6 remains latent in host cells, mainly in CD4+ T cells. Further research showed that the donor CD4+ T cells in the CD45RA+ depleted graft served as the reservoir for HHV-6 and caused encephalitis in the recipient[79]. Interestingly, when these cells were co-cultured with NK cells

in vitro, the NK cells were able to eliminate the virus. This concept was evaluated in a small study in 18 patients, who received NK cell infusions within 10 days of a CD45RA+ depleted HSCT and no HHV-6 encephalitis occurred[80].
Recent *in vitro* studies discovered that CD45RA-depleted grafts still had alloreactive properties that could provoke GVHD and identified CD276 as a marker for alloreactive Th1 T cells[81]. In a mouse model, depletion of CD276+ cells significantly delayed the onset of GVHD and decreased the severity. Results from clinical studies are not yet available.

Selective T cell depletion can be good platform for further cellular therapy as no further GVHD prophylaxis is needed[13]. In general, all haploidentical regimens serve as a good platform for further cellular therapy as the donor is easily available for extra cellular products. The effect of the administration of donor lymphocytes depleted of CD45+ cells after haploidentical HSCT with PTCy has been explored in a small phase II trial[82]. The procedure showed to be feasible and safe as none of the 19 evaluable patients developed severe GVHD. Furthermore, it was effective in reducing the risk of viral infections. However, a phase III randomised trial showed no effect on OS or relapse in children with leukaemia after αβ T cell depleted haploidentical HSCT[83]. Another option is the infusion of donor Tregs as they suppress alloreactive T cells but can improve T cell reconstitution[84]. In a recent clinical phase 2 study, Tregs were administered 4 days before a purified CD34+ haploidentical HSCT in 50 adult AML patients[85]. The study showed promising results with only 4% relapses and 2% of moderate/severe chronic GVHD[85]. However, NRM was still considerable (20%).

Another interesting concept is to administer T cells post-transplant that have been transduced with an inducible Caspase 9 suicide gene[86]. These T cells aid engraftment and immune reconstitution but can be switched off by an otherwise inert drug in case of GVHD. A small study in 12 children showed proof of principle and more studies are underway.

To increase T cells function against tumour cells, an attractive option is to administer specific CD8+ T cells, either selected for or transduced with an T cell receptor (TCR) that only has activity against leukaemia-specific antigens[87,88]. TCR-modified donor T cells against Wilms Tumour Antigen 1 (WT1), which is often seen in AML were prophylactically infused in 12 patients

post-transplant and 100% relapse-free survival (RFS) was seen after 44 months[87]. This was significantly higher than in a comparative cohort with similar disease characteristics (RFS 54%). Leukaemia-specific donor-derived, activated and expanded T cells against multiple AML related antigens were infused as monotherapy in eight relapsed patients. The response rate of 25% showed proof of concept in these patients with poor prognosis[88].

To prevent infectious complications, there is another cellular therapy option after haploidentical HSCT: infusion of specific antiviral cytotoxic T lymphocytes (CTL). At first, this was tried in 13 paediatric HSCT patients with adenovirus/Epstein-Barr virus-specific T cells from the same donor with good results[89]. However, these virus-specific T cells are only present in sufficient amounts in donors previously infected with these viruses, which is a limiting factor for this treatment. Further studies showed excellent results with the use of off-the-shelf multi-virus-specific T cells without causing GVHD[90].

Until now, none of the current graft manipulating strategies is perfect in preventing GVHD, keeping GVL at a maximum level, and showing anti-infectious properties. However, insights in our immune system are rapidly evolving. Furthermore, the technical possibilities for graft engineering and DLI manipulation are increasing. Because of these advances, I am convinced that more selective graft engineering, in combination with timing of the administration of different donor cell subsets post-transplant can further improve outcome. However, this graft manipulation is technically difficult, delicate and requires expensive equipment. Unfortunately, this makes these possibilities less accessible for resource-poor countries.

Another strategy for improvement can be adapting GVHD prophylaxis to improve the immunological balance after haploidentical HSCT *in vivo.* For this strategy, less equipment is needed, and it is easier to apply. A recent murine study investigated the possibility to reduce the dosage of PTCy to enhance GVL effects[91]. The GVHD preventing effect of PTCy was dose dependent, and a reduced dose was not sufficient to control GVHD. However, when α-galactosylceramide, a ligand of invariant NKT cells was added, this compensated for the insufficient GVHD prophylaxis by the reduced PTCy by stimulating donor-derived Tregs, without hampering the GVL. Another option would be to combine a lower dosage of PTCy with anti-thymocyte globulin

(ATG). A prospective phase II clinical trial showed a decreased incidence of acute and chronic GVHD, but OS and relapse rates did not improve[92].

A promising agent in GVHD prophylaxis is abatacept, a drug that creates a T cell co-stimulation blockade by binding to CD80/CD86 on antigen presenting cells and thereby preventing these receptors to bind their T cell activating ligand CD28. A randomised clinical trial involving both paediatric and adult patients in which a calcineurin inhibitor + methotrexate +/- abatacept were compared showed excellent results in mismatched unrelated donors as the incidence of severe acute GVHD was reduced from 30.2 to 2.3%[93]. Clinical results in haploidentical HSCT have been limited, but two small studies in children with hemoglobinopathies and severe aplastic anaemia using abatacept in combination with PTCy and sirolimus showed good effect on immune constitution, with a sustained increase of Tregs and NK cells and a decreased incidence of acute and chronic GVHD[94,95].

Several other novel compounds that target GVHD-inducing T cells are under investigation, but for these compounds there are hardly any clinical results yet[96].

Improvement of NK cell presence and function

Even though our study on NK alloreactive haploidentical HSCT in MM was not successful possibly because of the lack of mature NK cells due to the PTCy, there is still a lot of potential in NK cell-based immunotherapy. NK cells play a vital role in haploidentical HSCT since they do not induce GVHD but can mediate GVL effect. This effect can be potentially augmented by NK cell alloreactivity created by a KIR-ligand mismatch, a possibility in 70% of the patients. Even though we could not prove a beneficial effect of KIR ligand mismatch in our study in MM, this mismatch in haploidentical HSCT might be beneficial in other types of diseases (like AML), and with other immune suppressive regimens than PTCy, which hampers NK cell reconstitution, or with adaptation of graft composition.

Haploidentical HSCT is an excellent platform for NK cell therapy, since haploidentical donor NK cells can efficiently eliminate recipient tumour cells. As haploidentical donors are widely available for almost every patient, it is also possible to select a haploidentical family member with appropriate KIR repertoire for maximal effects, even though a KIR mismatch is not obligatory

for an effect of NK cells against cancer cells[97]. However, since KIR genes inherit independent from HLA type, KIR-ligand mismatch is also possible in HLA-identical HSCT.

There are many different other potential angles to improve the effect against tumour cells by manipulating NK cell function in haploidentical HSCT. These strategies can be used pre-emptively around the time of HSCT or shortly afterwards. They can also be used to augment the GVL effect if there are signs of relapse. Furthermore, some of these options can also be deployed as an alternative for HSCT.

Boosting NK cells

One of the most studied NK-mediated immunotherapeutic approaches is NK cell infusion. Several studies have been performed in the autologous setting with disappointing results. Autologous NK cells were not able to give potent responses against cancer cells because of inhibitory effects of self MHC I molecules[98]. After these findings, interest quickly shifted from autologous to allogeneic NK cell therapy. One study showed that infusion of primary NK cells of haploidentical donors after non-myeloablative chemotherapy were able to generate complete remissions in acute myeloid leukaemia (AML) patients[99]. It was noteworthy that there was only a response after lymphodepleting conditioning treatment. In these patients there was a marked increase in serum IL-15 levels, which did not have to compete with T cells that can otherwise function as cytokine sinks. IL-15 is essential for the expansion and survival of NK cells.

Immunotherapy using primary NK cells from the peripheral blood has several disadvantages, the most important of which is their limited numbers. A recent phase I/II study using donor-derived *in vitro* expanded NK cells showed remarkable results in AML patients after haploidentical HSCT (n=25) as only 4% relapsed in 2 years compared to 38% in historical controls[100].

More recently, extensive research has been performed to find alternative allogeneic NK cell sources. The most investigated are umbilical cord blood (UCB) NK cells, clonal NK cell lines and stem cell-derived NK cells. UCB consists of around 30% NK cells. These UCB NK cells have weaker cytotoxic activity but produce identical amounts of cytokines compared to primary NK cells[101,102]. Clonal NK cell lines, such as NK-92, are an alternative source of

allogeneic NK cells, as they are easily expanded. However, they are genetically unstable, which requires them to be irradiated prior to infusion. This limits the *in vivo* persistence and presents a major obstacle in achieving long-term clinical response. Stem cell –derived NK cells have many advantages as this approach allows one to produce virtually unlimited amounts of homogeneous NK cells, which are easier to reproduce, could be used as standard off-the-shelf therapy, and can be genetically modified.

Minimize inhibitory signals via NK cell receptors

To further optimize NK cell therapy, NK cells can be manipulated to increase their anti-cancer activity, possibly enhancing their effect also in other cancer types. Since NK cells express their cytotoxic function based on the balance between inhibitory and activating signals, another strategy to improve the anti-tumour effect of NK cells after HSCT is to block inhibitory receptors on NK cells (immune checkpoint inhibition). For instance, the effect of the anti-NKG2A antibody monalizumab is currently being investigated in clinical trials in various types of cancer, in both haematological and solid tumours. In preclinical studies, this agent enhances NK and T cell cytotoxicity[103]. Moreover, after the knockout of NKG2A in primary NK cells with CRISPR/Cas9 the NK cell cytotoxicity is enhanced even further in various tumour cell lines (unpublished results).

Another potential target is the T cell immunoreceptor with Ig and ITIM domains (TIGIT), which is overexpressed on human tumour-infiltrating NK cells[104]. Blockade of TIGIT in mouse models reversed NK cell exhaustion and promoted NK cell-dependent tumour immunity[104]. Several anti-TIGIT antibodies, such as ociperlimab are under investigation in clinical trials.

Similar to TIGIT, the inhibitory receptor CD96, a member of the same immunoglobulin superfamily, has been found in higher levels on NK cells in tumour tissues. Higher NK cell expression of CD96 predicts poorer clinical outcomes in hepatocellular carcinoma[105]. There are no clinical trials on CD96 inhibitors, but in mouse models anti-CD96 showed to inhibit the development of metastases[106].

Currently numerous other immune-checkpoint inhibitors are under investigation, such as anti- programmed cell death (PD)-1, anti- T cell immunoglobulin and mucin domain (TIM)-3, anti- lymphocyte activation gene

(LAG)-3, anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and anti-KIR[107-110].

Enhancing NK cell activity

Another way to enhance NK cell activity is to use monoclonal antibodies with antibody-dependent cellular cytotoxicity (ADCC)-triggering. Elotuzumab, a monoclonal antibody against SLAMF7, which is expressed on both NK cells as well as multiple myeloma cells and promotes NK cell cytotoxicity towards multiple myeloma tumour cells, is an example using this principle[111]. Daratumumab, a monoclonal antibody against CD38, also enhances NK-cell cytotoxicity *in vitro*, but the presence of daratumumab also lead to NK cell-fratricide, which led unfortunately to a higher number of death NK cells[112].

Over the last years, many bi-specific (BiKE) or tri-specific (TriKE) engager proteins have been created to bring immune cells in contact with tumour cells. A target domain could be an activating NK cell receptor whilst another binds a specific tumour-associated antigen[113,114]. At this moment, several of these molecules are under investigation in clinical trials and it will only be a matter of time before these antibodies are used in clinical practice.

The development of chimeric antigen receptor (CAR) NK cells is another way to augment NK cell activity. CAR NK cells are genetically modified to target a specific tumour-associated antigen and to activate cytotoxicity towards the target cell upon antigen binding. CAR T cells have already showed great clinical successes in paediatric acute lymphocytic leukaemia and aggressive B cell lymphomas. However, major side effects, such as cytokine release syndrome (CRS) and neurological toxicities, are frequently encountered[115,116]. It is thought that CAR NK cells could emulate these results, but without these problematic toxicities. The results of the first clinical trials are hopeful[117]. CAR NK cells could be extremely useful in the relapse setting after haploidentical HSCT. In this case, the donor can be used as cell source for donor-derived CAR NK cells.

In addition, a way to activate non-manipulated NK cells is by using cytokines. Most investigated is IL-2. A clinical study in AML patients showed that NK cell expansion after haploidentical NK cell infusions was improved when IL-2 was administered[118]. Moreover, IL-2 can also activate and expand host Tregs that are immunosuppressive for the NK cells[119,120]. IL-15 has also emerged as an

important cytokine to augment NK cell function as it stimulates the activation, proliferation, and expansion of NK and CD8+ T cells without increasing Tregs. As monotherapy, however, it failed to create objective responses [121,122]. In a phase I trial, IL-15 in combination with lymphodepletion and haploidentical NK cell infusions, was given to patients with relapsed or refractory AML, and generated 40% complete remission[123]. However, both cytokine release syndrome and rejection of the infused NK cells after day 14 were common. Nevertheless, IL-15 is an attractive candidate to influence NK cell activity. This is evidenced by the use of ALT-803, an IL-15 super agonist complex that has been designed to extend the *in vivo* half-life of IL-15[124]. In a phase I trial with 33 AML patients that relapsed after HSCT, infusion of ALT-803 created responses in 19% of the patients, without major side effects[124].

There are numerous ways tumour cells can escape immune cells, such as NK cells. A small phase I/II trial in older relapsed or refractory AML patients showed responses of allogeneic primary, preactivated memory-like NK cell infusions in five of nine evaluable patients[125]. These responses were only for a short duration as most of the patients relapsed within months. Researchers discovered that these relapses came from a small group of immature, chemo- and immune cell resistant AML cells, the leukaemia stem cells (LSC)[126]. These LSCs are defined by the absence of NKG2 ligand expression that other AML cells have, which makes it possible to escape from NK cells that carry NKG2D as an activating receptor. This NKG2D ligand expression in AML cells is suppressed by poly-ADP-ribose polymerase-1 (PARP-1). In a mouse model, PARP1 inhibition + allogeneic NK cells deletes human LSCs. A clinical trial in patients is still ongoing. In the same context, AML cells have also shown to upregulate CD47, a "don't eat me" signal[127]. The CD47- binding receptor signal regulatory protein (SIRP) alpha has recently been shown to be expressed not only by monocytes, but also by NK cells[128]. Upon binding to its ligand SIRP alpha CD47 inhibits NK cell mediated responses[129]. The use of the anti-CD47 antibody magrolimab could potentially enhance NK cell effector function. The clinical effect of magrolimab in combination with azacytidine with or without venetoclax is currently under investigation, but the initial results are promising[130].

These developments show that the improvement of pre-clinical knowledge about specific pathways on how different tumour cells evade our immune system can lead to better treatments.

Blocking the NK cell suppressive effect of the tumour microenvironment

NK cells are influenced by many immunosuppressive mechanisms of the tumour microenvironment (TME) and various substances are under investigation to counteract this. Transforming growth factor (TGF) β is one of the most studied molecules in this context. TGFβ is produced by immune cells as well as tumour cells in the TME and downregulates NK cells in many ways, for example by cytokine secretion, degranulation, cytotoxicity, and metabolism[131-133]. Studies in leukaemia and colon cancer cell lines show that the negative effect of TGFβ on NK cell cytotoxicity can be completely abrogated by adding an inhibitor of TGFβ[134].
Another barrier of the TME for NK cell cytotoxicity is hypoxia and its metabolic signature[112]. Furthermore, soluble ligands for NK cell activating receptors can act as suppressors of NK cell activity, like MICA and MICB[135]. In addition, IL-37 that is secreted by Tregs is another soluble factor to negatively influence the function of NK cells[136]. All these inhibitory pathways of the TME are potential targets to enhance the effectiveness of NK cell-based therapies and are currently under investigation.

A summary of the various approaches to enhance NK cell function is given in **Figure 9.2.**

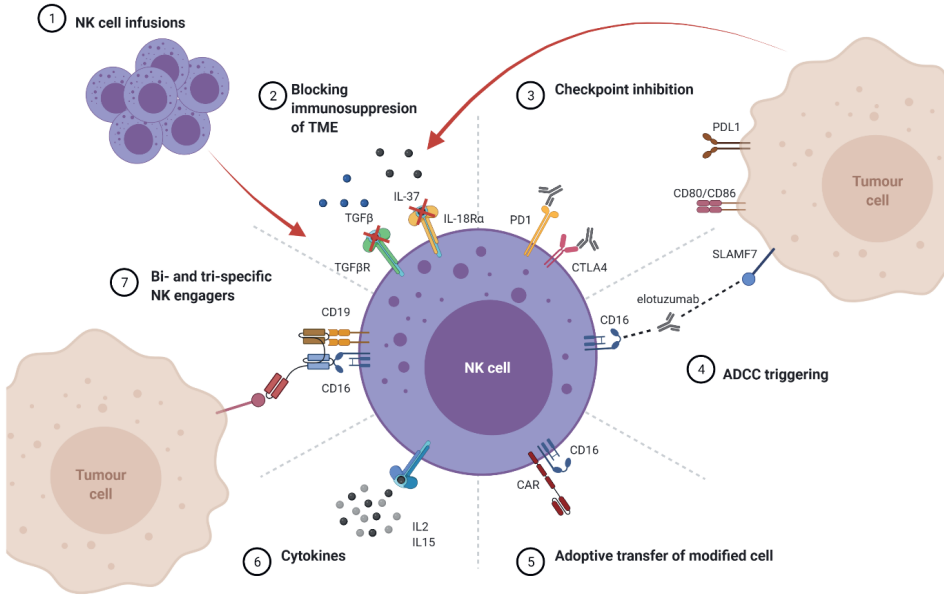
Fastening the immune reconstitution after hematopoietic stem cell transplantation with ascorbic acid

Slow immune reconstitution is an important problem after allogeneic as well as autologous HSCT, and can cause bacterial and viral infectious complications. Infections can delay the recovery of the patients, and decrease quality of life. They can even be lethal. In allogeneic HSCT, slow immune reconstitution can even lead to relapse as the GVL effect is diminished. When starting our research project we were convinced that boosting the immune system would be an attractive target to improve outcome in HSCT.

An optimal nutritional status is essential for a well-functioning immune system[137]. Large varieties of micronutrients play important roles in the innate and adaptive immune system. These include several vitamins, for example vitamin A, B6, B12, C, D, E and folate, and trace elements, for example zinc, iron, selenium, magnesium, and copper[137]. Frequently patients who are treated with HSCT have a poor nutritional status since they have generally been pre-treated with intensive chemotherapy. Furthermore, weight loss in

the period before HSCT is a strong predictor for a poor outcome with higher NRM and shorter OS, especially in allogeneic HSCT[138,139]. Presumably, this is not entirely related to the effects of malnutrition on the immune system, but it probably plays a role.

Figure 9.2 Overview of approaches to enhance NK cell function.



From the top left, clockwise: 1. Boosting the NK cell function by adding NK cells. 2. Blocking immunosuppressive cytokines from the tumour microenvironment. 3. Blocking inhibitory receptors on the NK cells by checkpoint inhibitors. 4. Monoclonal antibodies capable of binding to and activating CD16 on NK cells enable the targeting of these cells towards tumour cells expressing specific antigens. 5. Improving adoptive NK cell anti-tumour activity by genetically modifying them (for instance chimeric antigen receptor (CAR) NK cells). 6. Promoting NK cell proliferation and persistence with cytokines. 7. Increasing NK cell function by the use of NK cell engagers that bind and activate CD16 and specific antigens on tumour cells. NK: natural killer.

Vitamin C (AA) is an important vitamin for immune cells. For instance, it is necessary for the development of T cells[140], and it improves proliferation of both NK as well as T cells[140,141]. A recent *in vitro* study also showed that KIR expression, which is a sign of NK cell maturation and controlled by methylation in progenitor cells, could be induced by AA[142]. Phagocytic cells also rely on AA for their function[143]. In previous research, we showed that serum AA levels are low in patients with haematological malignancies[144]. It

would be tempting to supplement AA in HSCT patients to see if it improves immune reconstitution and thereby improves outcome.

Serum AA does not correlated with the total body storage of AA. However, leucocyte AA concentrations are correlated with body storage. Therefore, we first decided to develop a method to measure intracellular AA in various types of leucocytes. In **chapter 6**, this method is described in detail. During our clinical study, we wanted to use this method to investigate if a deficiency of AA really exists in these patients. In addition, we can measure if supplementation leads to normalisation of AA in immune cells, as these are the target of the intervention.

In **chapter 7**, the results of a systematic analysis about the effects of AA supplementation seen in cancer is presented. We considered this as a necessary step before using AA in the clinic. Concluding, we did not find any serious side effects or indications that it would be dangerous to use AA supplementation in cancer patients.

Recently, the interest of the therapeutic properties of AA is increasing, and at present, there are 226 active studies investigating its effects registered at clinicaltrials.gov. Several studies focus on infectious diseases, such as sepsis and COVID-19. However, until now, results of the published studies in severe sepsis and COVID-19 have been contradicting[145-150].

The studies concerning the effect of AA in cancer are performed in a large variety of cancer types. Furthermore, the potential pathways in which AA could have a beneficial effect are diverse. In my opinion, the biggest reason for the lack of progress in studies on AA is that there is no financial stimulus for pharmaceutical companies to finance these studies. Therefore, most of the studies on AA are investigator-initiated, and most clinicians lack the financial possibilities to facilitate a large, multicentre, randomized clinical trial to reach a conclusion on the efficacy of AA.

One of the most hopeful findings regarding AA in the treatment of haematological malignancies is that it has a synergistic effect with hypomethylating agents like decitabine. There was an increased overall survival in elderly AML patients that were treated AA in combination with decitabine plus low dose cytarabine and aclarubicin[151]. AA is essential for TET-

induced conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), the first step in active DNA methylation. Thereby, from a biological point of view, it is plausible that hypomethylating agents like decitabine and azacytidine, might function better when AA levels are normalized[152]. However, no randomised study has yet been published that confirms this concept (only in combination with chemotherapy), and since we lack conclusive evidence our patients on hypomethylating agents are not treated with AA. We regret that even after multiple attempts, it was impossible for us to raise enough funding for a study on this subject.

Nevertheless, we feel very excited that we could further explore the effect of AA on immune recovery after HSCT. For this goal, we developed a double-blind randomised placebo-controlled trial in which we investigated the effect of AA supplementation on immune reconstitution in autologous HSCT. The research protocol can be found in **chapter 8**. At this moment, this study has finished recruiting patients, but the follow-up period of the last patient is not completed. Since the randomisation is blinded, no results are available at this date. The primary endpoint of the study is neutrophil recovery after HSCT, as AA is proven to positively influence neutrophils. In addition, we investigate if the amount of AA supplementation is sufficient to increase AA in peripheral blood mononuclear cells (PBMCs). Furthermore, we look at the effect on T and NK cell development.

We hope in the future to be able to investigate the effect of AA on immune reconstitution in allogeneic HSCT. This will be especially worthwhile if we see an influence on immune recovery in autologous HSCT. Infections are a major problem after an allogeneic HSCT due to the slow immune reconstitution. This immune recovery in allogeneic HSCT is delayed even more by the use of necessary immunosuppressive agents. If patients develop GVHD, the immunosuppressive therapy can even be indicated for many years. Like other haematological patients who receive intensive treatment, patients treated with allogeneic HSCT have low serum AA levels[140,153]. This might be due to mucosal damage caused by the conditioning regimen in combination with side effects from the large amount of co-medication resulting in low intake and resorption of AA and other nutrients. Boosting their immune system with AA supplementation could be helpful to limit infectious complications. Furthermore, preclinical studies indicate that high doses of AA could have a direct effect on leukaemia cells by promoting apoptosis and inhibiting

proliferation though DNA hypomethylation[154,155]. In addition, AA is known to stimulate conversion of conventional T cell to Tregs[156,157]. Tregs can promote donor engraftment, protect from GVHD and increase graft-versus-tumour effect which could be of importance in allogeneic HSCT[158]. The relevance of Tregs in HSCT is proven in haploidentical HSCT + PTCy. Donor Tregs demonstrated to be resistant to PTCy-induced cytotoxicity because they express high levels of the enzyme aldehyde dehydrogenase that can inactivate cyclophosphamide[159]. It was shown in a murine model that it is not the elimination of alloreactive T cells but the suppression of these T cells by the leftover Tregs that prevents GVHD when using PTCy[160]. In other murine models on GVHD, it was shown that AA promotes the generation and stabilisation of CD8+ induced Tregs (iTregs) by increasing DNA methylation[161,162]. Those CD8+ iTregs were capable to control GVHD, while retaining the ability to control relapse[162]. On the other hand, the Tregs could be harmful in allogeneic HSCT as they can suppress NK cell function towards tumour cells[163]. Furthermore, changing the balance of different T cell subsets by AA could also influence the development of acute and chronic GVHD. Future research on AA supplementation in allogeneic HSCT should proceed with caution keeping the contradicting influences on the different immune cells in mind.

AA has an effect on tumour cells as well and thereby supplementation could have an additional benefit in cancer patients. One of the mechanisms tumour cells use to evade immunosurveillance is by epigenetic silencing of several genes by alteration of DNA methylation and histone modifications[164]. Amongst other things, these changes lead to a decreased gene expression and a decreased presentation of tumour-associated antigens. Hypomethylating agents, like AA can be used to counteract hypermethylation and restore gene expression. This could reinstate tumour recognition by the immune system as tumours express more neoantigens, and could increase T cell receptor repertoire.

Since AA is not the only micronutrient that is important for immune function, and HSCT patients often are malnourished, it could be beneficial to try to improve the complete nutritional status in these patients. Currently, however, there is no consensus on the optimal method of nutritional support and the limited randomised trials on this subject do not provide scientific evidence[165-167]. Further studies should be undertaken to examine which

nutritional interventions are effective to not only reduce malnourishment in these patients, but also optimally support the function of their immune system.

Expanding the deployment of cellular therapy

Even though most of these developments are connected to the treatment of haematological malignancies, NK cell therapy might also be of use in solid cancers.

In the distant past, in our institute, we performed a study on the effect of allogeneic HSCT in patients with solid malignancies, predominantly breast cancer[168]. Even though these studies had many shortcomings, such as heterogeneity of patient groups, small numbers, and a high rate of autologous recovery, it showed that some patients with metastatic breast cancer had disease response proving the potential for cellular therapies in this patient group. This graft-versus-tumour effect was confirmed by other investigators[169,170]. However, the effect was only temporary as most patients relapsed quickly and nowadays allogeneic HSCT, outside of a research setting, is not performed for solid malignancies. These studies, though, proved that cellular therapy is also feasible in patients with solid malignancies, and it might be worthwhile to develop strategies to improve the graft-versus-tumour effect.

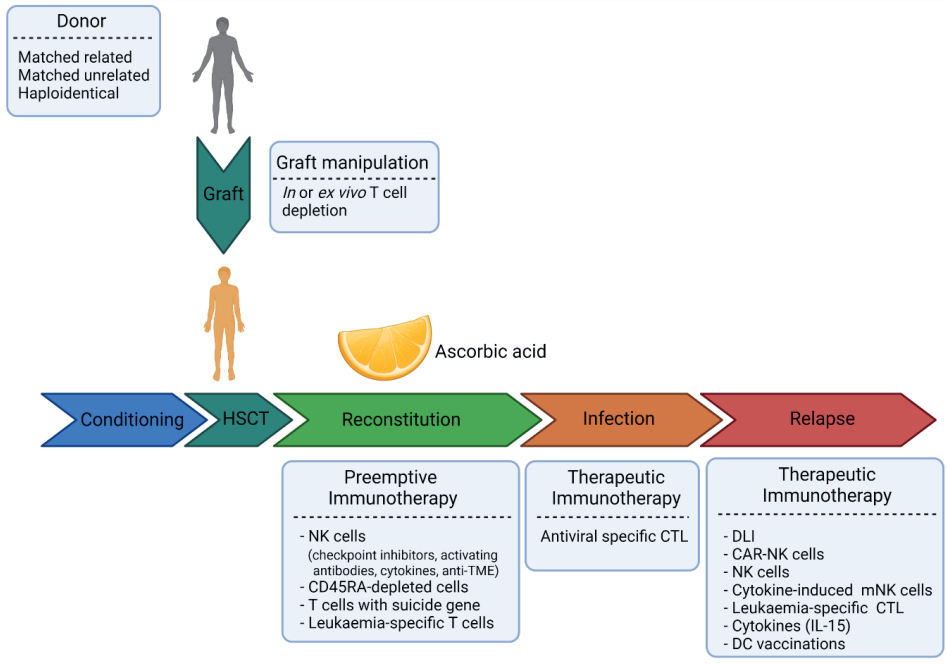
One option could be to enhance the NK cell function, just as it is investigated in haematological malignancies. In preclinical models, we and others have also shown cytotoxic activity of NK cells in solid cancers, like breast cancer and melanoma[171-173]. In addition, high density of tumour-infiltrating NK cells has been linked to a good prognosis in multiple solid tumours, proving their importance *in vivo*[174-177]. However, in the available clinical studies on NK cell therapy in solid tumours mostly no clear anti-tumour effect was showed. Nevertheless, there were promising results in children with neuroblastoma receiving allogeneic haploidentical NK cells after anti-GD2 therapy with an overall response rate of 29%[178]. It is thought that the lack of clinical responses in most solid tumours is related to the immunosuppressive TME, which inhibits the function of NK cells. Counteracting the immunosuppressive TME and engaging the NK cells with the tumour cells could also be beneficial in these solid tumours.

The division of cancer in solid or haematological malignancies in both the clinic and research feels illogical, as tumour development, molecular changes and treatment strategies are often closely connected. Perhaps in the future, the dividing line between oncologists and haematologists will fade away, as treatment strategies of solid and haematological cancers will become more aligned. In this way, haematologists could collaborate with oncologists in the treatment of solid cancer. They could initiate and support the use of more aggressive cellular treatments like HSCT, NK cell therapy or CAR T/NK cells in these patients.

In conclusion, various interesting options can be used to improve outcome after haploidentical HSCT. Important upcoming changes are to adapt GVHD prophylaxis, to improve GVL effect by NK or T cells and to enhance immune reconstitution by micronutrients like AA. **Figure 9.3** shows an overview of the possibilities to improve the outcome of HSCT as described in this general discussion.

The ultimate goal would be to have a treatment modality with very good anti-tumour characteristics that eliminates relapse, with a very good safety profile, accessible to most of the patients and suitable for many tumour types. I do not think this vision is reality yet with the existing possibilities, so for now the best strategy will be to combine different modalities and to adapt timing and treatment to the patient and the disease course. The most appealing course would be to use another post-transplant GVHD prophylactic *in vivo* regimen, as this is easier and cheaper than *in vitro* graft manipulation strategies, in combination with post-transplantation pre-emptive cellular therapy, like NK cell infusions in combination with either checkpoint inhibitors or blockers of the suppressive function of the TME. However, the exact ideal treatment regimen will depend on the biology of the specific tumour that is targeted, as some are more susceptible to cellular therapy than others. Patients should be observed closely so already at the first sign of relapse, new cellular therapy can be given.

Figure 9.3 Future treatment choices to further improve the outcome of HSCT.



HSCT: haematopoietic stem cell transplantation, NK: natural killer, TME: tumour microenvironment, CTL: cytotoxic T-lymphocytes, DLI: donor lymphocyte infusions, CAR: chimeric antigen receptor, mNK: memory natural killer, DC: dendritic cell.

There are multiple routes to improve outcome of HSCT. The use of haploidentical donors has improved the accessibility of HSCT for more patients, and it might be possible to influence the outcome further by the choice of specific donor characteristics. Graft manipulation can reduce the risk of relapse and increase the GVL effect and can lower the need for extra immunosuppressive medication post-transplantation. Ascorbic acid can improve immune reconstitution. There are several options to enhance the graft-versus-leukaemia effect by administering cellular products. Furthermore, NK cell function can be augmented by a broad range of medication. In the event of a serious viral infection post-transplantation, antiviral CTLs can induce viral clearance. In the case of relapse, or with minimal residual disease positivity, the donors can be used as a source of cells for additional cellular therapy options.

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10

Summary/Samenvatting

Summary of results

The aim of this thesis was to improve the outcome of patients after haematopoietic stem cell transplantation (HSCT), by the evaluation of alternative donors and optimisation of immune cell reconstitution after HSCT. One of the problems is donor availability, but our hope was that this could partly be solved by using haploidentical family donors that are widely available. Another major problem is the slow immune reconstitution after HSCT, which leads to infectious complications and relapses. Since we observed in previous studies that ascorbic acid (AA) is essential for the development of T cells and NK cells in vitro, and many of our patients had low serum AA concentrations, we aimed to explore the role of AA in boosting the immune system after stem cell transplantation.

Part 1 Haploidentical stem cell transplantation as alternative donor strategy

In **chapter 2, 3, 4** and **5** we assessed the role of the haploidentical family donor for allogeneic HSCT.

Chapter 2 describes the outcome allogeneic HSCT in chronic lymphatic leukaemia (CLL) when using a haploidentical donor. High-risk and relapsed CLL patients nowadays are mostly treated with novel agents, and outcomes have improved significantly over the last few years. However, when "fit" patients are refractory to one or more of these novel agents, allogeneic HSCT is a valuable option that can even be curative in these patients, who would otherwise have a dismal prognosis. We analysed the outcome of 117 patients, that received a haploidentical HSCT for CLL and whose data were available in the EBMT registry. Thirty-eight percent of these patients experienced long term (5 year) overall survival (OS), which makes a haploidentical donor a valid alternative in CLL patients if no HLA-identical donor is available. The outcome was not influenced by the use of post-transplantation cyclophosphamide (PTCy) as graft-versus-host disease (GVHD) prophylaxis. The most important risk factor for a poor prognosis was refractory disease at the time of the HSCT. In conclusion, this retrospective multicentre registry study shows that CLL patients, that have high-risk CLL and otherwise good risk transplantation characteristics, can be transplanted with a haploidentical donor, but preferably at the time of remission. However, at this time, because of the huge advances in CLL treatment and thereby highly increased overall

survival with the use of novel agents, allogeneic HSCT is hardly performed, and is only seen as last resort in fit and young patients that are refractory for novel agents.

In **chapter 3**, we evaluate the introduction of the haploidentical HSCT with PTCy as GVHD prophylaxis in our centre. Outcomes using this alternative donor type were similar to matched related donor (MRD) and matched unrelated donor (MUD) HSCT in our hands. However, non-relapse mortality (NRM) was high due to infectious complications. For this reason, we adapted our policy at the ward to prevent viral respiratory infections and added cotrimoxazole as prophylaxis against pneumocystis jivorecii pneumonia (PJP).

As we did not see any difference in clinical outcome between a MUD and haploidentical family donor, we investigated if costs and resource utilization could be an influence in donor choice in **chapter 4**. Costs pre-transplant for search and acquisition of the graft were significantly higher in MUD HSCT, but the costs in the transplant phase were highest in haploidentical HSCT due to longer hospitalisation, and subsequently use of more blood products, medication, and laboratory tests. This was due to slower immune reconstitution after PTCy. In total, there were no significant differences in costs between MUD and haploidentical HSCT.

Chapter 5 describes the outcome of a multicentre phase 2 clinical trial, in which we transplanted poor risk multiple myeloma (MM) patients using a killer cell immunoglobulin-like receptor (KIR)-ligand mismatched haploidentical donor. The aim was to investigate if natural killer (NK) alloreactivity could improve the anti-tumour response, as it was seen in vitro. Primary endpoint was progression-free survival (PFS) after 1.5 years, and stopping rules were installed in case interim results made a benefit unlikely. After the inclusion of 12 patients, the study was terminated prematurely, since all evaluable patients (9) relapsed within a median time of 90 days. However, we observed that haploidentical HSCT was safe in these patients, as there was a high engraftment rate, low NRM (18% at 1 year), and no unexpected adverse events.

Part 2 Ascorbic acid to boost the immune system after stem cell transplantation

In **chapter 6** and **7**, we developed a method for intracellular AA determination and reviewed the known effects of AA in cancer treatment to start further research to investigate the effect of AA on the immune system after stem cell transplantation.

In previous research, we discovered that serum AA levels were low in haematological malignancies. However, serum AA is not well correlated with the total body storage, while leukocyte AA levels do. Before starting a clinical trial to investigate the effect of AA supplementation on the immune system, we decided to develop a method to measure intracellular AA in various types of leucocytes. The validation of this method is described in **chapter 6**. The measurement of AA in peripheral blood mononuclear cells (PBMC's) and plasma was performed with hydrophilic Interaction Liquid Chromatography (HILIC) and UV detection and proved to be reliable and reproducible. As expected, there was no correlation between plasma and PBMC AA levels in healthy volunteers.

In **chapter 7**, we performed a systematic review regarding the effect of AA in the treatment of patients with cancer. A total of only 19 trials were included, as extensive research on this subject is lacking. Furthermore, there was a large heterogeneity in patient and disease characteristics, in interventions and in outcome measures. In only 4 of these studies, randomisation was used to determine treatment. Because of all these limitations, it was difficult to draw any definitive conclusions. Overall, we could not prove any clinically relevant effects of AA supplementation in cancer patients on OS, clinical status, quality of life and performance status. However, treatment with AA is likely to be safe since there were almost no serious adverse events and only mild side effects, even with very high doses of AA intravenously.

Chapter 8 contains the research protocol of a clinical intervention study in which we test the hypothesis that AA might improve immune reconstitution in autologous HSCT. The study is a double-blind randomized placebo-controlled trial in which patients that undergo an autologous HSCT for MM or lymphoma in the Maastricht University Medical Center (MUMC) are treated with either high dose AA or placebo for 6 weeks. Primary endpoint is the day of

repopulation (return of neutrophil level to at least 0.5 × 10^9/l) after autologous stem cell transplantation.

Samenvatting van de resultaten

Het doel van dit proefschrift was het verbeteren van de uitkomst van patiënten na hematopoëtische stamceltransplantatie (HSCT), door de introductie van alternatieve donoren en optimalisatie van de immuun-reconstitutie na HSCT. Een van de problemen is de beschikbaarheid van donoren, maar we hoopten dat dit deels zou kunnen worden opgelost door gebruik te maken van haplo-identieke familiedonoren die bijna altijd beschikbaar zijn. Een ander groot probleem is de langzame immuun-reconstitutie na HSCT, wat leidt tot infectieuze complicaties en recidieven. Omdat we in eerdere onderzoeken hebben waargenomen dat ascorbinezuur (AA) essentieel is voor de ontwikkeling van T-cellen en NK-cellen in vitro, en veel van onze patiënten lage serum-AA-concentraties hadden, wilden we de rol van AA bij het versterken van het immuunsysteem na HSCT onderzoeken.

Deel 1 Haplo-identieke stamceltransplantatie als alternatieve donorstrategie

In **hoofdstuk 2, 3, 4** en **5** hebben we de rol van de haplo-identieke familiedonor bij allogene HSCT onderzocht.

Hoofdstuk 2 beschrijft de uitkomst van allogene HSCT bij chronische lymfatische leukemie (CLL) bij gebruik van een haplo-identieke donor. CLL-patiënten met een hoog risico en recidief CLL worden tegenwoordig meestal behandeld met nieuwe middelen en de resultaten zijn daardoor de afgelopen jaren aanzienlijk verbeterd. Wanneer 'fitte' patiënten echter ongevoelig zijn voor een of meer van deze nieuwe middelen, is allogene HSCT een waardevolle optie die zelfs curatief kan zijn bij deze patiënten, die anders een sombere prognose zouden hebben. We analyseerden de uitkomst van 117 patiënten die een haplo-identieke HSCT voor CLL ontvingen en van wie de gegevens beschikbaar waren in het EBMT-register. Achtendertig procent van deze patiënten had een overleving (OS) op lange termijn (5 jaar), wat een haplo-identieke donor een acceptabele optie maakt voor CLL-patiënten als er geen HLA-identieke donor beschikbaar is. De uitkomst werd niet beïnvloed door het gebruik van post-transplantatie cyclofosfamide (PTCy) als profylaxe van graft-versus-host disease (GVHD). De belangrijkste risicofactor voor een slechte prognose was de refractaire ziekte ten tijde van de HSCT. Concluderend, deze retrospectieve multicentrische registratiestudie laat zien dat CLL-patiënten, die een hoog risico CLL hebben en anderszins goede risico

transplantatiekenmerken hebben, getransplanteerd kunnen worden met een haplo-identieke donor, maar bij voorkeur op het moment van remissie. Vanwege de enorme vooruitgang in de behandeling van CLL en daardoor de sterk verhoogde algehele overleving met het gebruik van nieuwe middelen, wordt allogene HSCT op dit moment echter nauwelijks uitgevoerd en wordt het alleen gezien als laatste redmiddel bij fitte en jonge patiënten die ongevoelig zijn voor nieuwe middelen.

In **hoofdstuk 3** evalueren we de introductie van de haplo-identieke HSCT met PTCy als GVHD-profylaxe in ons centrum. Uitkomsten met dit alternatieve donortype waren vergelijkbaar met gematchte gerelateerde donor (MRD) en gematchte niet-gerelateerde donor (MUD) HSCT in onze handen. De non-recidief mortaliteit (NRM) was echter hoog als gevolg van infectieuze complicaties. Daarom hebben we ons beleid op de afdeling om virale luchtweginfecties te voorkomen aangepast en cotrimoxazol toegevoegd als profylaxe tegen pneumocystis jivorecii pneumonie (PJP).

Omdat we geen verschil zagen in klinische uitkomst tussen een MUD en een haplo-identieke familiedonor, hebben we in **hoofdstuk 4** onderzocht of de kosten en het gebruik van middelen een invloed zouden kunnen hebben op de donorkeuze. De kosten vóór transplantatie voor het zoeken en verwerven van het transplantaat waren significant hoger in MUD HSCT, maar de kosten in de transplantatiefase waren het hoogst bij haplo-identieke HSCT vanwege langere ziekenhuisopnames, en vervolgens het gebruik van meer bloedproducten, medicatie en laboratoriumtests. Dit was te wijten aan een tragere immuunreconstitutie na PTCy. In het totale traject waren er geen significante verschillen in kosten tussen MUD en haplo-identieke HSCT.

Hoofdstuk 5 beschrijft de uitkomst van een multicenter fase 2 klinische studie, waarin we patiënten met een laag risico multipel myeloom (MM) transplanteerden met behulp van een killer cel immunoglobuline-like receptor (KIR)-ligand mismatched haplo-identieke donor. Het doel was om te onderzoeken of natural killer (NK) alloreactiviteit de antitumorrespons zou kunnen verbeteren, zoals in vitro werd waargenomen. Het primaire eindpunt was progressievrije overleving (PFS) na 1,5 jaar, en er werden stopregels ingesteld voor het geval tussentijdse resultaten een voordeel onwaarschijnlijk maakten. Na de inclusie van 12 patiënten werd de studie voortijdig beëindigd, aangezien alle evalueerbare patiënten (9) binnen een mediane tijd van

90 dagen recidiveerden. We hebben echter vastgesteld dat haplo-identieke HSCT veilig was bij deze patiënten, aangezien er een hoge engraftment was, een lage NRM (18% na 1 jaar) en geen onverwachte bijwerkingen.

Deel 2 Ascorbinezuur om het immuunsysteem te versterken na stamceltransplantatie

In **hoofdstuk 6** en **7** hebben we een methode ontwikkeld voor intracellulaire AA-bepaling en hebben we de bekende effecten van AA in de behandeling van kanker beoordeeld om verder onderzoek te starten naar het effect van AA op het immuunsysteem na stamceltransplantatie.

In eerder onderzoek ontdekten we dat de serum-AA-spiegels laag waren bij hematologische maligniteiten. Serum AA is echter niet goed gecorreleerd met de totale lichaamsopslag, terwijl leukocyten AA-concentraties dat wel doen. Voordat we een klinisch onderzoek startten om het effect van AA-suppletie op het immuunsysteem te onderzoeken, besloten we een methode te ontwikkelen om intracellulaire AA in verschillende soorten leukocyten te meten. De validatie van deze methode wordt beschreven in **hoofdstuk 6**. De meting van AA in perifeer bloed mononucleaire cellen (PBMC's) en plasma werd uitgevoerd met hydrofiele interactie-vloeistofchromatografie (HILIC) en UV-detectie, en bleek betrouwbaar en reproduceerbaar te zijn. Zoals verwacht was er geen correlatie tussen plasma- en PBMC AA-spiegels bij gezonde vrijwilligers.

In **hoofdstuk 7** hebben we een systematische review uitgevoerd met betrekking tot het effect van AA bij de behandeling van patiënten met kanker. In totaal zijn er slechts 19 studies geïncludeerd, omdat uitgebreid onderzoek over dit onderwerp ontbreekt. Verder was er een grote heterogeniteit in patiënt- en ziektekenmerken, in interventies en in uitkomstmaten. In slechts 4 van deze onderzoeken werd randomisatie gebruikt om de behandeling te bepalen. Door al deze beperkingen was het moeilijk om definitieve conclusies te trekken. Over het algemeen konden we geen klinisch relevante effecten aantonen van AA-suppletie bij kankerpatiënten op OS, klinische status, kwaliteit van leven en prestatiestatus. Behandeling met AA is echter waarschijnlijk veilig aangezien over het algemeen slechts milde bijwerkingen waren, zelfs bij zeer hoge intraveneuze doses AA.

Hoofdstuk 8 bevat het onderzoeksprotocol van een klinische interventiestudie waarin we de hypothese testen dat AA de immuun-reconstitutie in autologe HSCT zou kunnen verbeteren. De studie is een dubbelblinde, gerandomiseerde, placebogecontroleerde studie waarin patiënten die een autologe HSCT ondergaan voor MM of lymfoom in het Maastricht Universitair Medisch Centrum (MUMC) gedurende 6 weken worden behandeld met een hoge dosis AA of placebo. Primair eindpunt is de dag van repopulatie (terugkeer van neutrofielenspiegel tot ten minste $0,5 \times 10^9$/L) na autologe stamceltransplantatie.

11

Impact paragraph

Impact paragraph

In this thesis, we explored to role of a haploidentical stem cell donor and of ascorbic acid (AA) (=vitamin C) to improve the outcome of haematopoietic stem cell transplantation (HSCT). In this chapter, the relevance of the results described in this thesis and their scientific and social impact will be discussed.

Real-world data

In Europe, according to the European Society of Blood and Marrow Transplantation (EBMT), a total of 45,364 HSCT were performed in 2020 (autologous (=own) 26,568 and allogeneic (=donor) 18,796). The number one diagnosis for which an autologous HSCT is administered is multiple myeloma (47%), followed by non-Hodgkin's lymphoma (23%). In only 6% of the autologous HSCT the reason for the transplantation is a solid malignancy. The most common diagnosis in allogeneic HSCT was acute myeloid leukaemia (AML) (36%), followed by myelodysplastic syndrome (12%). These HSCT are performed by in total 690 centres.

In the Maastricht University Medical Center, in 2020 and 2021 we performed 105 and 106 autologous HSCT, respectively. Furthermore, we administered 43 allogeneic HSCT in 2020; 13 with HLA identical donor (MRD), 15 with haploidentical donor and 15 with matched unrelated donor (MUD). In 2021, we performed 41 allogeneic HSCT in total; 7 MRD, 21 haploidentical donor and 13 MUD. There has been a substantial increase in the number of haploidentical allogeneic HSCT we performed since the introduction of T cell replete haploidentical HSCT in 2016. We were also a strong advocate for the use of haploidentical donors in the rest of the Netherlands and the other centres followed our example[1]. With our research concerning the use of haploidentical donors, we proved systematically that it is a valid alternative donor option in various haematological diseases (acute myeloid leukaemia, chronic lymphatic leukaemia, multiple myeloma). Furthermore, haploidentical HSCT forms the perfect platform for other, newer forms of immunotherapy for which the potential anti-tumour effect is supported by biological mechanisms.

Outcome and costs analysis

HSCT is an intensive treatment for the patients and their family, and it also has a considerable impact on society as this treatment is very costly. This

holds especially true for allogeneic HSCT, which is only used with curative intent in fit patients. When performing these high-risk and expensive treatments it is important to critically review the quality of your treatment by evaluating your own results. This is part of what we did in this thesis. We discovered some shortcomings in our own protocol and by this analysis we could adapt our regimens to improve outcomes for our patients. This increased the cost-effectiveness of the HSCT in our centre. Moreover, we showed that in the real world it is not always possible to generate similar outcomes as published results when adopting a new regimen. Thereby, treatment should be adapted to local protocols. Furthermore, we analysed costs of the different treatment steps for different donor types. In this way we could explore if there were certain choices in treatment regimens to make, which would not influence outcome but could perhaps decrease costs.

Improvement of immune reconstitution with ascorbic acid

Several strategies are used to assist the immune recovery and to prevent infectious complications and relapses after HSCT, for instance by the use of granulocyte-colony stimulating factors (G-CSF) and donor lymphocyte infusions. Most of these treatments are expensive, and furthermore, medication and hospitalisation for these complications are costly. Moreover, infectious complications have a substantial impact on the patients, and can even be fatal.

Interestingly patients that receive a HSCT often have deficient levels of AA in their blood, and immune cells need AA to divide and develop. We argued that it could be beneficial to use AA for boosting the immune system after HSCT. This is a very simple and cheap option that could make a difference in outcome for transplanted patients, who are very susceptible for infections as their immune system is not working well shortly after transplantation. These infections are sometimes even fatal in this patient group. Furthermore, in allogeneic HSCT, the immune system is needed to fight against cancer cells. Boosting the immune system could lead to better disease control and less relapses. Supplementation of AA could improve cost-effectivity in HSCT.

Scientific impact

Before we performed our study on the use of haploidentical HSCT in chronic lymphatic leukaemia patients, it was not known if this type of donor was

suitable for this patient group. We discovered that it is a valid option, and this knowledge helps other haematologists making decisions for their patients.

We are optimistic that the use of AA can be beneficial in HSCT, but do not have the results of our randomised clinical trial (RCT) in autologous HSCT yet. There is a lack of well-designed and reliable RCT's on the effects of AA in cancer, so the knowledge gathered in this study will always be useful to build on for other clinicians in this field. In 1976 an article appeared co-authored by Nobel Prize winner Linus Pauling. It described a clinical benefit of intravenous AA in terminally ill cancer patients[2]. This study was strongly criticized because of methodological flaws and the results could not be repeated in other studies in which oral supplementation was used[3,4]. Even after this major setback, many studies regarding the effect of AA in cancer patients have been poorly designed. Most of the time there was a lack of supporting preclinical evidence for the specific role of AA in a certain setting. There was also a lot of heterogeneity in treated patients. The persistence of these qualitatively disappointing clinical studies surrounding AA in cancer undermines the real value of this nutrient and weakens efforts to fund additional well-designed RCTs. AA status and the effect of supplementation in these studies was most of the time followed in plasma, which correlates poorly with tissue AA[5]. In this thesis, we describe a method we developed to determine the AA concentration in lymphocytes that does correlate with tissue AA and that can be used in clinical studies. Currently, we are using this method to investigate the effect of tissue levels of AA in patients admitted in our hospital with a COVID-19 infection. In our RCT on the effect of AA supplementation, we examine a specific patient group treated with autologous HSCT for either lymphoma or myeloma, and the effects of AA on the immune system is already proven in preclinical research and can be explained by biological mechanisms.

All the results described in this thesis have been published in international peer review journals with open access. In this way, other researchers and haematologists can benefit from our findings without any limitations.

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ADDENDUM

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A

Curriculum vitae

Gwendolyn Nathanaël Yvette van Gorkom was born on June 18th, 1980, in Breda, the Netherlands. In 1998 she graduated from pre-university education at the Stedelijk Gymnasium in Breda. Afterwards she started medical school at the University of Utrecht and obtained her medical degree in 2004. Subsequently, she worked as a resident at the internal medicine department of Sint Elisabeth Hospital in Tilburg. In 2007, she started her training to become a specialist in internal medicine at the same hospital, under supervision of dr. P.L. Rensma. In 2011, Gwendolyn began her fellowship haematology under supervision of prof. dr. H.J. Schouten in the Maastricht University Medical Center, which she finished in 2013. In 2014, she worked as a haematologist at Medisch Spectrum Twente in Enschede. However, in 2015, Gwendolyn returned to Maastricht and started her work there as a haematologist, a position she still currently holds. In addition to patient care and teaching, she started her research concerning ascorbic acid and stem cell transplantation under supervision of prof. dr. G.M.J. Bos, from which this thesis is a result. In 2019, she became clinical program director of the stem cell transplantation program of the Maastricht University Medical Center. She lives in Maastricht with her husband Nicolas, and their three children Thomas, Aurélie, and Alexis.

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List of abbreviations

AA	Ascorbic acid
ADCC	Antibody-dependent cellular cytotoxicity
AE	Adverse event
aGVHD	Acute GVHD
ALL	Acute lymphatic leukaemia
AML	Acute myeloid leukaemia
APTT	Activated partial thromboplastin time
ASCT	Autologous stem cell transplantation
ATG	Anti thymocyte globulin
BEAM	BCNU, etoposide, cytarabine, melphalan
BM	Bone marrow
BMT	Bone marrow transplantation
BTK	Bruton's tyrosine kinase
Bu	Busulphan
CAR	Chimeric antigen receptor
cGVHD	Chronic graft-versus-host disease
CI	Confidence interval
CLL	Chronic lymphatic leukaemia
CNI	Calcineurin inhibitor
CR	Complete remission
CRF	Case report form
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CV	Coefficient of variation
CVC	Central venous catheter
Cy	Cyclophosphamide
CyA	Cyclosporine
CMV	Cytomegalovirus
CTL	Cytotoxic T cell
DC	Dendritic cells
DLI	Donor lymphocyte infusions
DN	Double negative
DP	Double positive
DRI	Disease risk index
DSA	Donor-specific antibodies
EBMT	European society for Blood and Marrow Transplantation
eGFR	Estimated glomerular filtration rate
EMA	European Medicine Agency
EORTC	European organization for research and treatment of cancer
EPD	Electronic patient documents
EPHPP	Effective Public Health Practice Project
G6PD	Glucose-6-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GFRS	GVHD-free relapse-free survival
GIAC	G-CSF priming; intensified immune-suppression; ATG
GRFS	GVHD-free relapse-free survival

GULO	Gulonolactone oxidase
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukaemia
Gy	Gray
Flu	Fludarabine
haploBMT	Haploidentical bone marrow transplantation
HCT-CI	Haematopoietic stem cell transplantation- Comorbidity Index
HHV-6	Human herpes virus-6
HILIC	Hydrophilic interaction liquid chromatography
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
HR	Hazard ratio
HRQoL	Health related quality of life
HSCT	Haematopoietic stem cell transplantation
ICU	Intensive care unit
IS	Immunosuppression
iTreg	Induced regulatory T cell
IVC	Intravenous vitamin C
KIR	Killer-cell immunoglobulin-like receptor
LAG	Lymphocyte activation gene
LLOQ	Lower limit of quantification
LoB	Limit of blank
LoD	Limit of detection
LSC	Leukaemia stem cells
MAC	Myeloablative conditioning
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility Complex
MM	Multiple myeloma
MMF	Mycophenolate mofetil
MMUD	Mismatched unrelated donors
mNK	Memory natural killer
MPN	Myeloproliferative neoplasia
MRD	Matched related donor
MRD-negativity	Minimal residual disease-negativity
MUD	Matched unrelated donors
MUMC	Maastricht university medical center
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
NMA	Non-myeloablative
NR	Not reported
NRM	Non-relapse mortality
OS	Overall survival
PAI-1	Plasminogen activator inhibitor-1
PARP1	Poly-ADP-ribose polymerase-1
PB	Peripheral blood
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PBSC	Peripheral blood stem cells

PD	Progressive disease
PFS	Progression-free survival
PJP	Pneumocystis jirovecii pneumonia
PR	Partial remission
PS	Performance status
PSA	Prostate-specific antigen
PT	Prothrombin time
PTCy	Post-transplantation cyclophosphamide
QC	Quality control
QCQ	Quality of Life Core Questionnaire
QOL	Quality of life
R/R	Relapsed/refractory
RCT	Randomised controlled trial
RIC	Reduced intensity conditioning
ROS	Reactive oxygen species
ROTEM	Rotational thromboelastometry
RP HPLC	Reversed phase high-performance liquid chromatography
SAE	Serious adverse event
SCT	Stem cell transplantation
SD	Stable disease
SIRP	Signal regulatory protein
SUSAR	Suspected unexpected serious adverse reaction
SVCT	Sodium-ascorbate co-transporters
TAFI	Thrombin activatable fibrinolysis inhibitor
TBI	Total body irradiation
TBF	Thiotepa, busuphan, fludarabine
TCD	T cell depleted
TCR	T cell receptor
TET	Ten-eleven-translocation
TEG	Thromboelastography
TGFβ	Transforming growth factor beta
Th	T helper cell
TI	Total imprecision
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM	T cell immunoglobulin and mucin domain
TME	Tumour microenvironment
tPA	Tissue plasminogen activator
Treg	Regulatory T cell
UCB	Umbilical cord blood
UCBT	Umbilical cord blood transplantation
VGPR	Very good partial response
VWF	Von Willebrand factor
WT1	Wilms tumour antigen 1