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Stem Cell Transplantation Unit

NON-TRANSFERRIN-BOUND IRON
IN HEMATOPOIETIC STEM CELL
TRANSPLANTATION

Leila Sahlstedt

Academic Dissertation
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My love, my friend, my husband
CONTENTS

LIST OF ORIGINAL PUBLICATIONS ............................................................................. 7
ABBREVIATIONS ........................................................................................................ 8
ABSTRACT ................................................................................................................ 10
1. INTRODUCTION .................................................................................................... 12
2. REVIEW OF THE LITERATURE ............................................................................. 14
   2.1. Hematopoietic stem cell transplantation (HSCT) .............................................. 14
      2.1.1. Conditioning .............................................................................................. 14
      2.1.2. Donor in allogeneic transplantation ....................................................... 15
      2.1.3. Graft ........................................................................................................... 16
      2.1.4. Complications of HSCT ........................................................................... 17
   2.2. Iron metabolism ............................................................................................... 20
      2.2.1. Iron in the human body ............................................................................ 20
      2.2.2. Mechanisms of iron regulation ............................................................... 21
   2.3. Iron overload ................................................................................................... 23
      2.3.1. Red blood cell (RBC) transfusions ......................................................... 23
      2.3.2. Evaluation of iron overload ...................................................................... 24
         2.3.2.1. RBC units ............................................................................................ 24
         2.3.2.2. Ferritin ............................................................................................... 24
         2.3.2.3. Liver iron content (LIC) .................................................................... 24
         2.3.2.4. Magnetic imaging ............................................................................. 25
   2.4. Iron toxicity ...................................................................................................... 25
      2.4.1. Iron and infections .................................................................................... 26
      2.4.2. Non-transferrin-bound iron (NTBI) ......................................................... 27
      2.4.3. Sources and status of NTBI in the circulation ........................................ 29
3. IRON AND HSCT ................................................................................................... 30
   3.1. Causes and impact of iron overload in HSCT .................................................. 30
      3.1.1. Iron and hepatic complications ............................................................... 33
      3.1.2. Iron and graft-versus-host disease ......................................................... 33
      3.1.3. Iron and infections ................................................................................... 34
      3.1.4. Iron and other transplant-related complications .................................... 35
   3.2. Treatment of iron overload .............................................................................. 35
4. TRANSFERRIN AND APOTRANSFERRIN ............................................................. 36
   4.1. Biochemistry .................................................................................................. 38
   4.2. Manufacture .................................................................................................... 39
   4.3. Characterization ............................................................................................. 41
   4.4. Potential clinical applications ....................................................................... 42
5. AIMS OF THE STUDY ........................................................................................... 44
6. PATIENTS, MATERIALS AND METHODS ........................................................... 45
   6.1. Patients ............................................................................................................ 45
      6.1.1. Patients treated with allogeneic HSCT (I) ............................................... 45
      6.1.2. Patients treated with autologous HSCT (II) ............................................. 45
      6.1.3. Patients treated with cytotoxic chemotherapy (II) ................................. 46
      6.1.4. Patients given a single apotransferrin dose (III) ..................................... 46
      6.1.5. Patients given repeated doses of apotransferrin in a dose-finding study (IV) ........................................................................................................ 46
      6.1.6. Patients given repeated doses of apotransferrin in a high-dose setting (IV) ........................................................................................................ 47
   6.2. Bone marrow samples for cell cultures (V) ..................................................... 47
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I Sahlstedt L, Ebeling F, von Bonsdorff L, Parkkinen J, Ruutu T

II Sahlstedt L, von Bonsdorff L, Ebeling F, Parkkinen J, Juvonen E, Ruutu T

III Sahlstedt L, von Bonsdorff L, Ebeling F, Ruutu T, Parkkinen J

IV Parkkinen J, Sahlstedt L, von Bonsdorff L, Salo H, Ebeling F, Ruutu T

V Juvonen E, Sahlstedt L, Parkkinen J, Ruutu T

In publication III, the two first authors contributed equally, and the publication has also been used as a part of the thesis of Leni von Bonsdorff.
ABBREVIATIONS:

aHSCT autologous hematopoietic stem cell transplantation
ALL acute lymphoblastic leukemia
AML acute myeloid leukemia
Asn asparagine
ATG anti-thymocyte globulin
BDI bleomycin-detectable iron
BEAC carmustine, etoposide, cytarabine, cyclophosphamide
BFU-E erythroid burst-forming unit
BHK baby hamster kidney
BPS bathophenantroline disulfonate
CFU-GM granulocyte-macrophage colony-forming unit
CI confidence interval
CML chronic myeloid leukemia
CMV cytomegalovirus
CPV canine parvovirus
Cy cyclophosphamide
DCI deferoxamine chelatable iron
EBV Epstein-Barr virus
EDTA ethylenediamine tetraacetic acid
EPO erythropoietin
Fe iron
FeNTA ferric nitrilotriacetic acid
G-CSF granulocyte colony-stimulating factor
GDF growth differentiation factor
GMP good manufacturing practice
GVHD graft-versus-host disease
HAV hepatitis A virus
H-ferritin heavy chain subunit
HLA human leukocyte antigen
HPLC high-performance liquid chromatography
HSCT hematopoietic stem cell transplantation
i.v. intravenous
I/R ischemia/reperfusion
Ig immunoglobulin
IL interleukin
IMDM Iscove’s Modified Dulbecco’s Medium
IPS idiopathic pneumonitis syndrome
L-ferritin light chain subunit
LIC liver iron content
MALDI-TOF matrix assisted laser desorption ionization-time of flight
MDCI mobilizer dependent chelatable iron
MDS myelodysplastic syndrome
MNC mononuclear cell
MRI magnetic resonance imaging
mTOR mechanistic target of rapamycin
NHL non-Hodgkin lymphoma
NK natural killer
NTA nitrilotriacetic acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>NTBI</td>
<td>non-transferrin-bound iron</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>PBSC</td>
<td>peripheral blood stem cell</td>
</tr>
<tr>
<td>RA</td>
<td>refractory anemia</td>
</tr>
<tr>
<td>RAEB</td>
<td>refractory anemia with excess blasts</td>
</tr>
<tr>
<td>RAEBt</td>
<td>refractory anemia with excess blasts in transformation</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RIC</td>
<td>reduced intensity conditioning</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCT</td>
<td>stem cell transplantation</td>
</tr>
<tr>
<td>SOS</td>
<td>sinusoidal obstruction syndrome</td>
</tr>
<tr>
<td>SQUID</td>
<td>superconducting quantum interference device</td>
</tr>
<tr>
<td>TAM</td>
<td>transplantation-associated microangiopathy</td>
</tr>
<tr>
<td>TBI</td>
<td>total body irradiation</td>
</tr>
<tr>
<td>Tf</td>
<td>transferrin</td>
</tr>
<tr>
<td>TWSG1</td>
<td>twisted-gastrulation 1</td>
</tr>
<tr>
<td>VOD</td>
<td>veno-occlusive disease</td>
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ABSTRACT

Hematopoietic stem cell transplantation (HSCT) is used in treating many diseases, mainly malignant hematological disorders. It is an intensive treatment often complicated by organ injuries. Non-transferrin-bound iron (NTBI), as an inducer of free oxygen radicals, is a potential factor in the pathogenesis of these complications. We studied the appearance and timing of NTBI in transplant patients and the possibility to prevent the occurrence of NTBI by binding it with apotransferrin administration.

Ten adult patients with hematological malignancy undergoing allogeneic HSCT with myeloablative conditioning were enrolled to study the timing of the changes in the serum levels of iron parameters in the peritransplant period. None of the patients were NTBI-positive at baseline. The bleomycin-detectable iron (BDI) -assay was used to assess NTBI. During the first two days after starting the conditioning the mean total iron concentration doubled from an initial level to a peak on day -4 before HSCT (day 0). The transferrin levels decreased continuously and fully saturated transferrin was observed by day -4, simultaneously with the occurrence of NTBI. The mean transferrin saturation remained above 80% thereafter. NTBI was detectable in the majority of patients until day +11, remaining present in half of the patients through the study period to day +14. Transferrin saturation level of 80% was found to be a significant threshold for the appearance of NTBI.

The timing and length of the appearance of NTBI were also studied in 16 patients who underwent autologous stem cell transplantation. The transferrin saturation levels were within the normal range at baseline. After a steep rise within the first days after the initiation of conditioning the maximum saturation levels were reached approximately on day 4-6 from the start of treatment. The levels started to descend one week after the beginning of each regimen. None of the patients were NTBI-positive at baseline. In all but three of the patients NTBI was detected variably during the transplantation process. The mean time from the first to the last NTBI-positive sample was 6.1 days. The disappearance or marked reduction of the NTBI-positive samples coincided with the recovery of the bone marrow function demonstrated by the rise of the leukocyte counts.

Eight patients with acute leukemia treated with conventional chemotherapy were also studied for the timing of the changes in iron parameters during the peritreatment period. The mean transferrin and transferrin saturation levels were below the reference range at the baseline, and the transferrin level remained subnormal thereafter. The transferrin saturation showed a steep rise above 90% by day 3 from the start of chemotherapy, and remained above 80% through the study period. NTBI was
detected already at baseline in nearly 40% of the samples. NTBI could be demonstrated in all patients during the study period of 20 days. The mean duration of NTBI-positivity was 15.6 days.

Six consecutive adult patients with acute leukemia, treated with myeloablative conditioning for allogeneic HSCT received a single dose of apotransferrin on day +3 after the transplantation. After the apotransferrin injection NTBI disappeared from the sera of all patients. NTBI reappeared 12-24 hours after the apotransferrin injection in 4 patients and after 6 days in one, one patient remained NTBI negative through the 12-day follow-up period.

Twenty allogeneic transplant patients were enrolled to receive repeated doses of apotransferrin. The ten patients in our first study who underwent HSCT but were not given apotransferrin were used as controls. Twelve patients were randomized to receive the study drug at two dose levels. A low-dose (340 mg/kg) group (n= 6) received a single loading dose (100 mg/kg) at the start of conditioning therapy on day -6 before HSCT, followed by nine maintenance doses (26 mg/kg) every other day. The medium-dose (610 mg/kg) group (n= 6) received four loading doses (100 mg/kg) on four consecutive days, starting on day -6, followed by eight maintenance doses (26 mg/kg) every other day. In the second part, the high-dose (1040 mg/kg) study, eight patients received a higher dose (115 mg/kg) given as four daily doses, from day -6 onwards, continuing every second day until day +7 after HSCT. In this group five of the eight patients had transferrin saturation < 80% and were NTBI negative through the study period. With lower doses there was a nonsignificant trend towards fewer days with demonstrable NTBI compared to the controls. Repeated doses of apotransferrin were followed by a steep rise in serum iron in a dose-dependent way.

In an in vitro-study we investigated the effect of NTBI on erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) colony formation and the effect of binding NTBI with apotransferrin. Hematopoietic progenitors were cultured with different concentrations of NTBI in the form of ferric nitrilotriacetic acid (FeNTA). Parallel cultures were performed after the preincubation of FeNTA with apotransferrin. NTBI inhibited colony formation and reduced the size of the colonies. Preincubation of FeNTA with apotransferrin diminished the inhibitory effect of FeNTA and increased both the number and the size of colonies.

In conclusion, we showed that NTBI appears regularly during the peritransplant period in HSCT. NTBI has been shown to cause cellular and organ toxicity, and thus it may contribute to the complications of HSCT. Therefore we assessed the possibility to bind NTBI during the peritransplant period with apotransferrin administration. The appearance of NTBI could be prevented or reduced by apotransferrin. The clinical utility of apotransferrin administration and its effects on transplantation outcomes remain to be investigated in further studies.
1. INTRODUCTION

Iron is an essential component of the human body. It is one of the most efficiently recycled agents. The largest proportions of iron are found as functional iron in hemoglobin and myoglobin, and it is also an important component of many enzymes. A substantial amount of iron is stored as ferritin and hemosiderin. Although essential to nearly all cells, iron can also be toxic due to its propensity to form oxygen radicals that damage cells.

Quantitative deviations of total body iron lead very often to morbidity. Iron deficiency anemia is the most common type of anemia. In disorders with ineffective erythropoiesis, e.g. thalassemias and congenital dyserythropoietic anemias (Ganz and Nemeth 2012b), or after repeated transfusions, excessive amounts of iron accumulate in the organism and may cause or contribute to organ damage. Iron uptake is strictly regulated, but there is no mechanism to actively export iron from the body; usage of iron is almost a closed circuit. Iron is passively exported out of the body only by shedding of intestinal epithelium or menstrual bleeding.

Hematopoietic stem cell transplantation is an intensive treatment, which may interfere with iron homeostasis in many ways. The underlying disease to be treated with HSCT can cause anemia, or sometimes iron overload. Many transplant patients have previously received substantial numbers of blood transfusions. Intensive cytotoxic treatment, conditioning, before the transplantation causes cessation of red cell production and reduction in the utilization of iron. Conditioning can also reduce the production of the main iron-carrying protein, transferrin, in the liver, and injury of liver cells may cause release of storage iron. Some patients need blood transfusions for a prolonged period after the transplantation.

The impact of iron overload in stem cell transplantation has been unclear, and there are still many open questions. Until recent years there were no effective and in practice feasible ways to treat iron overload if phlebotomy could not be applied because of anemia; this is the case in many transplant patients. For this reason clinical investigation on the impact of transplant-associated iron overload has not been very active until in recent years, when the situation has changed essentially.
In the 1990s it had become apparent in our own experience and in publications from elsewhere (Or et al 1987, Antila et al 1992, Angelucci et al 1997) that the interpretation of laboratory tests for iron load, especially when ferritin was used as a surrogate, was often problematic. Excessively high ferritin levels were sometimes seen in patients with only a limited number of red cell transfusions in the history, and the significance of such findings as an indicator of iron load was uncertain. The question of non-transferrin-bound iron and its possible role in transplantation complications gained increasing interest. At this time the Finnish Red Cross Blood Transfusion Service had developed a human apotransferrin concentrate that could be used clinically. This offered potential means to eliminate or reduce free iron in the process of stem cell transplantation and thereby diminish the risk of reactive oxygen radicals causing organ injury. Accordingly, we decided to evaluate the appearance of free iron in stem cell transplant patients and the possibility to bind it with exogenous apotransferrin.
2. REVIEW OF THE LITERATURE

2.1. Hematopoietic stem cell transplantation (HSCT)

HSCT is used to replace blood-forming tissue damaged by a blood disease or intensive cytotoxic treatment. In transplantations from another person, the effect of the donor’s immunological cells on the malignant cells, the so called graft vs. leukemia or graft vs. malignancy effect, is also utilized. The treatment of a malignant blood disorder is the most common indication for hematopoietic stem cell transplantation, but some non-hematological malignancies, as well as non-malignant blood diseases and some rare inherited immunological and metabolic disorders, have also been treated with HSCT.

In allogeneic transplantation the donor is another person, in syngeneic transplantation an identical twin. In autologous transplantation the graft has been earlier harvested from the patient him/herself.

2.1.1. Conditioning

Before the transplantation, the patient is given a preparative or conditioning regimen which consists of cytotoxic drugs and often also includes radiotherapy. The aim with the conditioning is to destroy the malignant or abnormal cells, and, in allogeneic transplantation, also to depress the immunological system of the patient to prevent rejection. In conventional full-intensity, myeloablative, transplantations the conditioning regimen has most commonly consisted of total body irradiation (TBI) or high-dose busulphan combined with large doses of cyclophosphamide although there have been many variants. In the recent years, the application of reduced intensity (RIC) or reduced toxicity conditioning has markedly widened the indications of allogeneic stem cell transplantation and the number of patients who can benefit from this treatment modality (Ljungman et al 2010). There is a wide spectrum of such regimens in use (Gratwohl and Carreras 2012).
In autologous transplantations for lymphoma, the conditioning usually consists of high doses of several cytotoxic drugs, for example carmustine, etoposide, cytarabine, cyclophosphamide (BEAC). The conditioning in autologous transplantations for myeloma is usually based on high-dose melphalan.

2.1.2. Donor in allogeneic transplantation

A prerequisite for the prevention of graft rejection is a good match of tissue antigens between the donor and the patient. A human leukocyte antigen (HLA)-identical sibling is usually an optimal donor. If such a donor is not available, an unrelated donor identical in HLA-A, -B, -C, -DRB1 and DQB1 antigens is searched for (Petersdorf et al 2007, Nowak 2008). Often a difference between the recipient and the related or unrelated donor in one of the ten antigens (alleles) of these five antigen pairs can be acceptable. An identity of DPB1 antigen is desirable. Generally, the better the match, the better the outcome, although this issue has become increasingly complicated with the development of tissue typing methods and the understanding of the significance of the findings. Until recent years, a majority of the donors have been HLA-identical siblings. In Finland a good sibling donor can be found for approximately 25% of the patients.

A donor can also be searched for in registries of voluntary unrelated donors. As the number of possible tissue antigen combinations is huge, the number of donor candidates has to be very large to permit a significant chance of finding a donor. There are donor registries in numerous countries, and the total number of donor candidates in these registries is about 25 million (Bone Marrow Donors Worldwide 2015). In international collaboration, a donor search for a patient can be carried out in most of these registries. As a result, a suitable donor can at present be found for a large majority of patients. If there is a choice, a sibling donor is in most cases still to be preferred, but with the development of tissue typing and increasing knowledge of the importance of tissue type match, the outcomes of transplantations from unrelated donors are now essentially similar to those from a sibling donor (Gluckman 2012).

Haploidentical transplantations, usually from a non-HLA-identical family member, are at present a topic of keen interest and clinical evaluation (Bashey et al 2013, Di Stasi et al 2014,
Martelli *et al* 2014). Strategies to minimize the adverse effects of tissue type mismatch in this kind of transplantations have been developed, and the results look promising. The next few years will show the role of haploidentical transplantations in the management of patients.

2.1.3. Graft

In earlier years the source of hematopoietic stem cells for transplantation was always bone marrow, usually harvested in general anesthesia from the iliac crests and sometimes additionally from the sternum. The total volume harvested for an adult patient is usually in the order of 700-800 ml, depending on the size of the recipient and the cell count of the graft. If the ABO blood groups of the donor and the recipient differ leading to an incompatible blood transfusion, red blood cells and plasma are removed from the graft.

At the present time, a majority of grafts are collected from peripheral blood (Gratwohl *et al* 2013). In steady state, there are very few hematopoietic stem cells in the blood and thus harvesting a sufficient graft is not in practice possible. However, with the administration of a hematopoietic growth factor, usually granulocyte growth factor G-CSF, enough stem cells can be mobilized from the marrow to allow a successful stem cell harvest from the blood. A cell fraction containing the hematopoietic stem cells is collected using a cell separator. Advantages for the donor with the use of blood stem cell grafts are that no anesthesia is needed and repeated bone marrow aspirates can be avoided. The recipient’s blood cell counts recover somewhat faster than after bone marrow transplantation, but the risk of graft-versus-host disease (GVHD), particularly chronic GVHD is higher when a blood stem cell graft is used (Apperley and Masszi 2012).

Umbilical cord blood contains large numbers of stem cells, and therefore it can be used as a stem cell graft (Gluckman 2012). An advantage of this method is that the criteria for tissue antigen match are less stringent than with bone marrow or peripheral blood stem cell grafts. The volume and thereby the number of stem cells in a cord blood graft is relatively small, and therefore cord blood grafts have been earlier mainly used in pediatric transplantations. However, with the use of two cord blood units the stem cell numbers are usually sufficient for transplantations in adults, and the numbers of such transplantations have markedly increased until recent years (Passweg *et al* 2012, Passweg *et al* 2015).
Autologous stem cells are usually collected from peripheral blood of the patients at a suitable time according to the treatment regimen. Bone marrow harvest may be used if peripheral collection is not successful or feasible.

2.1.4. Complications of HSCT

HSCT is an intensive treatment, which can lead to many complications. The principal mechanisms are infections, toxic effects caused by conditioning, and immunology (Figure 1). In autologous HSCT the complications are mainly based on toxic effects and infections.

**Figure 1.** Complications after allogeneic HSCT, typical timing.

CMV = cytomegalovirus, EBV = Epstein-Barr virus, VOD = veno-occlusive disease, TAM = transplantation-associated microangiopathy.
Rejection of the graft is uncommon when malignant blood diseases are treated using conventional fully myeloablative conditioning. If the donor is an HLA-matched sibling, the risk of rejection is in the order of two per cent (Anasetti et al 1989). If the donor is a non-HLA matched relative or unrelated, the risk is greater. If conditioning with strongly reduced intensity is used, the risk is higher than in conventional transplantation, depending on the details of the conditioning (Olsson et al 2013).

The central problem of allogeneic transplantation is GVHD (Apperley and Masszi 2012). The graft contains abundantly T-lymphocytes and natural killer (NK)-cells which can induce a reaction against the tissues of the recipient. The onset of acute GVHD is usually during the first few weeks after the transplantation. Its most typical features are rash, diarrhea, abdominal pain, and liver injury manifested as an increase in the serum levels of bilirubin and transaminases. There are other less common manifestations including lung complications. Also endothelium has been regarded as a target of acute GVHD; this links GVHD to microangiopathic complications which are a relatively common cause of morbidity and mortality after allogeneic transplantation (Tichelli and Gratwohl 2008). The incidence of acute GVHD is approximately 20-40% if the donor is a matching sibling, and higher with other types of donor (Ruutu et al 2000, Apperley and Masszi 2012).

In contrast to acute GVHD, the manifestations of chronic GVHD often resemble those of autoimmune diseases, but little is known about the pathophysiology. Chronic GVHD appears usually after several months from the transplantation (Lee and Flowers 2008). Most frequent manifestations are skin dryness and sclerodermatic skin, dry mouth and mucositis, and dry eyes. Other manifestations include liver injury, mainly affecting small bile ducts, and malabsorption, as well as restrictive and particularly obstructive lung problems, the latter manifesting as obstructive bronchiolitis. Long-term atherosclerotic cardiovascular problems bear a connection to chronic GVHD (Tichelli and Gratwohl 2008).

Approximately half of allogeneic transplant patients develop chronic GVHD, depending on the details of the transplantation procedure. For example T-cell depletion and the usage of anti-thymocyte globulin (ATG) decrease the risk of GVHD. On the other hand, higher degree of HLA-mismatch or unrelated donor, as well as peripheral blood stem cell (PBSC)-graft increase the risk of GVHD (Mohty et al 2003, Apperley and Masszi 2012, Baron et al 2012).
Prophylaxis and treatment of GVHD consist of immunosuppressive drugs (Ruutu et al. 2014). The most important drugs are calcineurin inhibitors, methotrexate, mycophenolate mofetil, corticosteroids and ATG. Other forms of treatment used are various monoclonal antibodies, mTOR inhibitors, extracorporeal photopheresis and mesenchymal stem cells. Although a large proportion of patients respond to treatment, GVHD is still, together with infections, the most important cause of mortality in allogeneic transplantation (Apperley and Masszi 2012).

Infections are an important cause of morbidity and mortality (Rovira et al. 2012). The cytotoxic conditioning and the immunological effects of the graft destroy the immunological system of the recipient. The rebuilding of the immunological system by cells from the graft takes several months; the immunological competence has usually mainly recovered by one year after the transplantation (Fujimaki et al. 2001, Seggewiss and Einsele 2010). Chronic GVHD delays the recovery (Rovira et al. 2012). Susceptibility to infections is most marked during the first months after the transplantation. Both bacteria, viruses and fungi can cause severe infections. The risk of pneumonia caused by *Pneumocystis jiroveci* is high but can be prevented with prophylaxis. Of viruses, the most important causative agent is cytomegalovirus. Epstein-Barr virus is also a frequent problem.

Allogeneic transplant patients need intensive follow-up and treatment during the first months and up to a year after the transplantation, but after the recovery of the immunological system all prophylactic treatment can often be discontinued.

Other important complications of allogeneic stem cell transplantation are veno-occlusive disease of the liver (VOD, also called sinusoidal obstruction syndrome, SOS) and transplantation-associated microangiopathy (TAM) (Ruutu et al. 2007, Coppell et al. 2010, Carreras 2012). In both the pathogenesis is based on injury of small blood vessels. Other less common syndromes belonging to the same group are diffuse alveolar hemorrhage and general capillary leak syndrome (Carreras 2012). In VOD, injury of endothelium in sinusoidal vessels of the liver leads to obstruction of the sinusoids manifesting as enlarging liver with right upper abdomen pain, icterus, fluid retention, and ascites. TAM manifestations include generalized microangiopathy with fragmentation hemolysis, consumption-based thrombocytopenia, and anemia, which can lead to multiorgan failure. VOD is mainly a complication of the first month after the transplantation; the onset of TAM is usually during the first hundred days. In both of these complications the mortality is high, although the
situation has been improving especially in VOD due to development in treatment (Coppell et al 2010, Carreras 2012).

Idiopathic pneumonitis syndrome (IPS) comprises a group of disorders that result in interstitial pneumonitis and/or widespread alveolar injury (Clark et al 1993). IPS was an important cause of morbidity and mortality in patients undergoing HSCT especially in the early years of transplantations. In recent years the incidence has somewhat fallen due to developing transplantation techniques (regimens not including TBI, RIC) (Panoskaltsis-Mortari et al 2011, Carreras 2012), but IPS is still a significant and feared complication.

The immune reconstitution after autologous stem cell transplantation occurs in general at a faster tempo than in allogeneic HSCT, although variation according to underlying disease has been reported (Damiani et al 2003, Laurenti et al 2004, Szodoray et al 2012).

### 2.2. Iron metabolism

#### 2.2.1. Iron in the human body

The total amount of iron in the body in men and women is approximately 50 and 40 mg/kg body weight, respectively. About 70% of the iron is found in red blood cell hemoglobin, ten percent in myoglobin, less than one per cent in enzymes, and about one fifth as storage iron, as ferritin and hemosiderin particularly in the liver, spleen and bone marrow (Muñoz et al 2009). The daily intake of iron in an ordinary diet is 15–20 mg. In normal conditions 1-2 mg of iron is absorbed from the diet through the duodenum and is bound by transferrin, forming diferric transferrin. The majority of transferrin-bound iron comes from senescent red blood cells and is re-utilized for erythropoiesis in the bone marrow, approximately 20 mg of iron daily (Shenoy et al 2014). Iron absorption is well regulated at the mucosal level (Fleming and Ponka 2012). There is no system to actively remove iron. Iron is passively exported out of the body only by the shedding of the intestinal epithelium (approximately 1 mg/day) or menstrual bleeding (1 mg/day) (Muñoz et al 2009). Therefore, systemic iron homeostasis is strictly controlled at the level of absorption by hormonal signals in response to the iron load (Ganz 2013, Shenoy et al 2014) (Figure 2).
2.2.2. Mechanisms of iron regulation

Hepcidin, a small peptide produced by the liver, is a central molecule that regulates iron metabolism (Krause *et al* 2000, Park *et al* 2001). It has been found to be a key regulator of the entry of iron into the circulation in mammals, including humans (Ganz 2013, Kautz and Nemeth 2014). The hepatic expression of hepcidin can be upregulated by at least two signals: iron loading (Krause *et al* 2000, Park *et al* 2001, Pigeon *et al* 2001, Nemeth *et al* 2004a, Nemeth *et al* 2004b, Kawabata *et al* 2007, Kanda *et al* 2011), and inflammatory...
stimuli, including interleukin-6 (IL-6), IL-17, oncostatin M (Nemeth et al 2004a, Nemeth et al 2004b, Lee et al 2005, Kawabata et al 2007, Kanda et al 2009, Kanda et al 2011) and lipopolysaccharide (Lee et al 2004). Hepcidin decreases iron absorption from the intestine and blocks its release from iron stores by downregulating the expression of the cellular iron exporter ferroportin (Nemeth et al 2004a, Nemeth et al 2004b, Kautz and Nemeth 2014), thereby maintaining circulating iron levels within the normal range.

Hepcidin can be downregulated by erythropoiesis-associated factors (Kattamis et al 2006, Kanda et al 2008), possibly including growth differentiation factor-15 (GDF) and a recently identified hepcidin regulator, erythroferrone (Kautz et al 2014). The mechanisms of hepcidin suppression are not fully understood, and may be disease specific (Shenoy et al 2014).

Hepcidin expression is markedly decreased in contexts in which erythropoiesis is increased, such as phlebotomy, hemolysis, and administration of erythropoietin. In hematopoietic stem cell transplantation, the serum hepcidin level was shown to peak one week after HSCT, during the deepest bone marrow depression, followed by a gradual decrease parallel with increasing reticulocyte counts (Kanda et al 2011). Erythropoietic activity has a greater influence on hepcidin expression than body iron status does (Kattamis et al 2006, Jones et al 2015).

Hepcidin deficiency causes iron overload in hereditary hemochromatosis and diseases with ineffective erythropoiesis (Ganz and Nemeth 2012a). Mice deficient in transferrin exhibit anemia and a paradoxical iron overload attributed to hepcidin deficiency (Bartnikas et al 2011). Similar findings in zebrafish have been reported by Fraenkel et al (2009). These studies indicate that transferrin may have an essential role as a regulator of hepcidin expression.

Several studies have shown that serum hepcidin levels are inappropriately low compared with the levels of iron load in some MDS patients, in line with observations in beta-thalassemia (Winder et al 2008, Murphy et al 2009), and high levels of GDF-15 with a suppressive effect on hepcidin (Tanno et al 2007) were reported in patients with refractory anemia with ringed sideroblasts (Ramirez et al 2009).
Increased circulating hepcidin contributes to anemia in inflammatory disorders, chronic renal disease, and cancer (Ganz and Nemeth 2012a). Anemia of inflammation is caused by the upregulation of hepcidin by IL-6 leading to decreased circulating iron levels. The high serum hepcidin levels frequently observed in patients with myeloma and Hodgkin’s lymphoma, related to IL-6 upregulation, may partly contribute to the anemia of these patients (Sharma et al 2008, Ganz and Nemeth 2009, Hohaus et al 2010).

2.3. Iron overload

In the treatment of malignant hematological diseases such as MDS and acute leukemia, multiple blood transfusions are required owing to ineffective erythropoiesis caused by the disease or to cytotoxic chemotherapy. However, iron overload is observed in some MDS patients without a history of transfusion (Cortelezzi et al 2000). Humoral factors with a suppressive effect on hepcidin may also play a role in iron overload (Kanda et al 2008), e.g. erythroferrone, as suggested by Kautz et al (2014).

2.3.1. Red blood cell (RBC) transfusions

RBC transfusions contribute strongly to the development of iron overload in patients with hematological malignancies as well as transplantation patients. A widely used threshold for RBC transfusion is the hemoglobin level of 80 g/l, in case there are no signs of anemia or clinically significant cardiac impairment (Pawson 2012). Although red cell transfusions are necessary to improve anemia-associated symptoms, multiple transfusions lead to iron accumulation because of the lack of an active iron export system. One red cell unit contains approximately 200-250 mg of heme iron, corresponding to the amount of iron normally absorbed in 6 months (Shander and Sazama 2010).
2.3.2. Evaluation of iron overload

2.3.2.1. RBC units

The simplest way to evaluate iron burden is the number of RBC units given. As one unit provides 200-250 mg of iron, a significant (4000-5000 mg) iron overload is reached with 20 transfused units.

2.3.2.2. Ferritin

Ferritin is the main storage form of iron. A proportion of ferritin is released into the circulation, and its serum levels, controlled by cellular iron status, correlate well with the body’s iron stores. However, because the serum level is also increased in patients with inflammation, it does not necessarily accurately represent the body iron load status in specific conditions, such as infection and autoimmune diseases (Lee and Means 1995, Olivé and Juncà 1996).

2.3.2.3. Liver iron content (LIC)

Hepatic iron content is considered to be a reliable indicator of total body iron stores (Angelucci et al 2000). Measuring the iron content of biopsy specimens from the liver has been a ‘gold standard’ for the evaluation of body iron overload (Emond et al 1999).

LIC is usually reported in μg/g (mg/g) dry weight with normal values 400-2200 μg/g (0.4-2.2 mg/g) in men, and 100-1600 μg/g (0.1-1.6 mg/g) in women (Batts 2007). Iron overload is considered to be severe when LIC is >7 mg/g (Tichelli 2012), or 10 mg/g (Batts 2007).

In allogeneic HSCT a histological evaluation of liver specimens provides useful information to differentiate iron overload from other causes of hepatic dysfunction such as GVHD and hepatic veno-occlusive disease (VOD). However, because iron distribution in the liver is often heterogeneous, multiple biopsies are required to accurately determine the liver iron concentration (Emond et al 1999). Furthermore, hematologic patients in the peritransplant
period are often thrombocytopenic and at a high risk of bleeding, and therefore a non-invasive evaluation of iron levels is preferred in the transplant setting.

2.3.2.4. Magnetic imaging

Using the paramagnetic properties of storage iron, the superconducting quantum interference device (SQUID) biosusceptometer was developed to measure iron levels (Sheth 2003). Iron content measured by this method has shown a good correlation with liver iron content measured by biopsy, but SQUID is available in only a few centers worldwide. Quantitative magnetic resonance imaging (MRI) enables the measurement of iron distribution and its averaged concentration for the entire liver and heart and has become a widely available modality for performing iron measurements (Anderson et al 2004, St Pierre et al 2005, Tanner et al 2006, Di Tucci et al 2008).

2.4. Iron toxicity

Highly reactive oxygen species (ROS) produced by free ferrous (Fe$^{2+}$) iron through the Fenton reaction are toxic to various internal organs. ROS oxidize lipids, proteins, and nucleic acids, resulting in premature apoptosis, cell death, and tissue and organ damage (Shenoy et al 2014). Under normal conditions, toxic reactions caused by free iron are prevented by circulating transferrin forming a compound with ferric (Fe$^{3+}$) iron.

Iron bound to transferrin is utilized in the bone marrow or transferred to the liver, where the cells express transferrin receptors and can take up transferrin by receptor-mediated endocytosis. In iron-overloaded conditions, such as primary or secondary hemochromatosis, plasma non-transferrin-bound iron (NTBI) levels increase to a measurable level because transferrin is almost or fully saturated with iron (von Bonsdorff et al 2002). The iron content of parenchymal cells in organs such as the liver, pancreas, heart and gonads increases due to uptake of the NTBI, potentially leading to irreversible organ damage and organ failure (Pietrangelo 2010).
There is evidence to indicate that iron overload impedes erythropoiesis. Iron overload inhibits burst-forming unit (BFU-E) colony formation and erythroblast differentiation of both murine and human hematopoietic progenitors *in vitro*, and cells exposed to excess iron exhibit dysplastic changes with increased intracellular ROS and decreased *bcl2* (anti-apoptotic gene) expression (Taoka *et al* 2012). Erythroid progenitors seem to be more susceptible to iron overload than myeloid progenitors (Hartmann *et al* 2013). Iron chelation has been shown to result in improved hemoglobin levels and reduced RBC transfusion requirements (Badawi *et al* 2010, Oliva *et al* 2010). In MDS, iron chelation improves blocked differentiation of hematopoietic cells and possibly reduces the risk of transformation to leukemia (Shenoy *et al* 2014). It is reasonable to expect that iron depletion is beneficial for erythropoiesis in iron overload states (Camashcella *et al* 2007).

### 2.4.1. Iron and infections

Iron is a necessary nutrient for the proliferation of bacteria and fungi, especially in a form easily accessible to the micro-organisms and not tightly bound to transferrin. They have developed several methods to obtain iron from the host, such as siderophores that acquire iron either from host proteins or from low-molecular-mass iron compounds (Weinberg 2009). *In vitro*, bacterial growth is faster after the addition of iron and the growth is slower after iron deprivation using an iron chelator (Weinberg 2009). An association between iron overload and bacterial or fungal infections has been demonstrated in hereditary and secondary hemochromatosis (Evens *et al* 2004, Khan *et al* 2007). In HSCT patients, increased pretransplant serum ferritin concentration or liver iron overload have been shown to be associated with an increased risk of bacterial or fungal infections (Altes *et al* 2004, Schaible and Kaufmann 2004, Altes *et al* 2007). Iron overload also has a negative impact on the functions of cells such as monocytes, neutrophils, macrophages, and natural killer cells that normally contribute to immunity to infections (Blijlevens *et al* 2000). Iron overload may also exacerbate the consequences of mucosal damage caused by chemotherapeutic agents and irradiation, allowing bacterial organisms to enter the circulation (Blijlevens *et al* 2000, Altes *et al* 2007).
2.4.2. Non-transferrin-bound iron (NTBI)

A central feature of NTBI is that it is not bound to the principal iron carrier transferrin. Such iron can be loosely bound to serum albumin, citrate and other undefined negatively charged ligands; because of its cationic nature iron cannot exist in absolutely free form (Breuer and Cabantchik 2001, Patel and Ramavataram 2012). The characteristic feature of such iron is that it retains its ability to catalyze the formation of ROS (Nilsson et al 2002).

Various terms have been used to address the same overlapping fraction of plasma iron: NTBI (Breuer and Cabantchik 2001), free iron (Nilsson et al 2002), catalytic iron pool (Halliwell and Gutteridge 1990, Kakhlon and Cabantchik 2002, Kruszewski 2003), and labile iron (Breuer and Cabantchik 2001). Based on the chelating properties of NTBI, different terms have been used: bleomycin detectable iron (BDI), deferoxamine chelatable iron (DCI), mobilizer dependent chelatable iron (MDCI), and bathophenantroline detectable iron (Breuer and Cabantchik 2001, von Bonsdorff, et al 2002, Patel and Ramavataram 2012). DCI has been later used by the same investigators to indicate the NTBI forms that can readily be chelated with deferoxamine without the assistance of mobilizers (Esposito et al 2003). MDCI has been used to indicate the NTBI forms which can be detected only after the action of a mobilizer such as oxalate or nitrilotriacetic acid (NTA) (Anderson 1999). Bathophenantroline detectable iron is applicable for NTBI forms which are directly chelated by the chromogenic chelator BPS (Nilsson et al 2002). Common for all these terms is that they refer to iron bound essentially to molecules other than transferrin.

Methods to measure the concentration of NTBI or any form of free iron have been developed and some are listed in Table 1.
Table 1. Assays for measurement of serum NTBI

<table>
<thead>
<tr>
<th>Assay type</th>
<th>Quantitation of iron</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Chelation of NTBI</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chelation of NTBI with EDTA, ultrafiltration of complex</td>
<td>Colorimetric</td>
<td><em>Hershko et al 1978</em></td>
</tr>
<tr>
<td>Chelation of NTBI with NTA, ultrafiltration of complex</td>
<td>HPLC</td>
<td><em>Singh et al 1990, Porter et al 1996</em></td>
</tr>
<tr>
<td></td>
<td>Colorimetric using bathophenantroline chromogen</td>
<td><em>Zhang et al 1995, Gosriwatana et al 1999</em></td>
</tr>
<tr>
<td>Chelation of NTBI with oxalate, transfer of oxalate to immobilized desferrioxamine</td>
<td>Fluorescence using metallosensor calcein</td>
<td><em>Breuer et al 2000b</em></td>
</tr>
<tr>
<td>Chelation of NTBI with hexadentate hydroxypyridinone-based beads</td>
<td>Beads containing both fluorescent and chelating moieties, flow cytometry</td>
<td><em>Ma et al 2014</em></td>
</tr>
<tr>
<td>2. <em>Biochemical reactions caused by NTBI</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleomycin assay. Ferrous iron complexed with bleomycin causes DNA degradation, degradation products are measured</td>
<td>Thiobarbituric acid reaction, spectrophotometry</td>
<td><em>Gutteridge et al 1981, Gutteridge and Hou 1986, Evans and Halliwell 1994</em></td>
</tr>
<tr>
<td></td>
<td>Ethidium-binding assay</td>
<td><em>Burkitt et al 2001</em></td>
</tr>
<tr>
<td>Activation of iron-sulphur cluster of aconitase</td>
<td>Enzymatic</td>
<td><em>Mumby et al 1998</em></td>
</tr>
</tbody>
</table>

Reprinted and adapted by permission from von Bonsdorff (2003).
2.4.3. Sources and status of NTBI in the circulation

The saturation of transferrin is about 30% at the normal serum iron level (approximately 20 μmol/l). Transferrin-bound iron is the only major form of non-heme iron present in plasma. In normal physiological conditions the transferrin concentration is sufficient for binding significantly greater doses of free iron and ensuring its absence in the internal milieu. This is the reason why NTBI is usually undetectable by most methods, and if found, its concentration does not exceed 1 μmol/l (Anderson 1999, Patel and Ramavataram 2012). In iron overload syndromes, such as thalassemia and hemochromatosis, the transferrin binding capacity is not sufficient and free iron is formed (Hershko et al 1978, Graham et al 1979, Batey et al 1980, Aruoma et al 1988, Porter et al 1996, Loréal et al 2000). The explanation for the existence of low concentrations of free iron even in case of low transferrin saturation is unclear (Gosriwatana et al 1999, Breuer et al 2000b, Loréal et al 2000).

In hemolytic anemia increased endogenous inflow of iron due to excessive hemolysis and/or compensatory blood transfusions may cause high saturation of transferrin leading to the appearance of NTBI (Fibach and Rachmilewitz 2008, Ozment et al 2013). NTBI has also been demonstrated in diabetes mellitus (Lee et al 2006). It has been suggested that glycated proteins, the concentrations of which are high in diabetics, do not only bind iron and copper with higher affinity but also have a tendency to extract iron from the traditional iron carriers. In cancer patients undergoing chemotherapy the reasons for the presence of NTBI are temporary shut-down of bone marrow function and associated reduced demand of iron. Other clinical conditions in which the presence of NTBI has been indicated are end-stage kidney disease patients treated with dialysis (Prakash et al 2005), alcoholism (De Feo et al 2001), myelodysplastic syndromes (Cortelezzi et al 2000, Mahesh et al 2008) and beta-thalassemia (al-Refaie et al 1992). In atransferrinemia or hypotransferrinemia it is pertinent that all or most of the plasma iron exists as NTBI. In the absence of transferrin, NTBI levels up to 20 μmol/l have been reported, whereas in the presence of insufficient transferrin concentrations the levels were found to be less than 10 μmol/l (Anderson 1999).

Various forms of NTBI may coexist depending on the degree and cause of iron overload. Clinically it has been proven that the efficacy of different iron chelators vary in various clinical conditions which indirectly supports the heterogeneity of NTBI. Careful segregation
of various isoforms of NTBI is essential in establishing the role of individual isoforms in specific pathological condition (Patel and Ramavataram 2012).

3. IRON AND HSCT

3.1. Causes and impact of iron overload in HSCT

Iron overload is frequently observed in patients with hematologic diseases before and after allogeneic stem cell transplantation (Sivgin and Eser 2013). Many transplant patients have a history of multiple red cell transfusions. Blood transfusions are often needed during the transplantation treatment, and some patients need transfusions for a significant period after the transplantation. In some diseases, such as thalassemia and MDS (Cortelezzi et al 2000), erratic absorption of iron may contribute to the development of iron overload.

In the peritransplant period, in addition to iron accumulation due to multiple red cell transfusions, bone marrow suppression caused by chemotherapeutic agents and irradiation substantially increases NTBI levels because erythropoiesis is the main route of utilization of circulating iron (de Witte 2008). Through the generation of ROS, increased NTBI may exacerbate organ and mucosal damage caused by chemotherapeutic agents and irradiation (Figure 2).
Figure 2. Possible mechanisms and interventions in iron-induced HSCT-toxicity.

GVHD = graft-versus-host disease, IPS = idiopathic pneumonitis syndrome, SOS = sinusoidal obstruction syndrome. Reprinted and adapted by permission from Nature Publishing Group: Evens et al, Bone Marrow Transplant, 2004; 34: 561-571.
The transplantation procedure affects the distribution of iron in the body. The conditioning
treatment before the transplantation causes cessation of red cell production and reduction in
the utilization of iron. Conditioning can also reduce the production of the main iron-carrying
protein, transferrin, in the liver, and injury of liver cells may cause release of storage iron. In
a large proportion of transplant patients the transferrin saturation reaches levels over 80%
during the bone marrow depression (Altès et al 2002). These effects may lead to the
appearance of NTBI.

The impact of iron overload for the outcome of transplantation has been evaluated in a
number of studies. In most of them ferritin has been used as a surrogate marker for iron load.

Generally iron overload has been found to have a negative impact on the outcome. Several
studies have shown that elevated pre-transplantation serum ferritin levels are associated with
Kataoka et al 2009, Mahindra et al 2009, Bazuaye et al 2012) or relapse-free (Armand et al
2007, Mahindra et al 2009) survival, and/or increased non-relapse mortality (Altès et al 2002,
Pullarkat et al 2008, Kataoka et al 2009, Mahindra et al 2009) after allogeneic transplantation. Bazuaye et al (2012) have shown that also other iron parameters, in addition to ferritin, especially soluble transferrin receptor, have similar and at least as good predictive value for transplantation outcome as ferritin. Higher than median soluble transferrin receptor levels, as well as lower than median ferritin levels, predicted better overall survival (OS).

The studies showing impaired outcome after HSCT in patients with iron overload have been
based on ferritin as a surrogate marker of iron. However, ferritin increases not only by iron
loading but also by inflammation caused by GVHD and infection, and that can lead to a wide
difference between the ferritin status and the actual iron load (Jastaniah et al 2008). Therefore, considerable effort has been invested to evaluate the iron load and its distribution in the main organs, particularly the liver and heart (Anderson 1999, Emond et al 1999, Sheth 2003, St Pierre et al 2005, Tanner et al 2006, Di Tucci et al 2008). The causal relationship between iron overload and adverse outcomes remains unclear and needs further investigation. In fact, Trottier et al (2013), using liver magnetic resonance imaging (R2-MRI) to quantify liver iron content, did not find any association between pretransplant iron overload and allogeneic stem cell transplantation outcomes. The same results came out in the meta-analysis by Armand et al (2014), where pre-transplant iron overload (>7 mg/g dry weight), assessed
by MRI estimation of LIC, was not associated with a significant increase in mortality and, as such, not a strong prognostic factor for OS.

3.1.1. Iron and hepatic complications

Liver being one of the organs in which iron preferentially accumulates, iron overload may particularly trigger and exacerbate hepatic injury after a conditioning regimen.

Liver enzyme elevations are very common in the first months after HSCT, and are also found in long-term survivors to varying degrees. The etiology of these liver abnormalities is multifactorial and may be related to hepatitis, graft-versus-host disease, or iron overload (Tomás et al 2000, de Witte 2008). An association between hepatic VOD and iron overload has been suggested by several studies (Morado et al 2000, Armand et al 2007, Maradei et al 2009).

Liver biopsies have shown that hemosiderosis occurs in most patients receiving chemotherapy with or without HSCT (Iqbal et al 1996), and hepatic iron and serum ferritin levels may be raised for several years after transplantation (Butt and Clark 2003). More than 80% of HSCT patients may be iron-overloaded at one year or later after the transplantation, as indicated by their serum ferritin levels (McKay et al 1996). Serum aminotransferase levels may be increased in association with iron overload for at least three years after HSCT, though the significance of this is not clear (Harrison et al 1996). Liver enzyme abnormalities caused by iron overload can mimic GVHD or hepatitis (Kamble et al 2006). An assessment of the cause of the liver enzyme abnormalities and iron loading status is necessary to avoid unnecessary treatment with immunosuppressive agents for chronic GVHD, which will expose patients to a higher risk of infection.

3.1.2. Iron and graft-versus-host disease

In acute GVHD, proinflammatory cytokines released from damaged tissues activate host antigen-presenting cells, enhancing the cross-presentation of minor antigens to donor T-cells and activating them (Blazar et al 2012). Therefore, tissue damage exacerbated by iron
overload might worsen acute GVHD. Elevated ferritin levels have been found to be associated with the incidence of acute GVHD or early transplant mortality (Platzbecker et al 2008, Pullarkat et al 2008).

Ferritin can sequester and store large amounts of iron, and may exert suppressive effects on the immune system (Recalcati et al 2008). Ferritin is composed of heavy chain subunits (H-ferritin) and light chain subunits (L-ferritin), both of which are regulated by post-transcriptional, iron-dependent machinery. Several studies have shown that ferritin can suppress the proliferation of T-cells in response to mitogens, impair the maturation of B-cells and inhibit the proliferation of myeloid precursor cells (Matzner et al 1979, Broxmeyer et al 1991, Cardier et al 1995). The immunosuppressive effect of ferritin has been proposed to been mediated by IL-10 (Gray et al 2001). It is suggested that H-ferritin induces the differentiation of dendritic cells, which stimulate IL-10 production from regulatory T-cells (Gray et al 2002).

Elevated pretransplant ferritin levels have been found to associate with a decreased incidence of chronic GVHD (Mahindra et al 2009). This may be related to the suppressive effect of ferritin on adaptive immune responses. However, these observations are based on ferritin levels and there is no clear evidence of the association of iron overload and acute or chronic GVHD (Majhail et al 2008, Trottier et al 2013).

3.1.3. Iron and infections

Iron is an important nutrient for bacteria and fungi, and therefore excess iron may enhance their proliferation (Weinberg 1978). In the HSCT setting, the ability of NTBI to enhance the proliferation of Staphylococcus epidermidis was shown in an in vitro study by our group using the serum of patients undergoing HSCT (von Bonsdorff et al 2003, von Bonsdorff et al 2004). Consequently, the high levels of NTBI observed in the post-transplant period may be a good environment for microorganisms to proliferate.
3.1.4. Iron and other transplant-related complications

In a study using T2-MRI, abnormally high iron levels were detected in the pancreas and pituitary gland in 40 and 55% of the patients, respectively (Au et al 2007). In 40 and 70% of the patients the pancreatic and pituitary function was found to be abnormal.

There is evidence to suggest that iron-induced ROS formation may contribute to the development of IPS. Free radicals may cause endothelial injury, both by direct cell damage and indirectly by inducing the expression of cytokines that amplify IPS, resulting in acute lung injury and pulmonary fibrosis (Crouch 1990, Balla et al 1993, Evens et al 2004).

3.2. Treatment of iron overload

Phlebotomy is the preferred way to reduce iron overload after the transplantation, if feasible. However, many patients have prolonged anemia which prevents the use of this method. Data of the utility of iron chelation therapy in stem cell transplantation is very limited. It has been shown, however, that patients with beta-thalassemia major who received regular chelation therapy in the pretransplantation period have improved outcomes after HSCT (Lucarelli and Gaziev 2008). Retrospective studies have also suggested that iron chelation in patients with MDS reduces morbidity (Takatoku et al 2007) and improves survival (Leitch 2007, Rose et al 2010). In a retrospective study by Lee et al (2009), patients with ferritin levels over 1000 ng/ml had a worse survival and higher transplant related mortality than those with lower ferritin levels. However, among patients with high ferritin levels who had had pretransplant iron chelation therapy and reached levels lower than 1000 ng/ml before the transplantation, the survival and transplant related mortality were not significantly different from the patients with initially low ferritin. One small study has reported that deferoxamine therapy after HSCT reduced serum ferritin levels faster than phlebotomy and was well tolerated without evidence of graft failure (Li et al 2000).

Currently there are two oral iron chelators in clinical use, deferiprone and deferasirox. Severe agranulocytosis can occur with the use of deferiprone, and it has not been investigated in the HSCT setting (Pullarkat 2010). The efficacy and safety of deferasirox have been shown in
thalassemia-patients (Taher et al 2012, Taher et al 2014). In a retrospective study, the OS of HSCT patients with iron overload was significantly better with pretransplantation use of deferasirox than that of the non-chelated patients (Sivgin et al 2013). In the first prospective post-transplantation study in HSCT patients with transfusional iron overload, the use of deferasirox for one year starting from 6 months after transplantation provided a significant reduction in serum ferritin and liver iron concentration, with mild or moderate adverse effects (Vallejo et al 2014).

In the light of the literature, iron chelation therapy before the transplantation, if logistically feasible, or after the transplantation may improve transplantation outcomes, but this remains to be carefully investigated.

4. TRANSFERRIN AND APOTRANSFERRIN

Transferrin is one of the most abundant proteins in human plasma, with an approximate concentration of about 2-3 g/l (von Bonsdorff et al 2013). It is the major iron transporting protein in the blood stream, and it plays an important role in the iron metabolism of the human body (Anderson and Vulpe 2009). Transferrin carries iron particularly for red cell production which requires high amounts of iron for heme synthesis, as well as to other rapidly dividing cells. It also sequesters iron released from the duodenal cells during dietary absorption and from macrophages which engulf senescent blood cells. Transferrin is of utmost importance in the transportation of iron; however, the 3-4 mg of transferrin-bound iron comprises only about 0.1% of the total body iron of which 60-70% is found in the red blood cell compartment and the rest in other iron proteins, such as ferritin and myoglobin. Transferrin-iron turnover is highly dynamic and about 30 mg iron is transported daily to the cells. Other iron binding proteins such as ferritin and hemosiderin are the intracellular iron storage proteins, which can store considerably higher amounts of iron per molecule (e.g. ferritin up to 4500 iron atoms) than transferrin with the maximum of two iron atoms (Sargent et al 2005).
Under normal physiological conditions, practically all iron in plasma is bound to transferrin, and the transferrin iron saturation is about 30%, constantly offering surplus of iron binding sites. Transferrin keeps iron in a form which makes it suitably accessible to the cells requiring iron. Under physiological pH iron ions easily form non-soluble hydroxide complexes; transferrin retains a soluble form of iron. By binding iron transferrin prevents the free radical reactions caused by free iron. An additional advantage is that iron bound to transferrin is not accessible to microorganisms (Weinberg 1999).

Transferrin is synthesized mainly in the liver. It is one of the acute phase proteins with suppressed production in inflammation. On the other hand, transferrin levels can be elevated, for example during pregnancy. As described above, in some disease or treatment conditions the iron binding capacity of transferrin is overwhelmed and the saturation level is exceeded, leading to the formation of non-transferrin-bound iron in the blood stream, which can result in iron depositions in non-hematological cells and lead to organ damage (Shander et al 2009, Hershko 2010).

The receptor-mediated endocytosis whereby transferrin-bound iron is taken up by cells is a well described process (Richardson and Ponka 1997, Cheng and Walz 2007). Transferrin carrying iron is bound to transferrin receptors on the cell surfaces. This transferrin receptor complex is internalized and iron is released through protonation, which lowers the pH, whereafter iron-free apotransferrin is recirculated to the blood stream. The half-life of transferrin is approximately 8-9 days (Katz 1961).

Of the two transferrin receptors, transferrin receptor 1 is the better characterized (Aisen 2004). It binds transferrin with high affinity. It has a much higher affinity for the iron-saturated form of transferrin than for transferrin carrying only one iron atom or for iron-free apotransferrin (Qian et al 2002). The transferrin receptor can be expressed in abundance, for example, on the surface of erythroid precursors with a high requirement of iron (Ponka et al 1998), and on other actively dividing cells, including cancer cells (Daniels et al 2006). A proportion of these transferrin receptors is soluble and circulating in the blood stream. As the concentration of soluble transferrin receptors is in proportion to the expression on the cell surface, the receptor level is used as a diagnostic tool for measuring iron deficiency anemia, where a high receptor expression is typical.
4.1. Biochemistry

Transferrin is classified as a beta-1 globulin. The transferrin molecule is a single polypeptide chain containing 679 amino acid residues (MacGillivray et al 1983). It is organized into two homologous lobes of about 330 amino acid residues, called the N- and the C-lobe (Figure 3). The lobes are linked by a short flexible spacer peptide and each lobe contains two dissimilar domains divided by a cleft, which is the binding site for Fe$^{3+}$ (Bailey et al 1988, Wang et al 1992). The synergistic binding of an anion, preferentially the carbonate molecule, is essential for the iron binding. Transferrin is capable of binding several other metal ions, but with a lower affinity (Harris and Aisen 1989).

Each molecule of transferrin can bind two Fe$^{3+}$ ions. The four iron forms of transferrin are the iron-free apotransferrin, the monoferric transferrin with iron in the C- or in the N-lobe, respectively, and the diferric holotransferrin. The affinity of transferrin for iron at the physiological pH of 7.4 is high (Harris and Aisen 1989). Upon binding of iron, the lobes

Figure 3. A ribbon diagram of a diferric rabbit serum transferrin molecule. Reprinted and adapted by permission from von Bonsdorff (2003).
undergo a conformational transition from the apo structure with an open interdomain cleft to a closed holo structure (Hirose 2000).

Transferrin is a glycoprotein and two N-linked oligosaccharides are found at the C-lobe at asparagine residues Asn$^{413}$ and Asn$^{611}$. The carbohydrates constitute about 6% of the mass of transferrin that has a molecular weight of 79750. The glycan chains are mainly biantennary (85%) and triantennary (15%) complex type glycans (Spik et al 1985, Fu and van Halbeek 1992). The number of sialic acid residues per transferrin molecule is between 4 and 6. The heterogeneity of the glycosylation is a determinant of the microheterogeneity of transferrin. Glycosylation variants can occur in different diseases or states. For example, in patients suffering from alcoholism, carbohydrate chains lacking two to four of the terminal trisaccharides, comprising the negatively charged sialic acid and the neutral N-acetylglucosamine and galactoside, are common (Stibler 1991). The presence of carbohydrate-deficient transferrin can be used as a marker of alcoholism (Turpeinen et al 2001). Another determinant of microheterogeneity is the genetic polymorphism. The most common variant was designated TFC, while the other common forms are TFB and TFD (Kamboh and Ferrell 1987). Of at least 38 variants, only 4 occur with a frequency of over 1% (de Jong et al 1990). Attempts to establish a relationship between the transferrin variant, serum transferrin concentration, and iron binding capacity have been inconclusive (Sikström et al 1993).

4.2. Manufacture

Transferrin is not a widely used plasma protein, and the process for commercial manufacturing has been set up only by some companies, whose aim is to provide transferrin as a cell culture or biochemical reagent. Transferrin can be produced from human plasma, and for this the widely used plasma fractionation procedures can be utilized as the basis. Transferrin precipitates into fraction IV, subfraction IV4, in the Cohn crude ethanol fractionation process for human plasma (Cohn and Strong 1946). Fraction IV has been a much used starting material to produce human transferrin as in many cases it is a surplus fraction in commercial fractionation process and thus discarded. In the first descriptions of large scale manufacturing of transferrin, precipitation, chromatographic and crystallization
techniques were employed to obtain highly purified transferrin (Kistler et al 1960, Inman et al 1961). Since then, the manufacturing methods have evolved and today they usually employ generally used techniques of protein purification and downstream processing. Current manufacturing processes aiming at clinical use include additional steps to achieve viral safety.

The transferrin preparations obtained by the different processes vary in regard to iron form. In some cases a process has been set up specifically to obtain iron saturated holotransferrin (Inman et al 1961, Van Beveren and Pappenhagen 1987, Rolf et al 1997) and others for iron-free apotransferrin (von Bonsdorff et al 2001, McCann et al 2005, Ascione et al 2010). Manufacturing methods can be or have been adopted so that a specific iron removal step or addition step is added to the process to ensure that transferrin of the desired iron form is obtained. A method for the production of transferrin was developed by the Finnish Red Cross Blood Transfusion Service in the 1990s (von Bonsdorff et al 2001), and based on this method a large scale production process has since been set up by a pharmaceutical company (Sanquin Plasma Products, The Netherlands). After the protein purification has taken place, transferrin-bound iron is removed by lowering the pH and adding the chelating agent EDTA so that apotransferrin is obtained as the final product. For this particular process, virus safety validation studies show high reduction results: overall reduction of enveloped viruses is $>19\log_{10}$, and in the nanofiltration step included in the process the reduction of non-enveloped viruses hepatitis A virus (HAV) and canine parvovirus (CPV) is $>5\log_{10}$ (von Bonsdorff et al 2013).

Transferrin has been presented in different formulations, for example a liquid 5% weight/volume formulation in sodium chloride (von Bonsdorff et al 2001), or a freeze-dried formulation in phosphate, sodium chloride and glycine (Ascione et al 2010). The former has been shown to be stable for up to 3 years when refrigerated (von Bonsdorff et al 2013), the latter for at least 18 months at room temperature (Ascione et al 2010). In addition to these manufacturing processes which focus on large scale manufacturing of human transferrin from plasma, there are numerous descriptions of small laboratory scale purifications schemes to obtain purified transferrin (Wu et al 2008). Many of these are described for recombinant transferrin molecules. Transferrin has been expressed in a large variety of recombinant systems, either as a full length molecule in mammalian baby hamster kidney (BHK) cells (Mason et al 1993), in microbial systems (Sargent et al 2006, Mizutani et al 2010,
Mukaiyama et al 2010), and in plants (Brandsma et al 2010, Zhang et al 2010), or as N- and C-terminal half-molecules in both eukaryotic expression systems (Funk et al 1990, Mason et al 1996 Mason et al 1997, Mason et al 2001), and prokaryotic systems (Hoefkens et al 1996). Such recombinant transferrin products have mainly been used in structural and functional protein analyses. Only one recombinant transferrin product is commercially available. This transferrin is a non-glycosylated (or partly O-glycosylated) form of transferrin expressed in a yeast (Saccharomyces cerevisiae) (Keenan et al 2006, Finnis et al 2010). Despite its abnormal glycosylation it is reported to have similar iron binding and transport abilities as human plasma transferrin and it can be used as a cell culture agent. So far, there is no description of its behavior in man (Brandsma et al 2011).

4.3. Characterization

Currently, transferrin is not commercially available as a pharmaceutical, and therefore the compendial pharmacopeia requirements available for other plasma protein therapeutics do not exist. A number of methods have been used to measure the concentration and purity, iron content, saturation and forms, as well as the iron binding capacity and biological activity (von Bonsdorff et al 2013).

The most obvious methods for visually distinguishing the iron forms of transferrin are the distinct colors; holotransferrin has a dark red “rusty” color while apotransferrin is pale yellowish or almost colorless. The colorimetric change can also be used as a tool to measure the iron binding capacity of transferrin as the spectrometric absorption at 450-460 nm changes when known amounts of ferric iron are added to a predetermined amount of transferrin (von Bonsdorff et al 2013). Another commonly used method for determining the transferrin iron form is polyacrylamine gel electrophoresis in urea, which is a good tool in distinguishing all transferrin iron forms (Makey and Seal 1976, Evans and Williams 1980).

As transferrin does not have enzymatic or similar properties, its function can best be established by its iron binding properties (Welch and Skinner 1989, von Bonsdorff et al 2001), its ability to bind to transferrin receptors (Fuchs and Gessner 2002), and its ability to
enhance *in vitro* growth of mammalian cells dependent on transferrin-bound iron for growth (Keenan *et al* 2006, Ascione *et al* 2010).

### 4.4. Potential clinical applications

Potential uses of transferrin are substitution therapy in transferrin deficiency and the utilization of its iron binding and regulatory properties in iron metabolism. It has also been used as a targeting molecule (von Bonsdorff *et al* 2013).

Due to the indispensable role of transferrin as an iron transport protein in plasma, patients with a genetic deficiency of transferrin are radically dependent on substitution therapy with human plasma transferrin. Hypotransferrinemia is a very rare hereditary disease and only a very limited number of cases have been reported worldwide (Goya *et al* 1972, Hayashi *et al* 1993, Beutler *et al* 2000). An ongoing clinical study by Sanquin investigates the treatment of hypotransferrinemia with apotransferrin.

Transferrin has been studied in several animal disease models in which a redox-active iron is considered to play a pathogenetic role (von Bonsdorff *et al* 2013). Apotransferrin administration was shown to effectively mitigate kidney injury in a mouse model of renal ischemia/reperfusion (I/R) injury (de Vries *et al* 2004). Apotransferrin reduced not only extracellular redox-active iron but also intracellular generation of reactive oxygen species. Subsequently, apotransferrin administration was studied in a porcine liver transplantation model, which is associated with severe I/R injury and a high incidence of primary graft non-function after transplantation. Apotransferrin was administered intravascularly to the recipients together with other anti-inflammatory and hepatoprotective agents. This treatment regimen remarkably protected ischemically damaged liver grafts against primary non-function and promoted the recovery of graft function (Monbaliu *et al* 2009).

The increasing understanding of the regulation of iron metabolism by hepcidin and the accumulating evidence on the involvement of transferrin in the regulation of hepcidin expression have given new insight into the possibilities of treating diseases with aberrant iron metabolism by transferrin.
It has been demonstrated that allogeneic stem cell transplantation is associated with liver iron accumulation (Iqbal et al 1996, de Witte 2008). Studies in a mouse model of GVHD showed that apotransferrin administration effectively mitigated iron accumulation in the liver and prevented liver injury and apoptosis (Bair et al 2009). In a previous study it was demonstrated that the hepatoprotective effect of transferrin was mediated by type 2 transferrin receptor, suggesting that transferrin may induce antiapoptotic signaling through the type 2 receptor (Lesnikov et al 2001). Recent findings in a mouse model of beta-thalassemia showed that treatment with transferrin injections markedly improved the disturbances in iron and red cell turnover characteristic for thalassemia (Li et al 2010, Gelderman et al 2015). Transferrin injections normalized labile plasma iron concentrations, increased hepcidin expression, normalized red blood cell survival, increased hemoglobin production, and concomitantly reduced reticulocytosis, erythropoietin level and splenomegaly. These results suggested that transferrin is a limiting factor contributing to anemia in thalassemic mice and suggested that transferrin therapy might be beneficial in human beta-thalassemia. Considering the beneficial effects of transferrin injections on inefficient erythropoiesis, transferrin could have therapeutic value in other diseases associated with inefficient erythropoiesis.

Another potentially useful action of transferrin would be the binding of iron to inhibit the growth of microbes dependent on iron supply. The binding of free iron by intravenous apotransferrin injections was shown to restore the growth inhibition of coagulase negative staphylococci in the sera of stem cell transplantation patients, suggesting that apotransferrin might protect patients against infections by opportunistic pathogens (von Bonsdorff et al 2003, von Bonsdorff et al 2004).

In addition to the clinical use of transferrin in the native form it has been employed as a targeting molecule. Transferrin has the potential to deliver guest molecules to cells expressing transferrin receptors. Cancer and tumor cells with a high expression level of transferrin receptors have been the target in most of these applications (Sato et al 2000, Inuma et al 2002, Suzuki et al 2008, Kim et al 2010).
5. AIMS OF THE STUDY

The aims of this study were to prospectively investigate the appearance and detailed timing of NTBI in patients undergoing hematopoietic stem cell transplantation and to study the effects of administered exogenous apotransferrin on NTBI in patients undergoing allogeneic HSCT. We also wanted to study in vitro the effect of NTBI on hematopoietic progenitor colony formation, and the possibility to counteract this with apotransferrin. The specific study goals were:

1. To determine the extent of the decline of serum transferrin and the liberation of iron, and the appearance and exact timing of the presence of NTBI during allogeneic and autologous HSCT. For comparison, a group of patients treated with intensive chemotherapy only was also studied.

2. To examine the kinetics and safety of a single i.v. dose of apotransferrin in allogeneic HSCT patients.

3. To examine the kinetics, safety and tolerability of repeated i.v. doses of apotransferrin in patients undergoing allogeneic HSCT.

4. To examine the effects of exogenous apotransferrin on the levels of serum total iron, NTBI, transferrin saturation, and on the different iron saturation forms of transferrin.

5. To study the effect of NTBI (in the form of FeNTA, ferric nitrilotriacetic acid) on erythroid and granulocyte macrophage colony formation in in vitro cultures, and the effect of preincubation of FeNTA with apotransferrin on the colony growth.
6. PATIENTS, MATERIALS AND METHODS

6.1. Patients

All patients were adults, over 18 years of age, and they were treated at the Division of Hematology, Department of Medicine of the Helsinki University Central Hospital for hematological malignancies between November 1996 and May 2000. For each study the patients were enrolled consecutively during the time of the respective study.

6.1.1. Patients treated with allogeneic HSCT (I)

Ten consecutive patients undergoing allogeneic stem cell transplantation were enrolled in this prospective study. There were equal numbers of men and women. All received myeloablative conditioning with cyclophosphamide (Cy) and total body irradiation (TBI). Two patients had acute myeloid leukemia (AML) and three acute lymphoblastic leukemia (ALL) in first remission; three had myelodysplastic syndrome (MDS), and two chronic myeloid leukemia (CML). Nine patients received a bone marrow graft, seven from a sibling, and two from a matched unrelated donor. One patient was transplanted with a PBSC graft from a sibling.

6.1.2. Patients treated with autologous HSCT (II)

Sixteen consecutive patients who were admitted for intensive therapy with autologous stem cell transplantation (aHSCT) were enrolled. Eleven had multiple myeloma (MM) (male n= 5, female n= 6), four non-Hodgkin lymphoma (male n= 2, female n= 2), and one female patient was treated for Hodgkin’s disease. The conditioning for myeloma consisted of melphalan and TBI (n= 7) or melphalan monotherapy (n= 4). All the lymphoma patients were conditioned with BEAC.
6.1.3. Patients treated with cytotoxic chemotherapy (II)

Eight consecutive patients treated for acute leukemia were enrolled. All patients were male. Four had AML, three ALL, and one had biphenotype acute leukemia. Two patients received cytotoxic chemotherapy as an induction and six as a consolidation treatment.

6.1.4. Patients given a single apotransferrin dose (III)

Seven consecutive patients undergoing conditioning with Cy/TBI for allogeneic HSCT and meeting the inclusion and exclusion criteria were enrolled. The inclusion criteria were serum transferrin < 2 g/l and transferrin saturation > 80% on day -3 before the transplantation. The exclusion criteria were clinically significant pulmonary or cardiovascular disease, or renal insufficiency (serum creatinine > 115 μmol/l). Four of the patients were female, three male. One female patient was excluded immediately prior to giving the study drug because of a then emerging exclusion criterion. One patient had ALL and one AML. Two of the patients had CML in first chronic phase. Of the two MDS patients, one had refractory anemia (RA) and the other refractory anemia with excess blasts in transformation (RAEBt) in first complete remission. One patient received a PBSC graft from a sibling; all others were transplanted with a bone marrow graft, two from a matched unrelated donor.

6.1.5. Patients given repeated doses of apotransferrin in a dose-finding study (IV)

Thirteen consecutive patients admitted for allogeneic HSCT with Cy/TBI and eligible for the study were enrolled. An inclusion criterion, in addition to age 18-65 years and Cy/TBI conditioning, was weight ≥ 40 kg. The exclusion criteria were clinically significant cardiovascular or pulmonary disease, renal insufficiency (serum creatinine > 115 μmol/l), proteinuria, complete IgA deficiency, or participation in another clinical trial. There were eight male and five female patients included in the study. One male patient was excluded after enrollment because the conditioning therapy and SCT were postponed for medical reasons. Five patients had MM, three CML in chronic phase, two had MDS (RAEB and RAEBt), one AML, and one patient had mantle cell lymphoma. Eleven of the patients
received bone marrow, and only one a PBSC graft. Nine had a sibling and three an unrelated donor.

6.1.6. Patients given repeated doses of apotransferrin in a high-dose setting (IV)

Eight consecutive patients, who met the same inclusion criteria as in the preceding study, were enrolled in this high-dose study arm. In addition to those of the previous study, the exclusion criteria also included serum alanine aminotransferase ≥ 100 U/l (men), ≥ 70 U/l (women), serum alkaline phosphatase > 275 U/l, serum bilirubin > 20 μmol/l, and plasma thromboplastin time < 70%. Six of the patients were male and two female. Five patients received a PBSC, and three a bone marrow graft.

6.2. Bone marrow samples for cell cultures (V)

The bone marrow samples used in the in vitro cell culture study were from healthy bone marrow transplant donors.

6.3. Transplantation procedures

All patients who underwent allogeneic transplantation were conditioned with intravenous cyclophosphamide 60 mg/kg body weight on two consecutive days (day -6 and day -5 in relation to the transplantation on day 0) and TBI 12 Gy, fractionated in six doses from day -4 to day 0. The immunosuppressive therapy consisted of i.v. cyclosporine 3 mg/kg/day as a continuous infusion starting on day -1, a short course of methotrexate (15 mg/m² on day +1, followed by 10mg/m² on day +3 and day +6), and oral methylprednisolone starting on day +8. The initial methylprednisolone dose was 0.5 mg/kg for 7 days, then escalating to the maximum of 1 mg/kg for 14 days. After that the dosage was reduced to 0.5 mg/kg for 14 days, and thereafter the dose was slowly tapered and discontinued on day 112 (Ruutu et al 2000).
The conditioning treatment for autologous transplantation in myeloma patients was either melphalan monotherapy (i.v. melphalan 100 mg/m² on two consecutive days), or i.v. melphalan 140 mg/m² + fractionated TBI 12 Gy. The preparative treatment for lymphoma was BEAC: carmustine 300 mg/m² on day 1, followed by etoposide 100 mg/m² x 2/day, cytarabine 100 mg/m² x 2/day, and cyclophosphamide 1500 mg/m² x 1/day, each for four days (from day 2 to day 5).

6.4. Treatment for acute leukemia

The treatment was based on the underlying disease and phase of therapy and followed the protocols in use at that time (Study II, Table 1).

6.5. Serum samples

The study samples, each a 10 ml vial, were mainly collected coinciding with routine sampling. The timing schedules were somewhat different and are described in detail in each study. Extra sampling was done only in Study III to measure the study parameters after the administration of the single dose of the study drug.

6.6. Laboratory methods

Serum total iron was measured by a colorimetric ferene-S method (reference range 8-30 μmol/l for women and 10-31 μmol/l for men). Serum transferrin was determined by an immunoturbidimetric method (reference range 1.75-3.13 g/l). Serum ferritin was measured using the Ciba Corning ACS ferritin chemiluminometric immunoassay with a reference range 10-150 μg/l for women and 15-230 μg/l for men. Transferrin saturation was calculated using the formula: serum iron (μmol/l)/serum transferrin (g/l) x 3.98  (Morgan 1992), without a
cutoff at 100%. The iron forms of transferrin were analyzed by urea polyacrylamide gel electrophoresis (6% acrylamide gel with 6 mol/l urea) according to Williams and Moreton (1980). Serum ferritin was measured using the Ciba Corning ACS Ferritin chemiluminometric immunoassay.

6.7. NTBI

NTBI in serum was determined using the bleomycin detectable iron (BDI) assay described by Evans and Halliwell (Evans and Halliwell 1994). In our studies it was performed according to a modification for small serum volumes modified by our group. In this modification all reactions were carried out in one-half the volumes using a spectrophotometric microplate reader (von Bonsdorff et al 2002). A detection limit of 0.1 μmol/l for NTBI was established based on the exclusion of interference of nonvisible hemolysis and on accuracy studies. In the validation study BDI was found only when transferrin saturation exceeded 80% (von Bonsdorff et al 2002).

6.8. Apotransferrin

The human plasma-derived apotransferrin product was developed in the Finnish Red Cross Blood Transfusion Service by means of purifying the Cohn fraction IV of human plasma by two ion exchange chromatography steps and ultrafiltration in conformance with good manufacturing practice (GMP) requirements for an investigational new product (von Bonsdorff et al 2001). The process comprises solvent detergent treatment as the main virus inactivation step and 15 nm virus filtration and polyethylene glycol precipitation as removal steps for physico-chemically resistant infectious agents. Product characterization by electrospray and MALDI-TOF mass spectrometry indicated no other chemical modifications than N-linked glycan chains and disulphide bonds, except minor oxidation. The purity of the product was more than 98%, main impurities being immunoglobulin (Ig)G, IgA and hemopexin. The product had intact iron binding capacity and native conformation. The apotransferrin solution was sterile, non-pyrogenic and suitable for i.v. use (von Bonsdorff et
al 2001). A liquid formulation with 50 mg/ml apotransferrin stored at cold room temperature was used throughout the clinical study.

6.8.1. Dosage of apotransferrin

6.8.1.1. Single dose (III)

During the myeloablative conditioning the transferrin levels are usually decreased, on average 1.4-1.5 g/l on day +3 (Sahlstedt, unpublished data). It was reasoned that a possible clinical benefit could be achieved with a serum transferrin elevation by 2 g/l to reach the upper reference limit of transferrin. As the plasma volume of an adult is approximately 45 ml/kg, the goal would be reached with a dose of 100 mg/kg (0.1 g/kg divided by 0.045 l/kg). The actual doses were rounded up to the nearest whole gram, and were given on day +3.

6.8.1.2. Repeated doses (IV)

In the dose-finding part, the higher apotransferrin dose, later in this review referred to as medium-dose, was planned to increase the serum transferrin level and maintain it at 5-6 g/l. That was estimated to be high enough to prevent the appearance of free iron in most patients. The lower dose was planned to maintain the serum transferrin level at 2-3 g/l, which approximately corresponds to the reference range in healthy humans.

The doses were calculated based on the results of Study III. The loading doses of 100 mg/kg we rounded up to the nearest whole gram. According to weight steps of 5 kg, starting from 40 kg up to > 104 kg, the actual loading dose varied between 91-111 mg/kg. The maintenance dose was 1 g for patients < 50 kg, 1.5 g for patients weighing between 50-59 kg, 2 g for patients 60-99 kg, and 3 g for patients ≥ 100 kg. The average maintenance dose was 26 mg/kg (20-33 mg/kg).

The medium-dose arm received 4 loading doses from day -6 to day -3, and 8 maintenance doses every other day from day -1 to day +13. The lower-dose arm received one loading dose on day -6, and 9 maintenance doses every other day from day -4 to day +12.
In this high-dose part, the dosage regimen was based on the results of the preceding dose-finding part of the study, and the transferrin level was planned to reach 5-8 g/l on day -2. With the five maintenance doses the basal transferrin level was anticipated to vary between 3-6 g/l. Peak levels after apotransferrin infusions were estimated to be on the average 2.6 g/l higher.

The average apotransferrin dose of 115 mg/kg was given to patients as a loading dose on four consecutive days from day -6 to day -3, and as a maintenance dose every other day between day -1 and day +7. The dose was rounded up to fit the package sizes of the study drug in the different weight ranges: for patients weighing 40-54 kg the apotransferrin dose was 5.0 g, for patients 55-74 kg 7.5 mg, and for patients ≥ 75 kg 10.0 g. Thus the actual dose varied between 93 and 136 mg/kg.

6.9. Assessment of adverse effects of the study drug

The immediate tolerance was monitored before and during the two hours following the injection by observing the blood pressure, pulse, temperature and respiratory rate. All other adverse events were recorded at the same time points, and then 1, 2 and 3 days after the study drug administration. The study drug safety was analyzed by monitoring blood counts, liver and renal function and electrolyte balance in routine samples until day +10.

In patients receiving the low and medium dose of study drug, blood pressure and heart rate were recorded just before, 15 minutes after the beginning of the loading infusion, immediately after, and 1 hour after the loading infusion. Body temperature was recorded before and 1 hour after the loading infusion.

In patients receiving the high dose of the study drug, blood pressure and heart rate were recorded at the first four infusions before, 15 minutes after the beginning and immediately after the completion of each infusion. Body temperature was recorded before and immediately after the first four infusions.
Blood counts, liver and renal function and electrolyte balance, plasma thromboplastin time and serum lactate dehydrogenase were monitored in the routine samples until day +17 after the dose-finding study, and until day +14 after the high dose study.

All events that impaired the well-being of the patient during the study period were recorded. The causality in relation to the study drug was evaluated by the clinical researcher, and graded to be non-related, improbable or possible. The maximum intensity of the adverse effects was recorded as mild, moderate or severe.

According to the study protocol, in the high dose study-arm some symptoms or changes in laboratory results, known to occur very frequently in HSCT patients, were not recorded as adverse events. The symptoms and laboratory parameters not recorded as adverse events were: fatigue, dry skin, mucositis, irritation of the insertion site of the central venous catheter, or temperature increase up to 37.5°C, all changes in the blood counts, and serum levels of bilirubin < 30 μmol/l, alanine aminotransferase ≤ 150 U/l, alkaline phosphatase ≤ 600 U/l, LD ≤ 700 U/l, and CRP ≤ 30 mg/l, as well as thromboplastin time ≥ 50%.

6.10. Ferric nitrilotriacetic acid (FeNTA)

Ferric nitrilotriacetic acid (FeNTA) was prepared from an iron atomic absorption standard solution and 5mM nitrilotriacetic acid (NTA) dissolved in 0.1 M Tris buffer, pH 8.0. The final solution contained 3 mM Fe³⁺ pH 7.4. The final concentration of free iron in the culture medium was confirmed by the BDI-assay described above. The detected levels of free iron were about 70% of the added concentration of FeNTA, but the results are given according to the added FeNTA concentration.
6.11. *In vitro* cultures of hematopoietic progenitors

Erythroid burst-forming units (BFU-E) and granulocyte-macrophage colony-forming units (CFU-GM) of healthy bone marrow transplant donors were cultured in a methyl cellulose assay according to Juvonen *et al* (1993). Three to five ml of bone marrow was aspirated and diluted to Iscove’s Modified Dulbecco’s Medium (IMDM) containing heparin (Iscove *et al* 1974). Mononuclear cells (MNC) were isolated by gradient centrifugation, washed and resuspended in IMDM. The culture medium consisted of 0.8% methyl cellulose, 20% fetal calf serum, 1% delipidated and deionized bovine serum albumin, $10^{-4}$ M mercaptoethanol, 310 μg/ml fully iron saturated transferrin (Sigma-Aldrich), 20% human leukocyte conditioned medium prepared in IMDM, and IMDM. The growth of erythroid progenitors was stimulated with recombinant human erythropoietin 2 U/ml (EPO, Cilag AG). The growth of CFU-GM was stimulated with bladder cancer cell line 5637-conditioned medium described by Coutinho *et al* (1990). Plates were incubated at 37°C in a fully humidified atmosphere with 5% CO₂. Both BFU-E and CFU-GM were scored on the 14th day of culture. The final concentration of free iron in the culture medium was confirmed by the BDI-assay (von Bonsdorff *et al* 2002). For cultures with apotransferrin FeNTA was preincubated for 60 min in 37°C with 4 mg/ml of apotransferrin.

6.12. Statistical analyses

In study II the differences of the transferrin saturation levels and the proportion of the NTBI-positive samples between the different study groups were analyzed by using the Kruskal-Wallis test. In Study IV the statistical differences were determined by two-way Anova for repeated measurements to test null hypothesis regarding the effects of between-subject factor (dose group), the within-group factor (day), and the interaction between them. *Post hoc* tests were used to evaluate differences between the dose groups. The influence of apotransferrin infusion on serum iron levels were tested by paired *t*-test. The Wilcoxon signed ranks test was used in the cell culture study (Study V) to determine the significant level of the nitrilotriacetic acid (FeNTA) concentration which has an effect on the number of erythroid burst-forming unit (BFU-E) colonies.
6.13. Approvals

The studies were approved by the Ethics Committee of the Helsinki University Central Hospital. The apotransferrin studies were also approved by the National Agency for Medicines. All patients were enrolled after giving their informed consent. The use of the donated cells also in scientific studies was approved by the bone marrow donors.
7. RESULTS

7.1. NTBI during allogeneic HSCT (I)

The baseline samples were collected on day -10 to -6, and the daily samples from the day after the start of conditioning, i.e. from day -5 on, until day +14 in relation to the transplantation (day 0).

During the first two days after starting the conditioning the mean total iron concentration doubled from the initial level to a peak on day -4 before transplantation. The transferrin levels decreased continuously and fully saturated transferrin was observed with the occurrence of NTBI by day -4. The mean transferrin saturation remained above 80% thereafter. NTBI was detectable in the majority of patients until day +11, remaining present in half of the patients through the study period until day +14. NTBI was detectable on average for 14.2 days (6-18 days), representing 77% (30-100%) of the samples. In the three patients who were NTBI-positive already at baseline, the transferrin saturation was already then above 80%.

NTBI was detected in 88% of the samples with transferrin saturation above 80% and only in 8% of the samples with transferrin saturation below 80%. Thus we found the transferrin saturation of 80% to be a significant threshold for the appearance of NTBI.

7.2. NTBI during autologous HSCT (II)

The serum samples referred to as baseline were collected 0-3 days before the start of the preparative regimen. Further samples were collected daily with routine sampling during the first 20 days after the onset of each regimen; the mean number of samples per patient was 18.5 (range 16-21).
The mean transferrin levels were within the normal range at baseline, started to descend from the beginning of chemotherapy, and decreased through the study period in all the different aHSCT-groups.

The mean transferrin saturation levels were also within the normal range at baseline. After a steep rise within the first days after the initiation of conditioning, the maximum levels were reached approximately at day 4-6 in all groups. The levels started to descend approximately one week after the beginning of each regimen. The transferrin saturation level of the melphalan-group was significantly lower than in the BEAC-group ($p= 0.0002$).

None of the patients were NTBI-positive at baseline, and three of them (1 melphalan monotherapy, 2 melphalan/TBI) remained NTBI-negative through the whole study period. In all the other patients NTBI was detected variably, the mean time from the first to the last NTBI-positive sample in the aHSCT-group as a whole being 6.1 days. Overall, NTBI was detected in 22% of the samples. The disappearance or marked reduction of the NTBI-positive samples coincided with the recovery of the bone marrow function demonstrated by the rise in the leukocyte counts.

7.3. NTBI during chemotherapy (II)

The baseline samples were collected 0-3 days before initiating the treatment and the samples were taken thereafter daily with routine sampling for 20 days. The mean number of samples per patient was 15.5 (7-21). The mean transferrin and transferrin saturation were below the reference range at baseline, and the transferrin level remained subnormal thereafter. The mean transferrin saturation showed a steep rise above 90% by day 3 from the start of chemotherapy, and remained above 80% through the study period.

NTBI was detected already at baseline in nearly 40% of the samples. The proportion of the NTBI-positive samples remained high during the whole study period, with an overall proportion of 59%. The average duration of NTBI-positivity was 15.6 days. No leukocyte recovery was seen during the study period.
7.4. Effects of a single intravenous dose of apotransferrin (III)

A single dose of 100 mg/kg of human apotransferrin was given as a slow (30 min) injection in a deep vein catheter on day +3 post-HSCT. Serum transferrin, total iron, NTBI, transferrin saturation and transferrin iron forms were determined from blood samples drawn immediately prior to the study drug administration, and at 15 min, 2 h and 12 h after the study drug injection. Thereafter the samples were drawn daily in the morning for the first 6 days and then on day +8, +10 and day +12 after the study drug administration.

7.4.1. Transferrin

The serum transferrin level before the apotransferrin injection was below the lower reference limit in all patients, the mean being 1.51 g/l (1.07-1.74 g/l). After the apotransferrin administration, the serum concentration increased by an average of 1.95 g/l (1.48-2.42 g/l). The mean serum transferrin at the 15 min sample was 3.46 g/l (3.06-4.04 g/l), with a rapid decline during the first day and slower decline thereafter. The calculated provisional elimination-phase half-life between days 1 and 5 after the administration was 4.8 days.

7.4.2. Iron

The mean serum iron level rose from an initial 35 μmol/l (24-38 μmol/l) to a peak value of 53 μmol/l (26-66 μmol/l) at 24 hours after the injection.

7.4.3. Transferrin saturation

The transferrin saturation decreased from the mean of 91% (87-94%) to 42% (30-49%) 15 min after the apotransferrin injection. After 2 hours the transferrin saturation started to increase in all patients, but in an individually variable course. The saturation exceeded 80% in three patients 12-24 hours after the injection, in one patient 2 days, and in another patient 6 days after the apotransferrin dose. In one patient the transferrin saturation remained below 80% during the whole 12 day follow-up period.
7.4.4. NTBI

Before the apotransferrin injection all samples were positive for NTBI. Immediately after the apotransferrin dose, serum NTBI became undetectable in all patients. One patient remained NTBI negative through the 12-day follow-up period. NTBI reappeared 12-24 hours after the apotransferrin injection in 4 patients and after 6 days in one. In these five patients NTBI reappeared showing a close association with the transferrin saturation increase above 80%. NTBI remained undetectable in the one patient whose transferrin saturation levels did not exceed 80%.

7.4.5. (Apo)transferrin conversion

The conversion of the given apotransferrin into monoferric and diferric transferrin forms was monitored by urea gel electrophoresis in sequential serum samples after the injection. At 15 min iron-free and monoferric forms appear in the sera of all patients. The amount of the diferric transferrin remained relatively unchanged in five patients, but was reduced in one. Later the iron-free form disappeared first, the monoferric form remained detectable longer, while the amount of the diferric form increased, indicating apotransferrin conversion first to monoferric and further to diferric form. The conversion coincided with the increase in the transferrin saturation.

The amount of iron bound by the administered apotransferrin during the first 15 minutes was, on average, 16 μmol/l (11-22 μmol/l), as calculated from the amount of formed monoferric transferrin.

7.4.6. Ferritin

Four of the six patients had their serum ferritin levels above the reference range before the beginning of the myeloablative regimen. Three of them had serum ferritin level > 1000 μg/l. A correlation was found between the baseline serum ferritin and the occurrence of NTBI after the apotransferrin dose. The patients with ferritin > 1000 μg/l had NTBI in 72% of serum
samples, whereas only 19% of the samples were NTBI-positive when baseline ferritin was <1000 μg/l \( (p < 0.0001) \).

7.5. Effects of repeated apotransferrin administrations (IV)

Twenty patients received repeated doses of apotransferrin. The ten patients in our first study (Study I) who underwent HSCT but were not given apotransferrin were used as controls.

7.5.1. Low and medium dose of apotransferrin

In the first part of the study, twelve patients were randomized to receive the study drug at two dose levels. The low-dose group \( (n= 6) \) received a total dose of 340 mg/kg, given as a single loading dose of 100 mg/kg on day -6, followed by nine maintenance doses of 26 mg/kg (mean) every other day, until day +12. The medium-dose group \( (n= 6) \) received a total dose of 610 mg/kg, given as four loading doses of 100 mg/kg on four consecutive days starting on day -6, followed by eight maintenance doses of 26 mg/kg (mean) every other day until day +13.

7.5.2. High dose of apotransferrin

In the second part of the study, eight patients received a total dose of 1040 mg/kg, given as four daily doses of mean 115 mg/kg from day -6 until day -3, continuing every second day until day +7 after HSCT.

7.5.3. Transferrin

As a result of the apotransferrin administration, the transferrin levels during the administration period were significantly higher in the high-dose group than in the groups given lower doses or in the controls, whereas there were no significant differences between
the low and medium-dose groups and the controls. In the high-dose group, the initial average level of transferrin was 2.2 g/l, after the first two to three apotransferrin doses it increased to > 4.0 g/l and remained between 3-6 g/l during the 10-day period when infusions were given every other day. This maintenance level of transferrin was in average 3.0 g/l (95% confidence interval (CI) 2.2-2.8 g/l) higher than in the control group during the dosing period until day +7 after transplantation.

7.5.4. Iron

In the high-dose group the mean serum iron level increased from the mean pretreatment level of 11 μmol/l to 32 μmol/l after the first infusion and to 80 μmol/l after the second infusion. Thereafter there was individual variation, the levels reaching >100 μmol/l in five patients, and remaining constant or decreasing in the other three patients. The iron concentrations were at their peak on the second day after the start of conditioning, with the mean increase in serum iron being 48 μmol/l/d.

The serum iron levels rose significantly higher in the high-dose group than in the low-dose group (p <0.05) and controls (p <0.01). Although there was a dose-dependent trend in the mean iron levels in the low- and medium-dose groups, the groups did not differ significantly from each other or from the controls.

After the initial rise, the serum iron levels remained on a highly elevated level for 10 days (from day -4 to day +5), mainly returning into the reference range by day +14. This coincided with the appearance of reticulocytes in the peripheral blood, which took place between days +6 and +14 in most patients.

7.5.5. Transferrin saturation

In the high-dose group the transferrin saturation was significantly lower than in the controls. The mean transferrin saturation was 70% during the period when the serum iron was highly elevated (from day -4 to day +5). During this period the average difference to the control group was 22.6% (95% CI 3.7- 41.4%). The average increase in transferrin saturation in the
high-dose group was 49% during the 4 days after the initiation of the conditioning. Calculating from the increment of iron saturation and the amount of endogenous and administered apotransferrin, an average 180 μmol of iron per day was bound to transferrin during the first 4 days after the start of conditioning regimen.

7.5.6. NTBI

In the control group the patients were NTBI-positive for most of the study period, the mean proportion of NTBI positive days being 75% (95% CI 60-92%).

In the high-dose group five of the eight patients had transferrin saturation < 80% and were NTBI negative through the study period. The average proportion of NTBI-positive days was 20% (95% CI 0-43%), which was significantly lower than in the control group ($p=0.001$).

7.6. Tolerability and adverse effects of apotransferrin administration (III, IV)

7.6.1. Single dose

No serious adverse effects during the 12-day follow-up period were noted. There were no clinically significant changes in the vital signs in association with the injection, and the immediate general tolerability was good. One improbable association with the study drug in the form of a mild, spontaneously subsided chilly feeling in one patient was discovered.

7.6.2. Repeated doses

No serious adverse events were encountered. In the high-dose group 228 non-serious adverse effects were recorded during the 21-day follow-up period. The maximum intensity was graded severe in six events and moderate or mild in all the other. The causal relationship with the study drug was assessed as not related in 223 events, improbable in four and possible in
one (serum alanine aminotransferase elevation). In none of the events was the causality assessed as probable.

7.7. Effect of NTBI on erythroid and granulocyte-macrophage colony formation; counteraction with apotransferrin (V)

Erythroid colonies of 17 and granulocyte-macrophage colonies of nine healthy bone marrow transplant donors were cultured from bone marrow samples without or with varying concentrations of free iron (FeNTA). In the cultures without FeNTA the median number of BFU-E colonies was 138 (range 60-251) and that of CFU-GM colonies 293 (range 163-514) per $1 \times 10^5$ cells.

7.7.1. Effect of FeNTA

The addition of FeNTA to the cultures suppressed both BFU-E and CFU-GM colony formation in a dose dependent manner. Besides colony numbers, also the size of the erythroid colonies decreased in the presence of free iron. Most colonies seen in the cultures with FeNTA concentrations $>30$ μmol/l were very small, though bright red indicating hemoglobinization. Free iron also reduced the number and size of granulocyte-macrophage colonies in a dose-dependent manner.

7.7.2. Effect of apotransferrin

The possible protective effect of transferrin was studied in seven BFU-E and five CFU-GM cultures with different iron concentrations, with or without the preincubation of FeNTA with apotransferrin. The binding of free iron to transferrin reduced statistically significantly the inhibition of colony formation in erythroid and granulocyte-macrophage cultures; the colony numbers and the size of the colonies normalized.
In the present series of studies we evaluated prospectively the appearance of non-transferrin-bound iron and its timing during the peritransplantation period in HSCT. For comparison, we also investigated the occurrence of NTBI after conventional combination chemotherapy. To measure NTBI, the bleomycin-detectable iron method was used. We studied whether it is possible to eliminate or reduce the appearance of NTBI with apotransferrin administration, what the kinetics of apotransferrin in this context is, and whether this novel approach is safe. The effect of NTBI on hematopoietic progenitors in *in vitro* cultures was also studied.

We found NTBI in all patients shortly after the start of conditioning for allogeneic stem cell transplantation and the appearance of NTBI to coincide with completely saturated serum transferrin. In our study we could demonstrate, for the first time, the exact timing and duration of the changes in transferrin saturation and the appearance and the length of the presence of NTBI in the peritransplantation period. We could also show and confirm the 80% saturation of transferrin to be a reliable threshold for NTBI.

We used the BDI assay for NTBI measurement, because it has a high specificity for NTBI, does not measure ferritin-bound iron, and because it measures the biochemical reaction between bleomycin and the redox-active iron. This method gives clearly lower NTBI levels than methods based on the mobilization of NTBI with a chelator and determination of the ultrafiltered iron-chelator complex. Therefore our results for NTBI concentrations were lower than those shown in previous reports (Bradley *et al* 1997, Dürken *et al* 1997). In these reports NTBI was measured using chelation with nitrilotriacetic acid, a step that also can remove a fraction of transferrin-bound iron, and therefore may show false positive results.

In our studies NTBI was also detectable in the majority of patients undergoing autologous stem cell transplantation and the intensity of the conditioning also seemed to have an effect on the appearance of NTBI. Patients conditioned with melphalan monotherapy had a lower proportion of NTBI-positive samples than those given melphalan with TBI or BEAC.
A clear finding was that the presence of NTBI coincided with bone marrow suppression, and NTBI largely disappeared at the recovery of the bone marrow function and reutilization of iron by the recovering marrow. Our observation of the normalization of the transferrin saturation with the same timing, despite the continuous decrease in the transferrin concentration, also supports this conclusion.

Patients who were treated with intensive cytotoxic chemotherapy, but did not receive stem cell rescue, experienced the longest bone marrow suppression and the most prolonged presence of NTBI, and showed complete saturation of transferrin. The coinciding presence of NTBI and neutropenia has also been reported by Harrison et al (1994). The appearance of NTBI after high-dose chemotherapy as a consequence of halted erythropoietic activity has been originally suggested by Bradley et al (1997). Other possible causes for extracellular iron accumulation after myeloablative conditioning include the destruction of malignant cells and hepatocellular injury (Evens et al 2004).

The cytotoxicity of NTBI, which has been suggested to play a role in the pathogenesis of many injuries associated with intensive cytotoxic therapy, has been confirmed by several lines of evidence (Anderson 1999, Smirnov et al 1999). Ferric iron has been shown to cause cytotoxicity after a few hours in liver cell cultures (Sakurai and Cederbaum 1998, Morel et al 1990). In our in vitro-study we found that NTBI caused a significant suppression of the colony formation of normal hematopoietic progenitor cells. Preincubation with apotransferrin partly prevented the toxic effects of free iron on erythroid and granulocyte-macrophage colony formation.

The effect of binding NTBI with apotransferrin and the possible influence on iron-induced complications was the rationale behind our studies with exogenous apotransferrin given to patients undergoing allogeneic stem cell transplantation. To our knowledge, this setting was the first use of any iron-chelating agent in HSCT patients.

We found that with a single apotransferrin dose NTBI disappeared from the sera of all patients, indicating that it was effectively bound. The effect was also demonstrated by the conversion of transferrin into monoferric and diferric forms in the sequential serum samples after the apotransferrin injection. The stable appearance of diferric transferrin suggested that the iron bound by the given apotransferrin represented NTBI and was not shuttled from fully
saturated endogenous transferrin. NTBI remained undetectable for a variable time, ranging from a few hours to several days. In most patients the reappearance of NTBI occurred 12-24 hours after the apotransferrin injection.

NTBI probably comprises heterogeneous iron complexes which may differ in their binding to chelating agents and whose proportions may differ in different clinical conditions (Breuer et al 2000a, Breuer et al 2000b). The disappearance of bleomycin-detectable NTBI after the apotransferrin injection indicated that the redox-active iron in the sera of the HSCT patients was in a form that was effectively bound by transferrin.

The apotransferrin injection resulted in a temporary rise in the serum total iron concentration, which continued at a variable rate and leveled off at 24 h in most patients. This may indicate that iron was not only released from the catabolism of senescent erythrocytes, but also mobilized from insoluble complexes by the given apotransferrin.

With repeated doses of apotransferrin, the highest doses given prevented full transferrin saturation in five of the eight patients studied. Based on the increment of iron saturation and the amount of endogenous and administered apotransferrin, it could be concluded that an average of 180 μmol/d of iron was bound to transferrin during the first 4 days after the onset of conditioning. Subsequently, the iron accumulation leveled off in most patients indicating that a new equilibrium was reached.

Repeated apotransferrin doses were followed by a much steeper rise in serum iron than the increment that occurred in the control patients. The increase in serum iron was in concert with the amount of apotransferrin given, which may suggest that apotransferrin induced iron release from tissues. However, during the period of highly elevated serum iron, apotransferrin doses were followed by a significant increase in serum iron only in patients with fully saturated transferrin and NTBI in their serum, whereas no significant change in serum iron took place in the patients with partially saturated transferrin. This suggests that, rather than inducing iron release from cells, apotransferrin may have rendered the poorly soluble NTBI into a transferrin-bound, soluble form, thus causing the increase in measurable iron.
Supporting the conclusion that apotransferrin did not induce iron release from macrophages, most (Esparza and Brock 1981, Brock et al 1984, Saito et al 1986), but not all (Rama et al 1988) in vitro studies with monocyte/macrophages have failed to show any effect of excess apotransferrin or transferrin or transferrin saturation on iron release. Furthermore, giving large amounts of apotransferrin to animals did not modify iron donation by tissue (Lipschitz et al 1971, Finch et al 1982). In human studies no correlation between transferrin saturation level and release of radioactive iron to plasma has been observed (Stefanelli et al 1984, Fillet et al 1989).

The results of our studies underline the transient erythroid arrest as the main reason for iron accumulation, as the majority of the patients studied were in complete remission and most had no signs of liver injury. Furthermore, the rapid normalization of the highly elevated serum iron levels concomitantly with the start of graft erythropoiesis supports the close association between extracellular iron accumulation and erythropoietic activity.

It remains unknown whether maintenance of the released extracellular iron in transferrin-bound form is less harmful than the accumulation of NTBI during the erythropoietic arrest following myeloablation. The maintenance of iron in the transferrin-bound form should prevent its rapid intake by the liver and other non-hematopoietic tissues and the consequent iron-induced toxicity. Apotransferrin binds NTBI into a physiological form that can be utilized by the recovering erythroid marrow. As the erythroid progenitors take up a lot of transferrin-bound iron and, as we have shown, the HSCT patients have low endogenous transferrin level, increase in the circulating transferrin pool might promote the recovery of the graft. Also, in murine in vivo-models pretreatment of mice with transferrin protected marrow cells against gamma-irradiation-induced death (Lesnikov et al 2001) and prevented Fas-mediated hepatocyte death and liver failure (Lesnikov et al 2004). The cytoprotective effects were also shown in our own in vitro hematopoietic colony formation studies with excess iron without and with preincubation with apotransferrin. Furthermore, transferrin withholds iron from most opportunistic bacteria and fungi, thus protecting patients from infections (Harrison et al 1994, Iglesias-Osma et al 1995, Matinaho et al 2001). We have also shown that apotransferrin prevents the growth of Staphylococcus epidermidis in vitro by binding the NTBI in the sera of HSCT patients (von Bonsdorff et al 2003, von Bonsdorff et al 2004). Therefore, there are several mechanisms by which the administration of apotransferrin might
prove beneficial to patients treated with intensive cytotoxic treatment, including HSCT patients.

The feasibility, efficacy to prevent complications, administration policy and dosing of apotransferrin in the clinical setting remain to be evaluated. Our study with repeated apotransferrin administrations showed that the highest dosage used was effective in binding NTBI in most patients, and this administration policy might offer a baseline for such studies.
9. SUMMARY

We showed that NTBI appears regularly during the peritransplant period in HSCT. This is largely due to reduced utilization of iron by erythropoiesis and the reduction of serum transferrin levels caused by the transplantation process. As NTBI might contribute to the complications of HSCT, we assessed the possibility to bind NTBI during the peritransplant period with apotransferrin infusions. We showed that the appearance of NTBI could be prevented or reduced by apotransferrin. Our in vitro study demonstrating the toxic effect of NTBI on hematopoietic progenitors and the beneficial effect of binding NTBI with apotransferrin on colony growth supports the possibility that binding of NTBI with apotransferrin infusions may turn out to be clinically useful. The clinical utility of apotransferrin administration and the effects on transplantation outcomes remain to be investigated in further studies.
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