From the Division of Clinical Immunology and Transfusion Medicine at the Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

The panorama of infections in immunocompromised patients and in patients with an increased susceptibility to infections

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From
THE DIVISION OF CLINICAL IMMUNOLOGY AND TRANSFUSION MEDICINE
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THE PANORAMA OF INFECTIONS IN IMMUNOCOMPROMISED PATIENTS AND IN PATIENTS WITH AN INCREASED SUSCEPTIBILITY TO INFECTIONS

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ABSTRACT

The main objective of this thesis was to contribute to immunomodulatory interventions that are important for the clinical outcome in patients undergoing haematopoietic stem cell transplantation (HSCT) and in patients with antibody deficiency or increased susceptibility to infections.

Graft-versus-host disease (GVHD), infections, and relapse of the underlying disease are the main complications after allogeneic haematopoietic stem cell transplantation. In study I, we retrospectively examined 179 patients who had undergone HSCT, with the aim of evaluating the effects and kinetics of IgG levels after HSCT. We concluded that IgG levels increased after transplantation throughout the five-year study period. Patients with low IgG levels (< 4 g/L) on two occasions during the first year after HSCT showed a reduced survival rate and an increased incidence of transplant-related mortality compared with patients with normal levels. Persistently low levels of IgG were found to be a risk factor for death after HSCT.

In vivo T-cell depletion with anti-thymocyte globulin is a commonly used strategy for the prevention of GVHD and to avoid rejection after transplantation. In study II, we retrospectively compared 36 patients given Campath as part of the conditioning with a matched cohort of 72 patients receiving thymoglobulin (TMG). The objective was to compare the two different drugs with regard to clinical outcome after HSCT. No differences in transplant related mortality, overall survival, or relapse-free survival were found between the two groups. Furthermore, Campath was associated with less overall acute GVHD but more chronic GVHD. Finally, we noted a trend towards more fungal infections in the patients treated with Campath. In conclusion, TMG and Campath as part of the conditioning appear to result in a similar clinical outcome.

Low serum levels of vitamin D are associated with an increased risk of respiratory tract infections (RTIs). To date, clinical trials with vitamin D against various infections have not been conclusive. Thus, our objective was to investigate whether supplementation with vitamin D could reduce infectious symptoms and antibiotic consumption in patients with antibody deficiency or increased susceptibility to RTI. 140 patients with > 42 days of symptoms from the respiratory tract over a 12-month period prior to inclusion were included in study III. They were randomized to receive vitamin D (4,000 IU) or placebo daily for 1 year. The primary endpoint was an infectious score based on five parameters: symptoms from the respiratory tract, ears and sinuses, malaise, and antibiotic consumption. Secondary endpoints were serum levels of 25(OH)D, microbiological findings, and levels of anti-microbial peptides (AMPs) in nasal fluids. The key message from this study was that vitamin D supplementation reduced symptoms and antibiotic consumption in patients with an increased frequency of respiratory tract infections.

The role of vitamin D in HSCT is not fully understood. To address this issue, we designed a study which included 123 children who were followed retrospectively for up to 8 years after HSCT. The aim of study IV was to determine whether vitamin D levels at baseline were associated with short- and long-term outcome parameters. Vitamin D

deficiency was defined as a pre-transplant level of 25(OH)D below 50 nmol/L. We found that the frequency of acute GVHD was higher and that of chronic GVHD lower in patients with sufficient vitamin D levels (> 50 nmol/L) compared with those with vitamin D deficiency. In patients transplanted due to malignancies, overall survival was higher in the group with sufficient vitamin D levels. We also found that relapse was more common in the insufficient-level group. Since vitamin D deficiency was associated with an increased risk of death, relapse, and chronic GVHD, we concluded that baseline vitamin D status appears to influence the clinical course in children undergoing HSCT. However, randomized and placebo-controlled trials will fully clarify this issue.

The main conclusions are that (1) low IgG levels are a risk factor for death after HSCT, (2) TMG and Campath as part of the conditioning before HSCT result in similar outcome, (3) supplementation with vitamin D may reduce disease burden in patients with frequent RTIs, and (4) vitamin D appears to affect the clinical outcome in children undergoing HSCT.

LIST OF PUBLICATIONS

I. A-C Norlin, D Sairafi, J Mattsson, P Ljungman, O Ringdén, M Remberger. Allogeneic stem cell transplantation: low immunoglobulin levels associated with decreased survival.
Bone Marrow Transplantation 2008;41:267-273.

II. A-C Norlin, M Remberger. A comparision of Campath and Thymoglobulin as part of the conditioning before allogeneic hematopoietic stem cell transplantation.
European Journal of Haematology 2010;86:57-66.

III. P Bergman, A-C Norlin, S Hansen, R S Rekha, B Agerberth, L Björkhem-Bergman, L Ekström, J D Lindh, J Andersson. Vitamin D_3 supplementation in patients with frequent respiratory tract infections: a randomized and double-blind intervention study.
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CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
AMP	Anti-microbial peptide
APC	Antigen-presenting cell
ATG	Anti-thymocyte globulin
Bu	Busulfan
CI	Confidence interval
CMV	Cytomegalovirus
CsA	Cyclosporine
CTL	Cytotoxic T-lymphocyte
Cy	Cyclophosphamide
DC	Donor chimerism
DCs	Dentritic cells
DLI	Donor lymphocyte infusion
GC	Vitamin D binding protein
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukaemia
EBV	Epstein Barr virus
Flu	Fludarabine
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
HSV	Herpes simplex virus
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IU	International units
IVIG	Intravenous immunoglobulin
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAC	Myeloablative conditioning regimen
MBL	Mannose-binding lectin
mHAg	Minor histocompatibility antigen
MTX	Methotrexate
MUD	Matched unrelated donor
Ns	Not significant
OR	Odds ratio
OS	Overall survival
PAMP	Pathogen-associated membrane patterns
PCR	Polymerase chain reaction
PRR	Pattern-recognition receptor
PTH	Parathyroid hormone
PTLD	Post-transplant lymphoproliferative disorder
RIC	Reduced-intensity conditioning
RTI	Respiratory tract infection

SNP	Single nucleotide polymorphism
TBI	Total body irradiation
TCR	T-cell receptor
TLR	Toll-like receptor
TMG	Thymoglobulin
Tregs	Regulatory T-cells
TRM	Transplant-related mortality
URD	Unrelated donor
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VOD	Veno-occlusive disease
VZV	Varizella-zoster virus

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system consists of a variety of cells and molecules that mediate defence against all sorts of pathogens and growth of virus-induced tumour cells. One can think of the immune system as “two lines of defence”. These two parts are called innate and adaptive immunity. While the innate immunity acts immediately, it takes a longer time for the adaptive immune response to fully mature. The adaptive immunity is characterized by clonal specificity and memory.

1.2 INNATE IMMUNITY

This is the first line of defence that is activated after an infection. When there is intrusion by a pathogen, the innate response is very quick, and it was originally thought to react in a similar way irrespective of how many times we become infected. In recent years, it has been shown that the innate system has an ability to remember previously encountered antigens (1). The innate immune system is a universal and ancient form of host defence against infection, and it relies on a limited number of germline-encoded receptors. These pattern-recognition receptors (PRRs) are made to recognize conserved products from different microorganisms and endogenous molecules such as uric acid and hyaluronic acid. The innate immune system is able to distinguish “infectious” non-self from non-infectious “self” (2). Toll-like receptors (TLRs) are a family of PRRs that are expressed by several different cell types such as dendritic cells (DCs) and macrophages. Activation of a TLR by recognition of a pathogen leads to activation of genes encoding inflammatory molecules, cytokines, and anti-microbial peptides (AMPs). The innate immune system also includes intracellular mechanisms as helicases (enzymes that recognize nucleic acids from for example viruses), and the protective barrier composed of the mucosa, cilia in the respiratory tract, and skin.

If a barrier is destroyed, an inflammatory response is initiated. The definition of inflammation is based on classical clinical signs such as elevated temperature, redness, pain, swelling, and loss of function. These symptoms arise due to the effect of inflammatory mediators, including cytokines and complement fragments, that cause dilatation of the vessels and increased permeability. The cytokines change the properties of the vessels in such a way that the circulating leukocytes can easily adhere to the walls of the vessels and later on—attracted by inflammatory mediators—migrate to the injured site.

Fever is a complex systemic response to infection and injury (3), and is mediated by IL-1, IL-6, and prostaglandins (PGE2) for example. Increasing body temperature confers protection by improving pathogen clearance *in vivo* (4-6). A body temperature above 39°C activates cellular signalling and mobilization of cells to sites of inflammation.

1.2.1 Antimicrobial peptides (AMPs)

AMPs are considered to be effector molecules of the innate immune system, acting as a first line of protection against invading pathogens (7-9). They protect the mucosal and

dry epithelial surfaces of all multicellular organisms (10). The fundamental structural principle underlying all classes of AMPs is the ability of the molecule to adopt an amphipathic character, i.e. to have clusters of hydrophobic and cationic amino acids spatially organized in discrete sectors of the molecule. In humans, two major classes of AMPs have been described: defensins and cathelicidins (10). Alpha-defensin -1, -2, and -3 (also known as HNP1-3) are AMPs found in human neutrophils (11). The targets for most AMPs are the membranes of invading microbes. Bacterial membranes are organized in such a way that the outermost leaflet of the bilayer, the surface exposed to the outer world, is covered by lipids with negatively charged phospholipid head groups. In contrast, membranes of eukaryotic cells are mostly composed of lipids with no net charge (neutral). This model proposes interaction of the peptide with the pathogen membrane, followed by displacement of lipids and in some cases entry of the peptide into the invading pathogen. It has also been demonstrated that some AMPs can promote epithelial growth and angiogenesis (12). Both cathelicidins and defensins have been shown to have the capacity to act as chemokines, substances that attract circulating and migrating cells. Defensins can attract dendritic cells and T-lymphocytes to sites of infection, and LL-37—the sole human cathelicidin—has the ability to attract neutrophils (13, 14).

1.2.2 The complement system

The complement molecules represent a large group of plasma proteins that act in a self-activating way. These proteins are secreted by liver cells and monocytes. Activation can occur by several means: (1) direct activation on a microbial surface, which is known as the alternative pathway, (2) by antibodies bound to foreign surfaces, which is known as the classical pathway, and finally (3) by the lectin pathway, which is induced by an interaction between microbial carbohydrates and mannose-binding lectins (MBLs) in plasma.

The nomenclature is derived from the fact that the proteins help (“complement”) the antibody response. C3 is the main complement protein and its activation is required for both classical and alternative complement activation pathways. When C3 binds to an antigen, an enzymatic conversion to C3b occurs. Since macrophages have receptors for C3b, the foreign microbe will either be opsonized or C3b can act as a focus for other soluble plasma factors. C3b induces formation of a membrane attack complex (MAC), which kills the microbe by cellular lysis. Other results of complement activation can be recruitment of inflammatory cells and stimulation of B-lymphocytes to produce antibodies (15).

1.2.3 Cells of the innate immune system

Neutrophils and macrophages are two main types of phagocytes in the innate immune system. They share the same function—to engulf and kill microbes. The phagocytic process is enhanced by stimulation of receptors for antibodies and complement. The characteristics of neutrophilic granulocytes are a multilobed nucleus and the presence of granules in the cytoplasm. The granules contain molecules required for successful elimination of microbes. The macrophage, which is the mature form of a monocyte, is mononuclear and has migrated from the blood to a tissue. Unlike neutrophils, which survive only a few days, the macrophages are long-lived cells and can survive for

several weeks. In addition to engulfing particles (cellular debris and microbes), macrophages have an ability to present antigen to T-cells. A successful immune response depends on a series of specific interactions between a T-cell and an antigen-presenting cell (APC). Although the first essential step in activating a T-cell is the binding of an MHC-peptide complex on an APC (signal 1), this interaction alone is not sufficient. Co-stimulatory signals (signal 2) are necessary to ensure an effective immune response and they are provided by molecules such as CD80 and CD86 expressed on the surface on APCs (16). Stimulation by cytokines (signal 3) is also essential for T-cell activation.

1.2.4 Dendritic cells

DCs, named for their probing, treelike or dendritic shapes, are pivotal for both recognition of a universe of antigens, and control of an array of responses (17). They are specialized to capture and process antigens, converting proteins to peptides that are presented on MHC molecules and recognized by T cells. DCs are an important bridge between innate an adaptive immunity, and can be divided into different groups. Plasmacytoid DCs are found in, for example, bone marrow, blood, and thymus. Migratory DCs are found in skin, for example, and resident DCs are mostly located in the spleen. All types of DCs are professional APCs since they present antigenic peptides to T-helper cells. DCs carry receptors that recognize antigen patterns (18). They process the antigens and present it to T-cells in a neighboring lymph node. Mature DCs can produce cytokines that affect both the innate and the adaptive immune response. Depending on the context, DCs produce different cytokines that in turn affect the function of the T-cells. When DCs ingest neutrophils infected with bacteria, they secrete TGF-β, IL-6, and IL-23 cytokines, leading to the differentiation of Th17 cells, which promote inflammation. In addition, engulfment of uninfected, apoptopic neutrophils leads to secretion of TGF-β and IL-10 by DCs, thus promoting *in vitro* differentiation of T cells to induced regulatory T-cells, which suppress immune responses (19, 20).

1.2.5 NK-cells

Natural killer cells are lymphocytes that comprise 5-10 % of the total lymphocyte fraction. They are characterized by the surface expression of CD56 and CD16, while lacking CD3. The role of this cell type is to attack and kill virus-infected cells in addition to certain types of tumour cells. NK-cells differ from other lymphocytes since, without antigen specific recognition, they can kill the target (21). Their activation is controlled by inhibitory receptors on their cell surface, which recognize molecules of MHC class I (22). This means that NK-cells are prevented from killing healthy host cells but they do kill cells that are infected by a virus or that are cancerous, since those cells do not express MHC class I molecules in a normal way. For killing, NK-cells use the granzyme and perforin granule exocytosis pathway or they express ligands for death receptors on the target cell (23). Another killing mechanism used by NK-cells is antibody-dependent cellular cytotoxicity (ADCC). NK-cells express a low-affinity receptor for the Fc part of the IgG molecule and can therefore destroy cells via antibodies (24). In addition, NK-cells can stimulate the immune response by release of different cytokines.

1.2.6 Inflammasomes

The inflammasome is expressed in myeloid cells, and is a component of the innate immune system. It is a large cytoplasmic complex responsible for activation of inflammatory processes (25), and has been shown to induce pyroptosis, a programmed cell death that requires the function of caspase-1 (26).

1.3 ADAPTIVE IMMUNITY

The adaptive immune system comprises two major cell types: B-lymphocytes and T-lymphocytes. Compared to innate immunity, the adaptive immunity often needs several days to fully mature. The memory of the adaptive immune system persists after the infection has been eradicated, which guarantees a more effective and rapid immune response if the host is reinfected with the same type of microbe.

1.3.1 Cytokines

Most cytokines are small- to medium-sized proteins or glycoproteins, which have potent biological effects on many cell types. They are released by several cells in the immune system and in general the function of cytokines is to coordinate the action of different cell types participating in the immune and inflammatory responses.

1.3.2 T-lymphocytes

T-cells can be broadly divided into T-helper cells (CD4+) and cytotoxic T-cells (CD8+). Other important T- lymphocytes are Th17 cells and regulatory T-cells. All T-cells express the co-receptor complex CD3. The development of T-cells does not take place in the bone marrow. Instead, undifferentiated progenitors migrate from the bone marrow to the thymus, where they develop into T-cells. The immunological function of the thymus was first described in 1961 (27). During development in the thymus, T-cells undergo positive and negative selection. In positive selection, the T-cells are licensed to be able to recognize but not react against autologous molecules. In negative selection, potentially self-reactive T-cells are destroyd (28). After this selection, T-cells that survive are able to recognize “self” molecules but will not react against themselves (i. e. there is a tolerance). The T-cells that have passed the selection will migrate to the periphery and to the secondary lymphoid organs. The T-cell receptor is special in that it is only able to identify an antigen when it is associated with a major histocompatibility complex (MHC) molecule on the surface of the cell. MHC class I molecules are found on all nucleated cells and class II molecules are found on all APCs.

1.3.3 CD4+ cells

CD4+ cells have the capacity to recognize MHC class II-associated peptides—for example, foreign antigens presented by a dendritic cell. After stimulation, CD4+ cells start to produce cytokines that will initiate an immune response. T-helper cells are subdivided into two distinct populations, Th1 and Th2 types, defined by the array of cytokines being produced (29). The Th1 type mainly secretes IL-2, TNF-β, and interferon (IFN)-gamma, and the Th2 type IL-4, IL-5, IL-6, IL-10, and IL-13. Th1 cells have the capacity to activate macrophages and therefore the cell-mediated immune defence. Th2 cells activate B-cells and enhance production of antibodies. Each of these Th subtypes suppresses the other, because IFN-gamma from Th1 cells inhibits

proliferation of Th2 cells while IL-10 from Th2 cells impairs cytokine secretion from Th1 cells (30, 31).

Th17 cells (32) are a CD4+ cell type that produces IL-17 and IL-22. These cytokines induce tissue cells to produce AMPs and chemokines, which leads to recruitment of inflammatory cells such as granulocytes and macrophages (33). Th17 cells develop from naïve CD4+ cells under the influence of a number of inflammatory cytokines such as IL-1, IL-6, and TGF-β (34). Th17 cells were first defined by their expression of IL-17 (IL-17A), but during the last few years they have also been reported to preferentially express IL-22, as well as IL-17F, IL-21, and GM-CSF (35, 36). Data have accumulated to suggest that Th17 cells play an important role in autoimmune diseases, different types of infections, and the adaptive immune response (37, 38). The polarization of Th17 cells is dependent on the action of IL-23 secreted by APCs (39, 40). An example of a primary immunodeficiency with a defect in differentiation of Th17 cells is hyper-IgE syndrome. This disease is mainly the result of a mutation in STAT3 (signal transducer and activator of transcriptor 3) and leads to an increased susceptibility to fungi and extracellular bacteria.

1.3.4 CD8+ cells

This is a type of T-lymphocyte that provides defence against intracellular pathogens and different types of virus infections. They eliminate target cells that present foreign peptide molecules (cytosolic pathogens) bound to MHC class I molecules on the cell surface. The release of granule contents accounts for most of the cytotxic activity of CD8 effector T-cells and Th 1 cells play an important role as helpers during CD8 T-cell responses.

1.3.5 Regulatory T-cells (Tregs)

To prevent autoimmune reactions in the body, most of the self-reactive T-cells are destroyed in the thymus. Nevertheless, some of these cells escape from the thymus and start to circulate out to the periphery. In mediation of peripheral tolerance and for control of the adaptive immune responses that may cause damage to self-tissues, regulatory T-cells have an important role (41). Naturally occurring CD4+CD25+ Tregs, characterized by the transcription factor FOXP3, are essential for prevention of autoimmune diseases by inhibiting T-cell proliferation. Type 1 Tregs, a subtype of Tregs that are inducible, exert their suppressive activity mainly through the release of IL-10. The primary task of type 1 Tregs is to inhibit T-cell responses (41).

1.3.6 B-lymphocytes

B-cells are lymphocytes that produce antibodies and recognize foreign antigens. Immature B-cells develop from lymphoid progenitor cells in the bone marrow. Later on, they migrate to secondary lymphatic tissue and an interaction with antigens and T-cells takes place. Resting mature B-cells express IgD and IgM on their surface. After activation, the B-cells switch to a specific isotype production of IgM, IgA, IgG, or IgE. The different antibodies serve as B-cell receptors that recognize antigens. When an antigen binds to an antibody on the B-cell surface, it will induce transmission of signals into the cell. The B-cell then processes the antigen, delivers it to intracellular sites, and

then presents the peptide bound to MHC class II molecules on the surface for detection/recognition by T-cells. Most antigens are not able to stimulate B-cells without the help of CD4+ T-cells. The foreign peptides bound to MHC class II molecules on the B-cell surface are recognized by T-cells, which became activated and express molecules, such as CD40L on their surface. The B-cell molecule CD40 binds to CD40L and stimulates antibody switching in the B-cell.

After activation, some B-cells become memory cells, resting in the bone marrow, while other cells turn into plasma cells, which can produce and secrete large amounts of specific antibodies (42). It is important that a significant proportion of them remain as memory B-cells since these cells are vital when the body becomes re-exposed for the antigen. Common variable immune deficiency (CVID) is a heterogeneous group of primary immunodeficiencies characterized by insufficient serum levels of immunoglobulin and a high incidence of repeat infections.

1.4 TRANSPLANTATION IMMUNOLOGY

There are several different types of transplantation, named after the relationship between the recipient and the donor. An autologous transplant uses the recipient's own cells, whereas an allogeneic transplant is a transplant between two individuals from the same species. Syngeneic grafts are genetically identical with the host—for example, stem cell transplantation between monozygotic twins. Transplantation across species barriers is called xenotransplantation.

As mentioned above, the immune system has the capacity to distinguish self from non-self. In a transplantation situation, the graft will be considered as non-self by the immune system of the host. The main reason for the host immune defence reacting against the transplanted cells is non-self HLA/MHC molecules. The immunological mechanisms that affect the graft are mostly mediated by the adaptive immune response in the host, i.e. T- and B-lymphocytes. When an allogeneic haematopoietic stem cell transplantation is performed, there is a risk that the immune cells in the graft will react against the host, which is called graft-versus-host disease (GVHD).

MHC genes are the most polymorphic of all mammalian genes. Since there are so many variants of each MHC gene, it is very difficult to find two unrelated individuals that share an identical set. The immune response to allogeneic MHC molecules is not fully understood.

There are two theories about the molecular mechanisms involved in direct allorecognition of MHC molecules. The “high determinant density” model proposes that alloreactive T-cells recognize the exposed polymorphic antigen directly on the allogeneic MHC molecule. If every allogeneic MHC molecule can serve as a ligand for an allospecific T-cell, then the density of antigen on the cell surface would be extremely high. The much higher ligand density available to the alloreactive T-cell means that receptors with a low affinity can respond to the foreign MHC molecule (43, 44). The second model, called the “multiple binary complex” hypothesis, suggests that the anti-MHC alloresponse is a conventional self-restricted immune response. This means that the response is mediated by T-cells that are specific for peptide/MHC

complexes. In this case, the peptide is a naturally processed peptide and the MHC molecule is allogeneic (45, 46). The set of peptides bound by an allogeneic MHC molecule often differs considerably from that bound by a self-MHC molecule. If each allo-MHC complex is recognized by a different alloreactive T-cell, then a single MHC incompatibility can stimulate numerous of alloreactive T-cells. Probably both of the above-mentioned models are involved in the overall allo-response.

Indirect allo-recognition is defined as the stimulation of T-cells by the recognition of processed peptides of the allogeneic MHC molecules themselves presented by self-MHC molecules (47, 48).

Apart from MHC proteins, other molecules are important in provoking a rejection or GVHD. Minor histocompatibility antigens (mHAg) are known to be derived from polymorphic non-MHC proteins that are presented by MHC molecules. Reactions to this type of antigen could be found as a transplant rejection or GVHD in MHC-matched transplants (49). An example of a well-known mHAg is a set of proteins on the male-specific Y chromosome.

The first year after HSCT is characterized by considerable immune deficiency, which is then followed by recovery. The deficiency starts with the conditioning regimen that destroys haematopoietic and mucosal progenitor cells. The patients lose T- and B-cell mediated immune memory, which results in a shortened duration of immunity unless a new antigenic challenge is given. The recovery of B- and T-cells will last for at least one or two years after transplantation (50, 51).

1.5 ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

The first allogeneic haematopoietic stem cell transplantation (HSCT) was performed over 50 years ago, and became feasible after the discovery of the HLA complex. E. Donnall Thomas et al. performed the first clinical HSCT studies in the 1950s, and they performed the first allogeneic bone marrow transplantation in humans in 1957 (52). Unfortunately, the early results were very disappointing (53). Many patients were in the late stages of leukaemia and did not survive long enough for evaluation. At the beginning of the era, it was also noted that engraftment could sometimes be obtained, but a lethal immunological reaction of the graft against the host emerged. Several years later, this lethal immunological reaction was known as GVHD. Dausset at al. (54) and van Rood et al. (55) discovered the HLA system in the 1960s and the ability to perform typing of HLA was a breakthrough in HSCT. Later on, in the 1970s, Thomas and his colleagues successfully performed bone marrow transplantations, using marrow from HLA-identical siblings, in patients with end-stage leukaemia (56). The occurence of GVHD was also discovered to have a positive effect in some cases, since it reduced the incidence of leukaemic relapse (57, 58).

The aim of transplantation is to replace a defective or diseased immune/ haematopoietic system with a new one from a healthy donor.

The procedure involves taking haematopoietic stem/progenitor cells from the donor, either from the bone marrow or from peripheral blood, and then transfusing them

intravenously into the patient. Haematopoietic stem cells (HSCs) are characterized by the ability to differentiate into all types of blood lineages. The most primitive HSCs express the molecule CD34 on their surfaces, and these are the target cells that are collected when both peripheral blood and bone marrow are used as the stem cell source.

Indications for HSCT include both malignant and non-malignant conditions, and more than 25,000 transplantations are performed every year with the purpose of treating lymphoma, leukaemia, immune-deficiency disorders, metabolic defects, sickle-cell anaemia, beta thalassaemia, and many other diseases (59).

Several serious main problems—GVHD, relapse, and infections — must be considered after HSCT.

1.5.1 Conditioning

Before HSCT, a conditioning therapy is given. The original aim of this treatment was to eradicate malignant cells but also to prevent rejection of the graft by suppressing the host immune system. Initial studies on animals investigated the threshold for total body irradiation (TBI), to achieve an immunosuppressive effect in order to permit allogeneic marrow engraftment. The conditioning regimens differ depending on the diagnosis and the local traditions of the hospital. In the 1970s, TBI and high-dose cyclophosphamide, which had been used earlier as two separate approaches, were combined to reduce the risk of relapse (60, 61). Follow-up studies showed promising results; more than half of the initial patients were free from disease five years after the transplant (62). In the 1980s busulfan offered an alternative to TBI (63). By individual dose adjustment and prophylactic treatment with anti-convulsants, side effects such as liver toxicity and proconvulsive effects were prevented (64). Attempts were made during the 1980s to combine different types of chemotherapy with TBI, to elevate the doses, and to add a third chemotherapeutic agent, but without success. Unfortunately, these efforts resulted in higher transplant-related mortality (TRM) and a significant increase in toxicity, and the overall survival of the patients did not increase at all (65-67).

Over the years, researchers have been able to show that transplantation of allogeneic stem cells—except the effect of myeloablative conditioning—also has an additional anti-leukaemic effect (57, 68). Studies have shown that the potent anti-leukaemic effect is due to an on-going reaction between malignant cells in the host and the transplanted allogeneic immunological cells (57, 58, 68, 69). Based on these results, a new era with reduced-intensity conditioning (RIC) started for older patients and for patients with poor medical conditions. RIC protocols where associated with significantly lower risk of TRM due to reduced organ toxicity. This protocol relies mainly on tumour cell killing (the graft-versus-leukaemia effect) and to a lesser extent on chemo-radiation therapy (70-73). However, the frequency of rejection of the graft increased when RIC therapy was introduced, and new combinations of chemotherapy drugs have therefore been tried (74).

There is an increased risk of GVHD and graft rejection when using unrelated donors (75, 76). The main reason is probably an increased alloreactivity due to differences in minor histocompatibility antigens and other tissue antigens. To overcome these

obstacles, at many centres the patients are treated with anti-thymocyte globulin (ATG) (77). These antibodies to T-cells reduce the number of—and modulate the function of—T-cells. Their main effect is to prevent rejection by inhibiting the host immune response, but they also reduce the risk of GVHD by their influence on donor T-cells. By adding ATG to the pre-transplant conditioning protocol, acute GVHD and early mortality of unrelated donor (URD) HSCT have been reduced to levels similar to those in matched, related HSCT (77, 78). Different doses and types of ATG are used today. However, the common mechanism of action is depletion of alloreactive T-cells in both the recipient and the graft, so-called *in vivo* T-cell depletion. Both the type and dose of ATG appear to be important for the outcome (79). There are basically three different types of ATG: thymoglobulin (TMG), Campath, and ATG-Fresenius.

TMG consists of rabbit immunoglobulins, and is produced by immunizing rabbits with fresh human thymocytes. The immunoglobulin fraction contains polyclonal antibodies to multiple cell-surface antigens (80, 81). TMG binds to T-cells and has a direct effect on them, resulting in T-cell depletion via opsonization and lysis following complement activation. To a lesser extent, TMG also binds to B-cells, macrophages, monocytes, and neutrophils (80, 81).

Campath is a humanized monoclonal antibody to human CD52, an antigen expressed on T-, B- and NK-cells, but (in contrast to TMG) not on other haematopoietic cells (82).

As with TMG, ATG-Fresenius consists of rabbit immunoglobulin. The rabbits are immunized with a lymphoblastic Jurkat T-cell line. Jurkat is a cell line of immortalized T-lymphocytes, originally from a 14-year-old boy with T-cell leukaemia. ATG-Fresenius, as TMG, binds not only to T-cells but also to other human cell-surface molecules (83). This agent is produced and mainly used in Germany.

Despite the proven effectiveness of the above-mentioned ATGs, their area of use is somewhat limited by the increase in infections and relapse that is associated with them.

1.5.2 Graft-versus-host disease

GVHD is one of the major challenges in patients after HSCT, and it is initiated by alloreactive donor lymphocytes recognizing the foreign histocompatibility complex of the host (84). Three criteria were defined by Billingham in 1966: (a) the graft must contain immunologically competent cells, (b) the host must—due to differences in transplantation antigens—be recognized as foreign to the graft, and (c) the host must be unable to initiate an immunological reaction against the graft (85).

The main organs that are usually affected are the skin, liver, gastrointestinal tract, and lymphatic organs (86). GVHD can be graded clinically according to the degree of involvement of the gut, liver, and skin. It occurs in up to 85% of patients, and is classified as either *acute* or *chronic*. Acute GVHD is graded I-IV and chronic GVHD is graded mild, moderate and severe.

Acute GVHD usually appears within the first 100 days after HSCT and can involve all the above mentioned tissues (86, 87). Disease stage, patient age, disparity in HLA antigens, GVHD prophylaxis, and donor-recipient sex mismatch (i.e. female donor for male recipients recognizing antigens associated with the Y chromosome) are risk factors for GVHD (75, 88, 89). The pathophysiology of acute GVHD is divided into three phases (90). The first phase involves damage of the tissue caused by underlying disease, conditioning treatment, and infections. In the second phase, activation of donor T-lymphocytes emerges. Recipient/host or donor APCs that have migrated to the lymph nodes present peptides from recipient antigens to donor T-lymphocytes and thereby induce donor T-cell activation (91, 92). The third phase of acute GVHD involves the inflammation and cellular damage caused by mature and activated graft T-cells. The result of this phase is sustained inflammation and further tissue damage.

Chronic GVHD is one of the most frequent late complications after HSCT, affecting 30–50% of patients. It differs from acute GVHD not only regarding timing but also in clinical appearance. Chronic GVHD develops with manifestations such as hepatic dysfunction, dermatitis, keratoconjunctivitis, and oral mucositis.

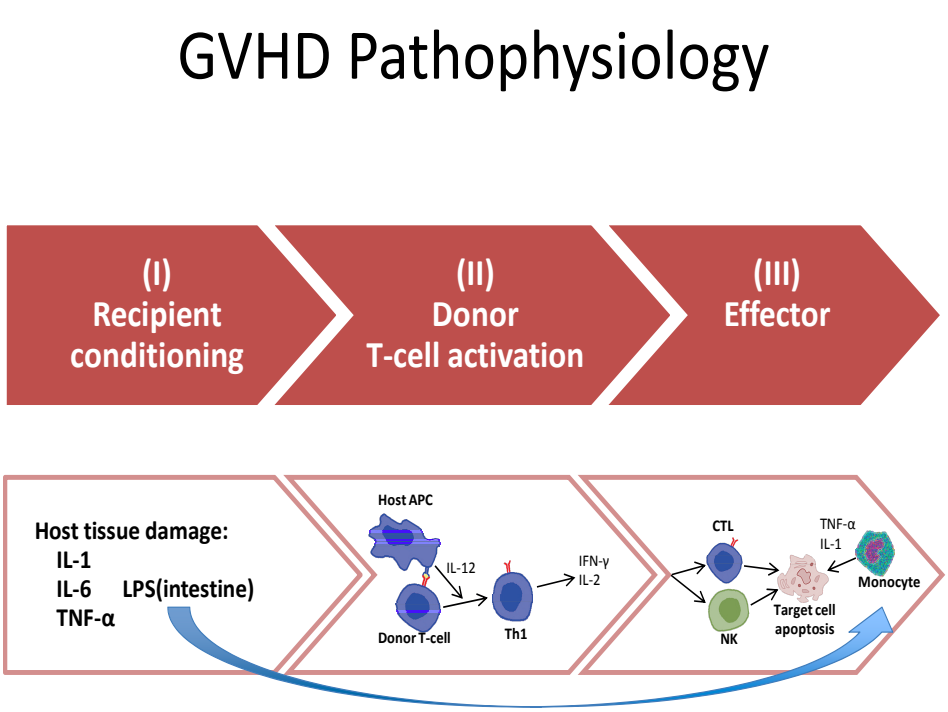

Figure 1. The pathophysiology of GVHD.

The immune pathogenesis of the two different diseases in not fully understood, although T-lymphocytes play a central role in both acute and chronic GVHD (93). Donor-derived T-cells may initiate a graft-versus-host reaction once they come into contact with antigen on the surface of the host APCs, such as macrophages and dendritic cells in the lymphoid organs, Langerhans cells in the skin, or Kupffer cells in the liver. The engrafted T-cells may recognize the hosts peptide-MHC complex (allo-antigens) as foreign, if they are different, or they may recognize minor histocompatibility antigens. Acute GVHD usually has a strong inflammatory component, whereas chronic GVHD shows a more fibrotic and autoimmune pattern. The important role of T-cells in acute GVHD is supported by the total abolition of GVHD following T-cell depletion of the graft, a treatment that is very effective in preventing acute GVHD. Acute GVHD was thought to be a process driven mainly by Th1 and Th17 cells, whereas chronic GVHD was thought to be predominately mediated by Th2-type immune responses (94). However, this paradigm has been challenged and is not absolute (95, 96).

Over the years, several research groups have provided evidence for the crucial effect of different cytokines in the context of GVHD:

Interleukin-2: This cytokine has been suggested to be a key molecule involved in the process of GVHD. It has a central role as a T-cell growth factor, and cyclosporine is known to inhibit IL-2 secretion. Experimental data have shown that IL-2 production by T-cells is an early event in GVHD (97). The precursor frequency of host-specific IL-2 - producing T-cells has been suggested to predict the occurrence of GVHD after transplantation between HLA-identical siblings (98).

Interleukin-1: One group has put forward evidence for a correlation between cytoplasmic IL-1 in peripheral blood and GVHD (99). Another model showed that administration of this cytokine to hosts in a murine model increased the mortality from what appeared to be an accelerated form of GVHD (100).

TNF-α: The first group to demonstrate that this cytokine is imperative as a mediator of GVHD was Piguet and colleagues (101). Infusion of TNF-α mimics most manifestations of GVHD. It has been shown that primed, activated macrophages in mice with GVHD secreted TNF-α, and that the mortality could be reduced by neutralization of TNF-α (102).

Interferon (IFN): During experimental GVHD, elevated IFN-gamma production has been noted (103, 104). Another study has shown that in the clinical situation, a large proportion of T-cells isolated from GVHD patients produce IFN-gamma (105).

Interleukin-10: This is an anti-inflammatory cytokine that inhibits the production of inflammatory cytokines such as TNF-α, IL-1, and IFN-gamma, and it also has the ability to inhibit MHC class II expression on APCs. Nevertheless, in experimental models IL-10 was not found to protect GVHD mice from GVHD mortality but caused a notable reduction in serum TNF-α or IL-1 (106, 107).

Interleukin-12: IL-12 is an inducer of both Th1 cell activity and NK cell function. One study has revealed that human macrophages stimulated with LPS *in vitro* produced increased levels of IL-12 in patients with GVHD, but not in those without (108). On the other hand, in a mouse model, another research group showed that a single injection of IL-12 inhibited GVHD but preserved the graft-versus-leukaemia (GVL) effect (109).

Despite its major side effects, the standard primary treatment for GVHD is systemic corticosteroid therapy. Other current therapeutic treatments that are routinely used to prevent GVHD, e.g. ATG, are broad-spectrum approaches that target T-cells. Unfortunately, these agents also have a negative effect on GVL responses and immune reconstitution.

More selective approaches that specifically influence the activation, function, or survival of allo-reactive T-cells should avoid some of the adverse side effects of global immunosuppressive therapy (94). In mouse studies, the depletion of B-cells from the graft resulted in a reduced incidence of acute GVHD, perhaps owing to the effect of B-cells on host APCs (94, 110). In the clinical setting, incorporating rituximab—a CD20-specific monoclonal antibody that depletes B-cells—in the conditioning regimen reduces the severity and incidence of acute GVHD (94). Recent investigations have demonstrated the importance of regulatory mechanisms, including Tregs. The results have suggested a relationship between the incidence and severity of GVHD and the levels of circulating Tregs (111, 112).

1.5.3 Relapse

The last three decades have witnessed a significant improvement in survival rates, probably due to improvements in supportive care (113, 114), greater availability of suitable donors, and modulation of conditioning regimens (115). Despite this, the risk of relapse has not changed considerably (116, 117). Patients transplanted at the early stages of acute leukaemia have an incidence of relapse of around 20–30%. In patients with more advanced disease, studies have shown a higher incidence of relapse—reaching 40–70% (118, 119).

Generally speaking, leukemic relapse mainly occurs in recipient-derived cells and this is probably due to incomplete killing of the leukaemic cells or inadequate graft-versus-leukemia effect. Some studies have demonstrated recurrent leukaemia in donor cells (120-124). Occasionally, leukaemia can occur in donor cells as a *de novo* event, masquerading as a relapse (125, 126). Transplant factors with an increased risk of relapse are high patient age (acute leukaemias), refractory disease at transplantation, HLA-identical donor, type of GVHD prophylaxis, and donor chimaerism (127). The lowest relapse rates are seen in patients who are transplanted in first complete remission (128). Other factors that are associated with an increased risk of relapse are less or no GVHD at all, and T-cell depletion of the stem cell product (129-131).

Traditionally, a morphological or haematological relapse is the case if the bone marrow or blood sample from the patient shows a significant amount of blast cells. Since the sensitivity of this method is low, new ways of detecting relapse at an early stage have been developed the last three decades. Some malignancies are characterized by specific

chromosomal changes and—with cytogenic analysis—cells containing these abnormalities can be identified. The translocation of the Philadelphia chromosome, t(9;22), is probably the most well-known example and it is present in chronic myeloid leukaemia, in some forms of acute lymphoblastic leukaemia, and in a few cases of acute myeloid leukaemia (132, 133). Certain combinations of surface antigens on malignant cells can be detected with flow cytometry and monoclonal antibodies in an immuno-phenotypic analysis. Certain leukaemias are accompanied by rearranged T-cell receptors and typical immunoglobulin genes, and these changes can be detected with real-time quantitative polymerase chain reaction (PCR). Compared to the other techniques, this method offers a very high sensitivity and gives the opportunity to detect a low number of transformed cells (134-136). Occurrence of residual recipient cells after HSCT can also be detected with real-time quantitative PCR. This specific assessment is referred to as a chimerism analysis, and studies have shown that an increased proportion of host-derived cells in the leukaemia-affected cell line strongly correlates with imminent relapse of disease (137-139). One study has concluded that monitoring of leukaemia lineage-specific chimerism is of utmost importance for DLI response after allogenic HSCT (140).

1.5.4 Infectious complications

Immunosuppressive treatment in combination with an immature immune system of donor origin give rise to several infectious complications that are important causes of morbidity and mortality after HSCT (141, 142). Engraftment after allogeneic HSCT occurs within 14 to 28 days. The risk of infection is related to the time period after transplantation, which according to some researchers can be defined as pre-engraftment, day 0–30 after HSCT; immediate post-engraftment, 30–100 days after HSCT; and late post-engraftment, at more than 3 months. Allogeneic recipients are at risk of infection during all of the above-mentioned phases.

To evaluate the patient's individual risk of infection, several factors have to be considered. Important elements that must be taken into account are anti-microbial prophylaxis, local patterns of antibiotic resistance, isolation routines, type of transplant, numbers of given cells, type of conditioning therapy, and the serological status for the donor and recipient regarding virus infections. During the first phase, the number of granulocytes in peripheral blood is of great interest and the total T-cell (CD3) or CD4 cell levels can be used as a surrogate marker for T-cell immunity. Even so, there are no definitive biomarkers for immune reconstitution that predict infection risk and the need for anti-microbial prophylaxis. Consequently, patients require careful monitoring for signs or symptoms of infection, and early intervention.

1.5.5 Viral infection

The most common viruses that cause infection after HSCT are herpes simplex virus (HSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and varicella-zoster virus (VZV).

1.5.5.1 Herpes simplex virus infection

During the aplastic period, reactivation of HSV is common. The virus is categorized into two types: herpes type 1 (HSV-1) and herpes type 2 (HSV-2). Infection with HSV has been shown to be associated with an increased risk of invasive bacterial infection (143, 144) and also an increased risk of persistent necrotic severe mucocutaneous ulcers and deeper infections. Thus, all patients who undergo HSCT and who are serologically positive for HSV should be considered for acyclovir prophylaxis (145).

1.5.5.2 Cytalomegalovirus infection

CMV infection has been one of the most dreaded infections after HSCT (146). Approximately 75% of the general population is infected with the virus. The immune response to CMV is mainly mediated by the cellular immune system. After the initial infection and immune response, the virus establishes life-long latency. Primary infection in immunocompetent individuals is often asymptomatic, but infection in immunocompromised patients can cause a life-threatening disease. CMV infection or reactivation after HSCT can cause pneumonitis, hepatitis, retinitis, colitis, and/or suppression of the bone marrow. Risk factors for CMV infection, apart from the receipt of a positive graft, are the presence of GVHD and prolonged and persistent neutropenia. The risk of CMV infection can be reduced by matching CMV-negative donors to CMV-negative recipients. Surveillance with quantitative plasma PCR for CMV and introduction of pre-emptive treatment has greatly reduced the risk of fatal disease (147-149). Early treatment is preferable to prophylaxis, since anti-viral drugs can be toxic, cause neutropenia, and delay recovery of CMV-specific lymphocytes (150, 151). For patients with CMV disease in which anti-viral therapy does not work, infusions with CMV-specific cytotoxic T-lymphocytes (CTL) may be a successful treatment (152, 153).

1.5.5.3 Epstein-Barr virus infection

90% of the population of the world are infected with EBV. In healthy individuals, EBV is a self-limiting primary infection, and the virus remains latent in B-lymphocytes. In the PTLD situation, there is an uncontrolled proliferation of EBV-infected B-lymphocytes. Post-transplant lymphoproliferative disorder (PTLD) is the name of numerous B-cell hyperproliferative states. Almost all of these lymphoid proliferations are associated with a T-cell dysfunction. Risk factors for PTLD are; HLA-mismatch, serological Epstein-Barr virus mismatch (recipient–/ donor+), use of reduced intensity conditioning, acute GVHD grade II to IV, splenectomy—pre-transplant, and infusion of mesenchymal stromal cells (154). PTLD has high mortality and the reported incidence ranges from 0.6% to 10% (155, 156). Histopathologically, it resembles malignant lymphoma. PTLD may be treated and prevented with adoptive cellular transfer therapy, rituximab, or donor lymphocyte infusion (DLI).

1.5.5.4 Varicella-zoster virus infection

Due to cellular deficiency after HSCT, there is an increased risk of both primary and reactivated VZV infection. Reactivation of latent VZV is observed in about 30% of HSCT recipients within the first year after transplantation (157). The most common

manifestation is dermatomal herpes zoster (65%), while other patients have a more disseminated infection such as meningoencephalitis or visceral infection (35%).

1.5.5.5 Community acquired viral infections

Mostly during epidemic seasons, respiratory syncytial virus (RSV), influenza A and B, parainfluenza (PIV), and adenovirus may be a crucial cause of morbidity and mortality, especially in lymphopenic HSCT recipients (158, 159). Patients infected during the aplastic phase are at high risk of development of pneumonia, and once pneumonia has developed, the mortality is high in spite of treatment with anti-viral drugs (160).

1.5.6 Bacterial infection

All patients, except those undergoing a non-myeloablative conditioning, will become totally aplastic during the first phase, and will lack measurable numbers of granulocytes in peripheral blood (161). During the neutropenic or pre-engraftment period, both Gram-positive and Gram-negative bacterial infections from the skin, mouth, and gastrointestinal tract are common (162). Gram-negative bacteria from the gastrointestinal tract can cross the intestinal barrier and cause severe infections. However, due to treatment with broad spectrum antibiotics in the case of fever or prophylactic antibiotic treatment, the incidence of these types of infections has decreased (141, 163-165).

In the initial post-transplant period, bacterial infections are usually caused by normal skin flora (coagulase-negative Staphylococcus) and bacteria from the gastrointestinal and oropharyngeal tract (*Streptococcus viridans, Enterococcus* species, and enteric Gram-negative bacilli) (166, 167). Other bacterial species that could be fatal are *Pseudomonas aeruginosa*, *Enterobacteriaceae,* and *Stenotrophomonas maltophilia*. Infectious diarrhea is also a problem that must be addressed after HSCT, and the most common pathogen is *Clostridium difficile.*

1.5.7 Fungal infection

Immunosuppressed patients are predisposed to fungal infection. Risk factors for invasive fungal infection are prolonged severe neutropenia, use of broad-spectrum antibiotics, the presence of acute GVHD, treatment with corticosteroids (168, 169), and mucocutaneous damage. The incidence of invasive fungal infection is around 10% in patients who have undergone HSCT (170, 171). Both *Aspergillus spp.* and *Candida spp*. must be taken under consideration during the first aplastic phase, when decisions about anti-mycotic treatment are made. Patients who deteriorate despite adequate anti-microbial treatment should always be considered for a possible invasive fungal infection. Invasive aspergillosis in particular is associated with a high mortality rate in HSCT patients (169, 172). Diagnosis of invasive fungal infection is difficult and complicated. Blood cultures become positive in only a few of the deep-seated infections. Positive cultures from other sites may represent colonization rather than invasive infection. The contributions from PCR and from serological antibody or antigen tests are unsatisfactory (169). In the past, fungal infections have been treated with conventional or liposomal amphotericin B with proven effect on mortality in neutropenic patients (173). Today, newer agents such as voriconacole (Vfend) and

caspofungin (Cancidas) are available for invasive infection and they have been shown to be effective against *Candida spp.* and *Aspergillus spp.* (174-177). At the Centre for Allogenic Stem Cell Transplantation (CAST), Karolinska University Hospital, the incidence of invasive fungal infection, has radically been reduced (the last years around 2.5%) probably due to the use of prophylaxis with posakonazol (personal communication with Dr Jonas Mattsson, Karolinska University Hospital, Stockholm). To prevent opportunistic infections such as *Pneumocystis jirovecii*, prophylaxis with trimetoprim-sulfametoxazol is used.

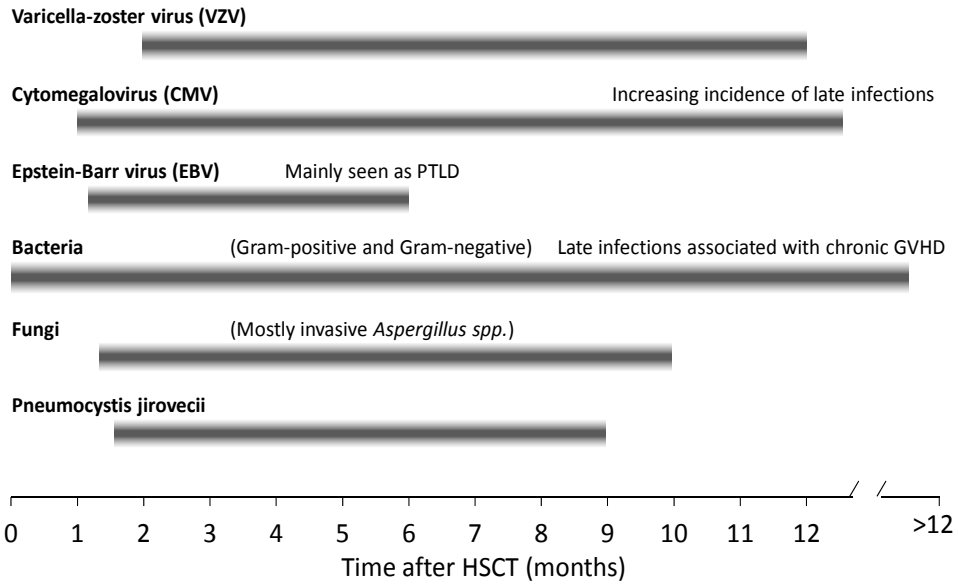

Figure 2. Common causes and timing of infections post HSCT.

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1.6 VITAMIN D DEFICIENCY

Most vertebrates including reptiles, birds, and lower primates need exposure to UVB radiation for their vitamin D production (178). For humans, the association between sunlight and health was first appreciated with the industrialization of northern Europe. During that time, there was markedly reduced exposure to sun, resulting in a childhood epidemic of a condition commonly known as rickets, with bone deformities and severe growth retardation (179-182). At the beginning of the twentieth century, observational studies showed a relationship between exposure to ultraviolet radiation and cure of rickets (183).

During the past decade, major advances have been made in vitamin D research that transcend the simple concept that the vitamin is only important in the context of bone health. Epidemiological studies have shown an association between low vitamin D levels and a variety of respiratory tract infections (184) (185). For example, vitamin D insufficiency is associated with increased risk of developing tuberculosis (186, 187). The relationship between vitamin D deficiency and susceptibility to viral respiratory tract infections is unclear. Nevertheless, several observational studies have found an inverse association between 25(OH)D levels and incidence of respiratory tract infections (188, 189).

1.6.1 Sources and metabolism

Humans get vitamin D mainly through exposure to sunlight and from small amounts in the diet or dietary supplements (190). It is almost impossible to obtain adequate levels of vitamin D only from dietary intake, but large amounts of oily fish can prevent vitamin D deficiency. There are two different forms of vitamin D, vitamin D2 and vitamin D3. Vitamin D2 is found naturally in mushrooms such as chanterelles and is manufactured through ultraviolet irradiation of ergosterol from yeast. Vitamin D3 is found in, for example, fatty fish and it is the metabolite produced when solar ultraviolet B radiation (of wavelength 290–315 nm) penetrates the skin. Long-term exposure to sunlight degrades pre-vitamin D3 and vitamin D3 into inactive photoproducts, so excessive sun-tanning does not cause intoxication. Vitamin D3 is produced commercially by exposing 7-dehydrocholesterol from lanolin to ultraviolet radiation.

When vitamin D is transported in the blood, it is bound to a vitamin D binding protein (VDBP), and after synthesis it binds to vitamin D receptor (VDR), a member of the nuclear receptor superfamily that is expressed by numerous cells in the body. A liganded vitamin D receptor mediates the action of active vitamin D by controlling the expression of sensitive genes. Vitamin D is fat-soluble and can be stored in and released from fat cells, which constitute an endogenous depot (191). Obesity is associated with vitamin D deficiency, and it is believed to be due to the sequestration of vitamin D by the large pool of body fat (192).

During exposure to UVB radiation from sun-light, 7-dehydrocholesterol in the skin is converted to pre-vitamin D3, which— in a heat dependent procedure—is immediately converted to vitamin D3 (cholecalciferol) (191, 193, 194).

With the help of VDBP, vitamin D3 enters the liver and is converted by the enzyme D-25-hydroxylase (encoded by the CYP2R1 gene) into 25-hydroxyvitamin D, 25(OH)D. This is the major circulating form of vitamin D and is the metabolite considered to best reflect the vitamin D status of an individual. 25(OH)D is inactive and must be converted a second time, with the help of the enzyme 25(OH)D-1α-hydroxylase (encoded by the gene CYP27B1), into 1,25-dihydroxyvitamin D [$1,25(OH)_2D$],which is the biologically active form. This last transformation mainly takes place in the kidneys, but it also occurs in other cells such as macrophages, monocytes, and epithelial cells. After synthesis, $1,25(OH)_2D$ is released into the general circulation and binds to the VDR. It is only the kidney CYP27B1 that contributes to the levels of $1,25(OH)_2D$ in the circulation.

Vitamin D is metabolized to its active form in various tissues and cells for regulating of cellular proliferation and differentiation as also to induce AMPs such as the cathelicidin LL-37 in macrophages. When a macrophage is stimulated through a Toll-like receptor (TLR1/2) by an infectious agent, there is a signal that up-regulates the production of the enzyme 25(OH)D-1α-hydroxylase and there is also an increase in the expression of vitamin D receptor. $1,25(OH)_2$ D reduces its own synthesis through negative feedback and reduces the synthesis of parathyroid hormone (PTH) by a direct effect on cells in the parathyroid glands.

Expression of the catabolizing enzyme 25(OH)D-24-hydroxylase (encoded by the gene CYP24A1) is enhanced by $1,25(OH)_2D$, and it converts both 25(OH)D and $1,25(OH)_2D$ into biologically inactive products. The elimination half-life of 25(OH)D is approximately 15 days, whereas that of $1,25(OH)_2D$ is about 2–4 h (195). The progressively hydroxylated metabolites, including lactones and calcitroic acid (more hydrophilic), allow both biliary and renal excretion (196). $1,25(OH)_2D$ enhances intestinal calcium absorption in the small intestine by interacting with vitamin D receptor, resulting in increased expression of epithelial calcium channels and production of calcium binding proteins (calbindin 9K) (191). Receptors on osteoblasts bind $1,25(OH)_2D$, which results in increased expression of receptor NFκB ligand (RANKL). RANKL binds to RANK on the osteoclasts, and this makes the osteoclasts mature (197). The task of a mature osteoclast is to remove calcium and phosphorus from bone while maintaining calcium and phosphorus levels in the blood, which is essential for healthy bone. Thus, vitamin D deficiency leads to deficient mineralization of bone.

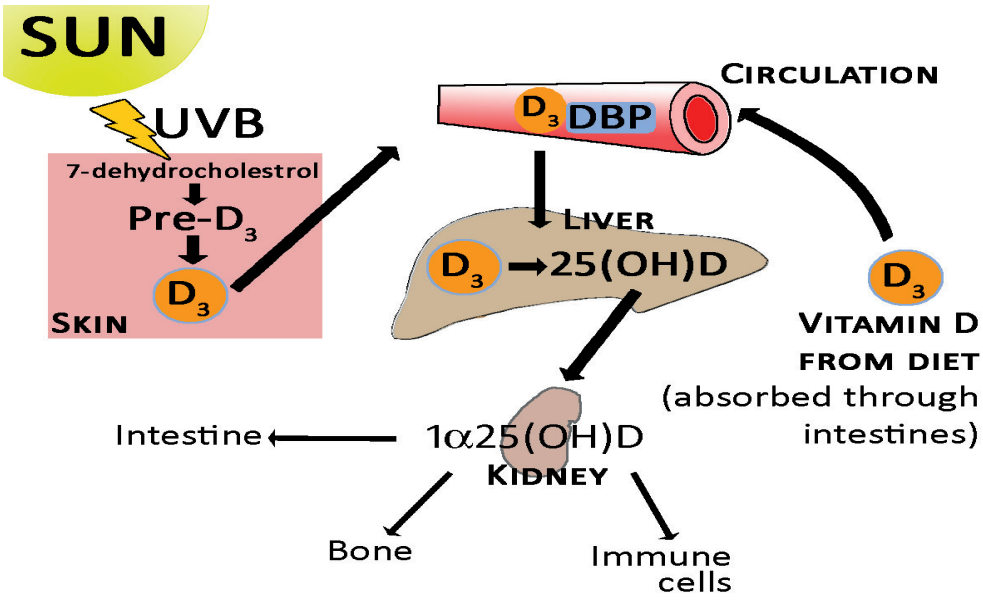

Figure 3. Vitamin D metabolism.

1.6.2 Prevalence, definition, and treatment

Since 25(OH)D comes from both endogenous and dietary sources of vitamin D, it reflects the vitamin D status of the patient in the most correct way. Vitamin D inadequacy appears to be a widely spread global problem in all age groups. The major cause of vitamin D deficiency is inadequate exposure to sunlight (198-201). Wearing a sunscreen with a sun-protection factor of 30 reduces vitamin D synthesis in the skin by more than 95% (202).

Much debate has taken place over the definition of vitamin D deficiency. Vitamin D deficiency could be defined as a 25(OH)D below 50 nmol/liter (20 ng/ml) and vitamin D insufficiency as a 25(OH)D of 50-75 nmol/liter (21-29 ng/ml) (203). This is based on the observation that intestinal calcium absorption is maximized above 80 nmol/liter, in postmenopausal women (204) and that parathyroid hormone (PTH) concentrations in adults continue to decline and reach their nadir at 75-100 nmol/liter (204-206). This means that it is an inverse relation between 25(OH)D and parathyroid hormone.

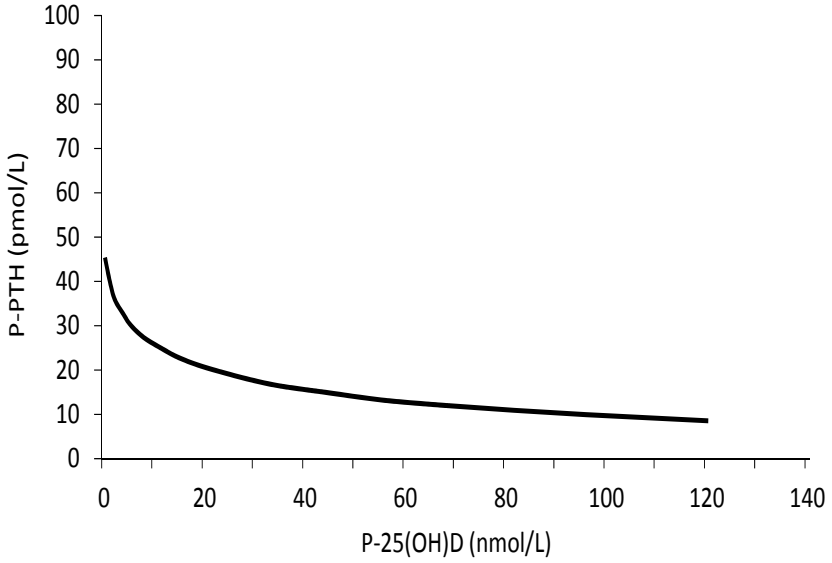

Figure 4. Correlation between P-PTH levels and P-25(OH)D levels.

Risk factors for vitamin D deficiency include dark skin pigmentation, living at a northern latitude, lack of direct exposure to sunlight, osteoporosis, kidney disease, malabsorption syndromes, obeseity, pregnancy, lactation, and hyper-parathyroidism.

The most common way to treat vitamin D deficiency in northern Europe is by oral supplementation of vitamin D3. $1,25(OH)_2D$ is not the first-line treatment, except in patients with chronic kidney disease, because its renal effects increase the risk of both cholelithiasis and hypercalcaemia due to its influence on calcium metabolism.

The optimal regimen for treatment of vitamin D deficiency is controversial because of an on-going discussion about the level of 25(OH)D at which there is a need for supplementation, and for what kinds of symptoms. There is also an on-going debate about which dose is optimal to achieve a high enough serum level to provide the numerous health benefits that can be associated with vitamin D. One must remember that individuals with malabsorption or other risk factors for vitamin D deficiency may require higher chronic doses to maintain optimal levels (207, 208). One study has suggested that healthy men consume/metabolize 3,000–5,000 IU vitamin D3 per day,

meeting > 80% of their winter cholecalciferol needs with cutaneously synthesized accumulations from solar sources during the preceding summer months (209). The recommendations from the Endocrine Practice Guidelines committee for patients at risk of vitamin D deficiency are as follows: in children up to 1 year of age, 2,000 IU/day; in children up to 13 years of age, 4,000 IU/day; and in adults, doses between 4,000 and 10,000 IU/day are required. For individuals with no risk factors for deficiency, the committee recommends 1,000–3,000 IU/day for children and 4,000 IU/day for adults (203).

It has been proposed that declining vitamin D levels during winter time may explain the increased prevalence of respiratory tract infections during that part of the year. One relatively recent prospective cohort study suggested that supplementation with vitamin D to raise the concentrations of 25(OH)D to 95 nmol/l could release the burden of illness from viral infections in adults living in temperate climates (210).

Toxicity due to excess vitamin D intake is rare, but it has been reported in a few cases and generally with doses exceeding 10,000 IU/day (211, 212) for extended periods. Acute vitamin D toxicity that involved hypercalcaemia has been found in cases with an intake of vitamin D exceeding 40,000 IU/day (211-213). It has been estimated that circulating levels of 25(OH)D of > 250–375 nmol/l could possibly give signs of hypercalcaemia (214), which can include weakness, nausea, bone pain, and headache. No adverse effects have been seen in healthy individuals consuming daily doses of vitamin D up to 10,000 IU (213, 214). Daily intake markedly exceeding this amount for several months is required to maintain a vitamin D level above 250 nmol/l (214).

1.6.3 Non-skeletal effects of vitamin D

There is increasing evidence to suggest that optimal vitamin D levels throughout one's whole life—even *in utero*—may be essential. It may be crucial not only in maintaining good bone health, but also in protection against, for example, autoimmune diseases, cardiovascular diseases, and cancers (215, 216). Many tissues in the body express the nuclear receptor for $1,25(OH)_2D$, including skin, brain, colon tissues, prostate, breast and also different cells in the immune system, such as T- and B-lymphocytes and macrophages (190, 194). Active vitamin D is present in the circulation for a very short time period only, and local activation in target immune cells is imperative for vitamin D-mediated effects on the immune defence (217). $1,25(OH)_2D$ is involved in regulation of more than 200 genes controlling cell proliferation, differentiation, and apoptosis (179, 218), and the non-skeletal biological effects of vitamin D are thought to be involved in several disease processes (194).

1.6.3.1 Association with susceptibility to infection

The link between vitamin D deficiency and susceptibility to infections of the respiratory tract has been suggested for many years, but it has not yet been definitively proven. A systematic review of clinical studies by Jolliffe *et al*. (2013) has shown broadly consistent associations between vitamin D deficiency and susceptibility to respiratory infection (219). In contrast, vitamin D supplementation trials have not demonstrated consistent protective effects against respiratory infection. Clinical trials that show no effect may have had a low prevalence of baseline vitamin D deficiency in

participants, or sub-optimal doses of vitamin D. Another meta-analysis and systematic review, published by Bergman *et al*. (2013), of randomized controlled trials indicated that vitamin D has a protective effect against respiratory tract infection and that dosing once-daily appears to be most effective (220).

As mentioned below, several clinical trials in different populations have given mixed results.

Murdoch *et al*. compared 100,000 IU/month of oral vitamin D for 18 months or placebo in 322 healthy adults in New Zealand (221). No effects on the incidence or severity of RTIs were seen. In this study, those included had a mean vitamin D level of 75 nmol/l at baseline and this means that the participants did *not* have a vitamin D deficiency (221). In addition, a recent study performed by Rees *et al*. concluded that supplementation with 1,000 IU/day did *not* significantly reduce the incidence or duration of upper RTI in adults with a baseline 25(OH)D level of 61 nmol/l (222). The finding that vitamin D has no effect on RTIs is consistent with two other randomized controlled studies that unfortunately had weaknesses in the design, with short duration or too small dose of vitamin D, and both of these studies were underpowered (223, 224). In the United States, Li-Ng *et al*. reported no effect on respiratory tract infections in 162 adults given 2,000 IU/day vitamin D for 12 weeks, compared with placebo (223). This trial may have been underpowered, and too short to allow vitamin D levels to reach a steady state. Laaksi *et al*. included 164 healthy Finnish soldiers who were randomly assigned to the intervention group, which received 400 IU vitamin D3 daily, or the control group, which received placebo. This study was performed for 6 months, between October to March (224), and the participants reported an average of 2.2 days missed from work due to respiratory tract infection in the vitamin D group and 3.0 in the placebo group. One weakness of this study was that there are indications that additional supplementation with 400 IU/day is not sufficient to maintain an adequate level of vitamin D through the winter (225).

It is possible that an effect will only be observed in a population with a high prevalence of vitamin D deficiency, as occurred in a recent trial of vitamin D substitution with the aim of reducing exacerbations of chronic obstructive pulmonary disease (226). In that study, supplementation of vitamin D significantly reduced exacerbations only in individuals with levels less than 25 nmol/l at the time of inclusion. Two other recent studies have shown contrasting effects of vitamin D supplementation in children. In one randomized trial, researchers gave vitamin D supplementation to Mongolian school children in winter time and noted a 50% reduction in acute respiratory infections in the study population that had an average vitamin D level of less than 25 nmol/l (227). In The Lancet, Semira Manaseki-Holland and colleagues reported contradictory results of supplementation of bolus-dose vitamin D (228). In that study, the addition of vitamin D did not influence the incidence of first episode of pneumonia in children in Afghanistan, which is actually a population with a high prevalence of vitamin D deficiency (228). The researchers randomly assigned 3,046 infants aged 1–11 months to receive a quarterly dose of 100,000 IU vitamin D3 or placebo over 18 months. An editorial comment, written by A.R. Martineau, about this study has been published in The Lancet (229). The author wrote that some issues have to be considered when interpreting the results of the Afghanistan study. One must consider the possibility that

the pharmacokinetics of vitamin D3 administered in large doses quarterly and malnutrition in the study population could interfere with the outcome of the study. Another explanation of the study result would be that a subgroup of participants might have benefited from the supplementation but that this effect was concealed by a larger group of non-responders.

A cross-sectional trial from Greenland showed that both low (< 75 nmol/l) and high serum concentrations of vitamin D (> 140 nmol/L) were associated with an increased risk of tuberculosis (230). Similarly, molecular studies have suggested the presence of feedback systems that effectively block the activation of vitamin D at several levels when large supraphysiological doses are given (231-233).

Low levels of 25(OH)D are associated with an elevated risk of tuberculosis (234-236). It has been found that through activation of a Toll-like receptor, *Mycobacterium tuberculosis* initiates expression of 25(OH)D-1α-hydroxylase and VDR genes in the cell. If there is not enough 25-hydroxyvitmain D available to the 1α-hydroxylase, as is the case in vitamin D deficiency (237), then insufficient $1,25(OH)_2D$ will be present locally. This, in turn, will reduce binding of $1,25(OH)_2D$ to the macrophage VDR, thereby limiting the activation of VDR-dependent anti-microbial genes and the subsequent killing of microbes (238).

In one study, β-defensin, which has a bactericidal effect on *S. aureus*, has been shown to be induced by vitamin D; moreover, it has been suggested that low levels of vitamin D are associated with an increased risk of being colonized with this bacterium (9, 239).

There is both mechanistic and clinical evidence to suggest that vitamin D can prevent viral infections (210, 240-243). The ability of viruses to activate TLR-induced pathways similar to those activated by mycobacteria and bacteria suggests that induction of immune responses to vitamin D may also promote anti-viral effects. It has been proposed that vitamin D has effects on cytokine production and suppression of inflammation (244), and it may therefore have the ability to reduce the severity of viral pneumonia (245).

The link between vitamin D deficiency and increased susceptibility to infections has grown stronger in recent years.

1.6.3.2 Muscle strength

Skeletal muscles have a vitamin D receptor, and probably require vitamin D for optimal function (179, 246). Lack of vitamin D has been shown to cause muscle weakness (194, 247, 248). Several studies have supported the hypothesis that vitamin D inadequacy contributes to age-related muscle weakness (249, 250) and falls (251, 252). A meta-analysis of randomized controlled trials on vitamin D supplementation showed an approximately 20% reduced risk of falls in ambulatory or institutionalized older individuals treated with vitamin D supplements (253).

1.6.3.3 Cancer

There is a latitude gradient for several different malignancies in adults, including Hodgkin's lymphoma as well as prostate, ovarian, colon, breast, pancreatic, and other cancers (246, 254-260). Both prospective and retrospective epidemiological studies have suggested that levels of 25(OH)D less than 50 nmol/l may be associated with a 30–50% higher risk of breast, prostate, and colon cancer (254, 257, 258, 261, 262). There are some experimental data to suggest that 1,25 $(OH)_2D$ in a malignant cell can induce apoptosis and prevent angiogenesis, and thereby reduce the capacity of cancerous cells to grow (194, 263).

1.6.3.4 Autoimmune diseases, diabetes, and inflammation

Some studies have indicated that living in areas with less sunlight increases the risk of type 1 diabetes, Crohn's disease, and multiple sclerosis (264, 265). Women whose intake of vitamin D was more than 400 IU/day showed a significantly reduced risk of developing rheumatoid arthritis (266, 267). In addition, 10,366 children in Finland received 2,000 IU vitamin D per day during the first year of life were followed for 31 years and showed a reduced risk of type 1 diabetes by approximately 80% (268). Vitamin D3 has also been shown to suppress the Th2 response in allergic broncho-pulmonary aspergillosis (269). In addition, experimental studies have shown that $1,25(OH)_2D$ has broad anti-inflammatory effects on the adaptive immune system by shifting the T-helper cell pool from a Th1/Th17 response to a mainly Th2/Treg-predominant response (270, 271).

1.6.3.5 Cardiovascular disease

Earlier studies have shown an association between living at a higher latitude and an increased risk of hypertension and cardiovascular disease (272, 273). In a group of patients with hypertension who were treated with UVB radiation 3 times a week for 3 months, it was found that 25(OH)D levels increased by approximately 180% and blood pressure, both systolic and diastolic, was reduced by 6 mmHg (274).

1.6.3.6 Mental health

One group has pointed out that it could be important for the foetus that pregnant women achieve a satisfactory vitamin D level in the circulation, since vitamin D is probably essential for transcriptional activity of receptors in the brain, for brain development, and for maintenance of mental function later in life (275). In different studies, lack of vitamin D has been associated with an increased incidence of depression, schizophrenia, and autism (276-278).

1.6.4 Vitamin D and HSCT

Vitamin D deficiency is common following HSCT. In both adults and children who have experienced HSCT, several studies have suggested that there is a high incidence of vitamin D deficiency or insufficiency at baseline (70–89%) (279-282).
For several reasons, HSCT patients are potentially at increased risk of having low vitamin D levels after transplant. Factors that could possibly contribute to post-HSCT vitamin D deficiency are reduced uptake from the intestines because of GVHD, bacterial overgrowth in the bowel, and increased catabolism because of the use of

glucocorticoids (281-285). IFN-gamma, a distinct stimulator of macrophage 1α-hydroxylase expression, is often down-regulated by glucocorticoids and other immunosuppressive agents (286). Transplanted patients may also have reduced exposure to direct sunlight because of hospitalization and convalescence.

How vitamin D interferes with the responsiveness of the human immune system, in the context of HSCT, is of great interest, but to date findings are contradictory. It has been described that vitamin D might prevent GVHD, and since GVHD is one major obstacle to successful HSCT, great efforts are being made to prevent—and to deepen our understanding of—this complication. T-lymphocytes and DCs are well known to play a central role in the pathogenesis of GVHD, and it is known that their function can be modified by vitamin D.

Several preclinical trials have been performed to investigate this subject in greater detail. It has been shown that vitamin D inhibits the maturation of DCs and polarizes the T-cell population towards the expression of Th2 cytokines rather than Th1 cytokines. This in turn gives a reduced allogeneic T-cell proliferation in response to DC stimulation. These data suggest that vitamin D supplementation could result in immature DC populations that skew toward tolerizing T-cells rather than stimulatory populations (287). One study has shown less signs of GVHD in mice treated with a vitamin D analogue, probably due to down-regulation of T-lymphocytes and weakened inflammatory response (288). In addition, *in vitro* studies have shown that active vitamin D inhibits mixed lymphocyte cultures in a dose-dependent manner (289). Active vitamin D has also been suggested to alter the surface phenotype and morphology of DCs, which may compromise contacts between DCs and T-lymphocytes and thereby diminish interaction and T-lymphocyte cytokine secretion (290). In a vitamin D receptor knock-out mouse model, it was shown that vitamin D-mediated suppression of DC maturation was lost, resulting in an altered ability to stimulate allogeneic T-cell proliferation (291). DCs that are generated in the presence of vitamin D have a reduced production of IL-12 and they induce hypo-responsiveness of allogeneic T-cells (292, 293). It has also been shown that vitamin D enhances apoptosis of mature DCs, resulting in inhibition of T-cell alloreactivity (294). One study has concluded that the net result of the action of $1,25(OH)_2D$ on T-cells, thereby also having a possible effect on GVHD, is to block the induction of Th1 cell cytokines, especially IFN-gamma , while promoting Th2 cell responses (295). Taking all these results together, the main effect of vitamin D on GVHD is modulation of the T-cell response.

A clinical study performed by Rosenblatt *et al*. found two cases of improvement in adult patients suffering from a steroid-refractory GVHD when the patients were given vitamin D supplementation (287). In addition, earlier clinical studies have shown evidence of a linkage between sufficient vitamin D levels and less chronic GVHD in adult patients (296, 297).

There is also emerging evidence that vitamin D may play a role in immunomodulation of other parts of the immune system. For more than 20 years, it has been known that vitamin D can suppress immunoglobulin production (298). In earlier studies, vitamin D

has been shown to suppress the proliferation of B-lymphocytes, the differentiation of plasma cells, and the secretion of antibodies (299, 300).

The possible effect of vitamin D on the outcome after HSCT is not well documented. Nevertheless, the effects of vitamin D in HSCT deserve greater attention.

2 AIMS OF THE PRESENT STUDY

The overall objective of the present thesis was to deepen our understanding of the panorama of infections in patients undergoing HSCT and in patients with antibody deficiency or increased susceptibility to infections.

Specific aims of the present thesis were:

1. To study the association between IgG levels and the outcome after allogeneic HSCT.

2. To compare the effects of conditioning with Campath and Thymoglobulin on the clinical outcome of allogeneic HSCT.

3. To determine whether treatment with vitamin D could reduce infectious symptoms and antibiotic consumption in patients with antibody deficiency or frequent RTI.

4. To determine the prevalence of vitamin D deficiency in children after allogeneic HSCT and to relate vitamin D status to clinical outcomes.

3 MATERIALS AND METHODS

3.1 PATIENTS AND MATERIALS IN STUDY I, II, AND IV

Patients included in studies I, II, and IV of this thesis were transplanted at Karolinska University Hospital, Huddinge, Sweden between 1995 and 2011. Studies I, II, and IV were approved by the local ethics committee at Karolinska University Hospital, Huddinge (DNR 425/97). The patient characteristics are summarized in **Table 1:**

	Study I	**Study II**	**Study IV**
Number	179	108	123
Sex (Male/Female)	102/77	54/54	83/40
Mean age	35 (10-65)	55 (<1-67)	9 (<1-19)
Children (<18y)	35 (20%)	4 (4%)	119 (97%)
Diagnosis:			
Acute Leukaemia	83 (46%)	25 (23%)	38 (31%)
Chronic Leukaemia	53 (30%)	21 (19%)	3 (2%)
MDS	5 (3%)	40 (37%)	20 (16%)
Other malignancy	22 (12%)	14 (13%)	5 (4%)
Non-Malignant	16 (9%)	8 (7%)	57 (46%)
Disease stage (Early/Late)	111/57	30/78	78/45
Donor:			
Sibling	91 (51%)	32 (30%)	43 (35%)
MUD	76 (42%)	67 (62%)	57 (46%)
MM	12 (7%)	9 (8%)	23 (19%)
Donor age	35 (5-62)	34 (21-71)	22 (0-55)
Female to Male	32 (18%)	11 (10%)	28 (23%)
Stem cell source:			
BM/PBSCs/CB	85/94/0	19/89/0	84/21/18
NC dose (x10^8/kg)	4.5 (0.5-27.6)	11.1 (1.0-54.5)	4.5 (0.3-36.9)
$CD34^+$ dose (x10^6/kg)	5.3 (0.3-25.5)	7.6 (0.6-26.0)	4.8 (0.1-43.8)
Conditioning:			
MAC/RIC	146/33	17/91	82/41
TBI-based	101 (56%)	19 (18%)	37 (30%)
Chemo-based	78 (44%)	89 (82%)	86 (70%)
ATG/Campath	109/1 (61%)	72/36 (100%)	94/2 (78%)
GVHD prophylaxis:			
CsA+MTX	157 (88%)	95 (88%)	81 (66%)
CsA+MMF	12(7%)	0	0
Tacrolimus+Sirolimus	0	13 (12%)	21 (17%)
Other	10 (6%)	0	21 (17%)

Table 1. Abbreviations: BM: bone marrow, CB: umbilical cord blood, CsA: cyclosporine A, MAC: myeloablative conditioning, MDS: myelodysplastic syndrome, MM: HLA mismatched donor, MMF: mycophenolate mofetil, MTX: methotrexate, MUD: HLA matched unrelated donor, NC: Nuclear cells, PBSC: peripheral blood stem cells , RIC: reduced intensity conditioning, TBI: total body irradiation

3.2 PATIENTS AND MATERIALS IN STUDY III

In study III, patients were included from the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, between March and June 2010. Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infection; that is, > 42 days with symptoms from the respiratory tract over a 12-month period prior to inclusion. Study III was approved by the local ethics committee in Stockholm (DNR 2009/1678-31/4 and 2010/498-32). Baseline data and patient characteristics are summarized in **Table 2:**

	Vitamin D	Placebo
Number	70	70
Mean age	55.4	50.8
Female	52/70	50/70
Male	18/70	20/70
IgG replacement	39/70	42/70
Smoking	4/70	6/70
25(OH)D levels (mean, nmol/l)	51.5	46.9
Immunological diagnosis		
IgA deficiency	9/70	9/70
IgG subclass deficiency	27/70	30/70
CVID	6/70	4/70
ND	28/70	27/70
Concomitant disease		
No other disease	16/70	18/70
Lung: Asthma	27/70	25/70
Lung: BE	5/70	7/70
Lung: COPD	5/70	4/70
Other disease*	17/70	16/70

Table 2. * other disease includes: hypertension, body pain, hypothyroidism and gastritis as most common diagnosis. Abbreviations: BE: bronchiectasis, COPD: chronic obstructive pulmonary disease, CVID: common variable immunodeficiency, ND: increased susceptibility to infections without a defined immunological disorder

3.3 METHODS IN STUDY I, II, AND IV

3.3.1.1 Conditioning

In study I, II and IV, Myeloablative conditioning regimens (MAC) consisted of cyclophosphamide (Cy) (60 mg/kg daily) for 2 days in combination with either TBI (single dose 10 Gy or fractionated 4x3 Gy) or busulfan (Bu) (4 mg/kg daily) for 4 days (301, 302). Patients with aplastic anaemia and sibling donor received 200 mg/kg of Cy and ATG.

Reduced intensity conditioning regimens (RIC) consisted of fludarabine (Flu) (30 mg/m^2 daily) for 3–6 days, combined with 2 Gy TBI, Cy alone (30 mg/kg daily) for two days, Cy (60 mg/kg) and 2x3 Gy TBI, Treosulfan (12-14g/m^2 daily) for 3 days or Bu (4 mg/kg daily) for 2 days (71, 303, 304).

Patients with unrelated or mismatched donors, and all patients with non-malignant disorders, were treated with ATG 2 mg/kg/day for 2–5 days or Campath 30 mg/day for 1–3 days before transplantation. (305). Both antibodies were given with the last dose on the day before infusion of the cells.

3.3.1.2 GVHD prophylaxis

Most of the patients received CsA combined with a short course of methotrexate (MTX) as prophylaxis against GVHD (306, 307). In the absence of GVHD, in patients with malignancies CsA was discontinued after 3 months when HLA-identical sibling donors were used and after 6 months when unrelated donors were used. CsA was discontinued after 12–24 months in patients with non-malignant disorders. No GVHD prophylaxis was given after syngeneic transplants. Other protocols included CsA alone, T-cell depletion, and CsA combined with either prednisolone or mycofenolate mofetil. A few patients received sirolimus and tacrolimus.

3.3.1.3 Supportive care

Supportive care has been described in detail previously (308-310). During the pancytopenic phase, adult patients received prophylactic treatment with ciprofloxacin in combination with anti-fungal therapy until the absolute neutrophil count exceeded 0.5×10^9/l. During the first six months after engraftment, trimethoprim-sulfametoxazol was administered against *Pneumocystis jirovecii* infection. In study IV, in children who developed plasma IgG < 4 g/L after HSCT, IgG substitution was used (311).

3.3.1.4 Definitions

Engraftment was defined as stable absolute neutrophil counts of $> 0.5 \times 10^9$/l for three consecutive days. Diagnosis of acute and chronic GVHD was made based on clinical symptoms and/or biopsies according to established criteria (86, 312). For the diagnosis of bacteraemia, at least one positive blood culture was required. Invasive fungal infection was defined as positive blood culture and/or positive cultures from at least two organs for *Candida* or *Aspergillus* species. *Aspergillus* pneumonia was defined as pulmonary infiltrates and positive cultures of bronchoalveolar lavage fluid, sputum, or autopsy samples. In study I, patients with IgG values below 4 g/L were defined as significantly deficient according to the existing guidelines for antibody-replacement therapy (313, 314). In study IV, vitamin D insufficiency was defined as ≤ 50 nM/L 25(OH)D at baseline.

3.3.1.5 Cell-surface markers and immunoglobulin

Relative numbers of T- (CD3+) and B- lymphocytes (CD19+) and NK-cells (CD56+) were determined by flow cytometry (FACS). Serum IgG was analyzed routinely by nephelometry at the Department of Clinical Chemistry, Karolinska University Laboratory.

3.3.1.6 Statistics

Continuous variables were compared using Mann-Whitney test or Kruskal-Wallis test, and proportions were compared using Fisher's exact test or chi-square test. The Kaplan-Meyer method was used to estimate the probability of overall survival and

relapse-free survival while TRM, relapse, and GVHD were calculated using a nonparametric estimator of cumulative incidence curves. Univariate and multivariate analyses were performed using Gray's test or Cox proportional hazard regression models. A p-value < 0.05 was considered to indicate a significant difference between the groups being compared. Only factors at the 10% level from the univariate analysis were assessed in the multivariate (stepwise) analysis.

3.4 METHODS IN STUDY III

3.4.1.1 Study design

Study III was a prospective, randomized, double-blind placebo-controlled study of vitamin D supplementation in patients with increased susceptibility to respiratory tract infections. The study was registered at www.clinicaltrials.gov (NCT01131858, eduraCT nr: 2009-011758-16) before inclusion of the first patient.

3.4.1.2 Sample size calculation

The sample size was based on the assumption that treatment with vitamin D would reduce the number of days with symptoms from the respiratory tract, from 42 to 28 days. Given this assumption, a sample size of 60 patients per study group was predicted to provide the study with 90% power at a significance level of $p = 0.02$. To compensate for exclusion of participants, the two arms were increased to include 70 patients per treatment arm. Importantly, the only reason for using $p = 0.02$ was to ensure that there was a sufficient number of participants. The conventional significance level of $p = 0.05$ was used for all statistical analysis of the results.

3.4.1.3 Interventions

The patients were randomized to 12 months of supplementation with vitamin D (4,000 IU daily) or placebo oil. The participants were instructed to mark their daily symptoms in a diary. Every month, they sent their filled-out diary to the study site by regular mail. The following data were recorded in the diary: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times of and reasons for visits to hospitals, frequency of travelling abroad, and adherence to the study drug.

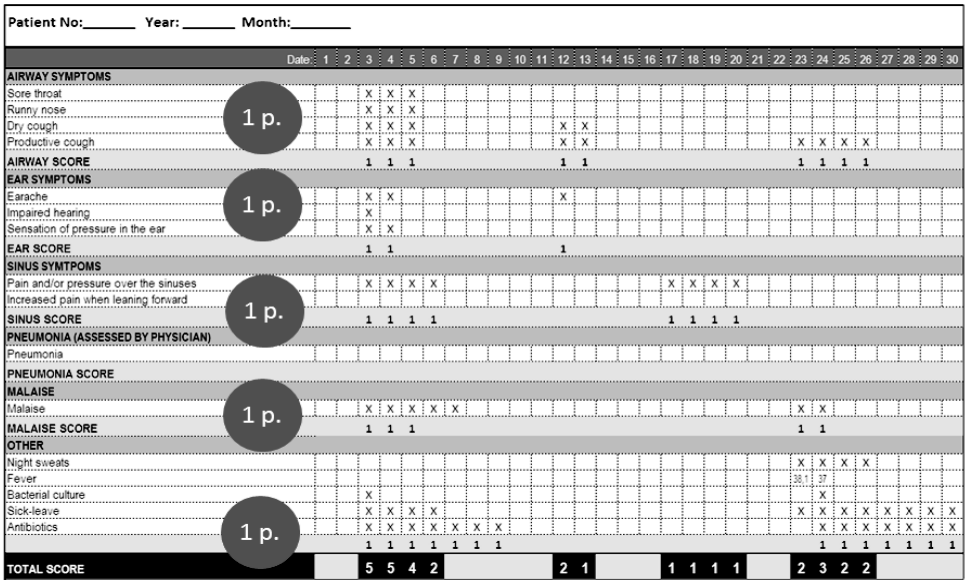

Figure 6. The infection diary used in study III.

3.4.1.4 Outcomes

The primary outcome was a composite infectious score, based on the diary mentioned above, and included five parameters: (1) symptoms from the respiratory tract, (2) symptoms from the ears, (3) symptoms from the sinuses, (4) malaise, and (5) use of antibiotics. All the participants were asked to only report those symptoms that they felt were caused by an infection in the respiratory tract (i.e. not allergy, wounds etc).

Secondary outcomes were serum levels of 25(OH)D (at baseline and after 3, 6, 9, and 12 months), numbers of bacterial cultures, microbiological findings, and amounts of AMPs (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, analyses for single nucleotide polymorphisms (SNPs) were performed for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1, and vitamin D binding protein (GC).

3.4.1.5 Statistics

In the statistical analysis, continuous variables were analyzed using Mann-Whitney U test or linear regression. Dichotomous variables were analyzed by Fisher's exact test or logistic regression. Regressions of log-transformed infectious scores were performed both unadjusted and with adjustment for potential confounders such as underlying diseases or differences in SNPs for VDR. Because of severe skewing, scores were log-transformed before analysis. Furthermore, the randomization had resulted in age distribution that could possibly impinge upon the study result, and a multivariate analysis was therefore added to the original analysis plan, adjusting for potential confounders.

A multivariate model was used in which an effect size of 1 indicated identical outcome in the study groups. To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual part of the infectious score. In addition, the temporal dimensions of the vitamin D effect were investigated by dividing the study period into four 90-day periods.

4 RESULTS AND DISCUSSION

4.1 PAPER I — IGG LEVELS AFTER HSCT

Because of complications associated with GVHD, infections, and patient age, the B-cell repertoire is often belated after HSCT. Patients who develop chronic GVHD may never have a revitalized, normal immune function (50, 315). It usually takes 3–6 months for serum IgM levels to return to normal ranges whereas recovery of serum IgG levels may be delayed up to one year or longer (316, 317). In earlier studies, impairment of antibody immunity after HSCT has been found to be correlated with an increased risk of infections, mostly due to encapsulated bacteria (318).

In paper I, we determined the clinical influence of IgG levels after HSCT in 179 patients who received transplants between 1995 and 2002. Only patients with at least two IgG levels measured during the first 12 months post-transplantation were included in the study. Because of difficulties in estimating immunoglobulin levels in patients with myeloma, in patients treated with intrevenous immunoglobulin (IVIG), and in children less than 10 years of age, these patient groups were excluded.

Serum samples for measurement of IgG were collected 3, 6, 9 and 12 months after transplantation and then annually until 5 years post-transplantation. In this study, IgG levels after HSCT increased throughout the study period, especially from 6 to 12 months.

Multivariate analysis was performed to evaluate different factors that could be associated with low IgG levels (< 4 g/L) after HSCT. Acute and chronic GVHD, patient age, donor age, HLA mismatch, treatment with ATG, and conditioning regimen were included as variables. Factors influencing IgG levels three months after transplantation were acute GVHD of grades I–IV and patient age ≤ 30. Six months after HSCT, acute GVHD of grades I–IV, patient age ≤ 30 years, and no treatment with ATG were associated with low IgG levels. One year after HSCT, three variables were associated with low IgG levels: acute GVHD of grades I–IV, female donor to male recipient, and GVHD prophylaxis with CsA + MTX.

In paper I, we also studied IgG levels and the relationship to survival and transplant-related mortality. Patients with IgG levels of < 4 g/L on at least two occasions during the first year after HSCT showed a reduced cumulative proportion of survival and an increased risk of TRM compared to patients with moderately low or normal IgG levels. Patients with low IgG levels had an increased incidence of infections when they died, but this finding was borderline-significant ($p = 0.056$).

Discussion

The combination of immunosuppressive treatment and an immature immune system gives rise to a variety of complications after HSCT. All patients undergo a period of both humoral and pronounced cellular immunodeficiency. Haematopoietic stem cells are characterized by the ability to self-renew and differentiate into all mature blood lineages. After the cells have been transfused into the patient's circulation, the

immunological function gradually increases. Most of the patients were immunologically competent within 2 years (316, 319). Even so, some patients—especially those who suffer from GVHD—do not show reconstitution of the immune system. In study I, we found that IgG levels after HSCT increased throughout the study period, which is in accordance with earlier studies (320). We found five factors that had a negative effect on IgG levels in our study: acute GVHD, patient age ≤ 30 years at the time of transplantation, lack of treatment with ATG, female donor to male recipient, and treatment with CsA and MTX as GVHD prophylaxis. Our hypothetical model is that many of these factors can increase GVHD and, because of this, decrease immunological revitalization. In addition, in multivariate analysis we found that low IgG levels were significantly correlated with higher TRM and inferior survival. These results indicate that a reasonably high level of IgG in peripheral blood after HSCT is one important factor for a good outcome after transplantation.

It is known that patients who have undergone HSCT are highly susceptible to all kinds of infections. The major indication for IVIG treatment is primary immune deficiency, but there are also indications for IVIG treatment in various autoimmune disorders and infections in immunocompromised patients. Different studies performed during the last few years have found contradictory results regarding beneficial effects of IVIG treatment. IVIG has been shown to have a positive effect on bacterial infections (321). It has also been suggested that IVIG could have immunomodulatory effects *in vivo*, a treatment that decrease the risk of acute GVHD after HSCT (322). In contrast, other studies have found no effect of IVIG treatment on either GVHD or survival after transplantation (317, 323). In a retrospective study, prophylactic IVIG treatment after HSCT was shown to have no effect on overall survival, but the incidence of death due to infections was lower in the patients treated with IVIG (323). In another study, IVIG was shown to reduce proliferation of B-lymphocytes and immunoglobulin production by co-ligation of the B-cell antigen receptor and the Fc-γ RIIb receptor mediating the Fc receptor off signal (324). In addition, in one study it was concluded that both IgM and IgG profiles become less heterogeneous in patients treated with IVIG after HSCT, and that this effect remains months to years after accomplished IVIG treatment (325). In summary, there is no consensus on whether or not IVIG treatment is beneficial after HSCT.

4.2 PAPER II — CAMPATH AND THYMOGLOBULIN AS PART OF CONDITIONING

GVHD is a major cause of morbidity and mortality after HSCT. Many centres add ATG to reduce the risk of GVHD and rejection. The risk of rejection is increased when an unrelated donor is used. By adding ATG to the pre-transplant protocol when using an unrelated donor, both acute GVHD and early mortality have declined to levels similar to those in HSCT with matched, related donors (78, 309).

In paper II, we retrospectively compared 36 patients given Campath (alemtuzumab) as part of the conditioning with a matched cohort of 72 patients who received thymoglobulin (TMG).

There was a higher incidence of invasive fungal infections in the Campath-treated patients in this study, but this was not statistically significant. The incidence of bacteraemia and CMV reactivation was the same in the two groups; nor could any differences be noted in HSV and VZV reactivation. Three cases of PTLD occurred in the Campath group and one case occurred in the control group.

The cumulative incidence of any grade of acute GVHD was 34% in the Campath-treated patients, as compared to 53% in patients given TMG ($p = 0.09$). The cumulative incidence of chronic GVHD was 46% and 25% in patients who received Campath and TMG, respectively ($p = 0.09$).

There was no significant difference in TRM in the two groups. No disparity was seen in the two groups when we compared the risk of relapse and survival. The 5-year cumulative incidence of relapse was 26% in the Campath-treated patients and 34% in patients given TMG (ns). The 5-year overall survival was 53% and 58% in patients who received Campath and TMG, respectively (ns).

No difference in erythrocyte and platelet transfusions between the two groups was seen. Of the Campath-treated patients, 17% were given granulocyte transfusion as compared to 4% in the TMG group ($p = 0.024$). Time to neutrophil and platelet engraftment was similar in the two groups. When we compared cell-surface molecules (CD3+ T-lymphcytes, CD19+ B-lymphocytes, and CD56+ NK-cells) and IgG levels in the two groups, no significant disparity was found. Chimerism data for the donor CD3+ cell line were available for 106 of the 108 patients. Of the TMG-treated patients, 60% reached full donor chimerism (DC) within 90 days, 67% reached full DC within 6 months, and 72% reached it within 1 year. For the Campath-treated patients, the corresponding numbers were 44% at 90 days, 53% at 6 months, and 56% at 1 year ($p = 0.12$). No significant differences were seen in median time to full DC in the CD3+, CD33+, and CD19+ cells in the groups compared.

Discussion

Both Campath and TMG have been developed to achieve so-called *in vivo* T-cell depletion. In study II, we were able to show that Campath was associated with less overall acute GVHD but more chronic GVHD. Whether this was due to a greater effect of Campath than of ATG, in terms of early depletion of alloreactive T-cells of graft origin, is not known. Our findings correspond well with an earlier study (326) in which GVHD was well controlled in the majority of patients treated with Campath. The finding of more chronic GVHD in the Campath-treated patients in this study could have been due to the small number of patients included. There may be a correlation between the cell dose of the graft and chronic GVHD, but this was not found in the present study. However, patients in the Campath group received a higher CD34+ cell dose and had more chronic GVHD.

Considering all types of infections, no difference was found between the Campath group and the TMG group. Nevertheless, there was a trend of more fungal infections in the patients treated with Campath ($p = 0.057$). This finding is supported by a study that showed that treatment with Campath increases the risk of invasive infection with *Aspergillus* after RIC HSCT (169). In addition, 17% of the patients in the Campath

group and 4% of the patients in the TMG group were given granulocyte transfusions as treatment for infections that had not responded to conventional anti-microbial therapy. These findings may indicate that patients treated with anti-CD52 antibodies are more susceptible to severe infections.

Our small study revealed no differences in T- and B-lymphocyte levels during the first year after HSCT. This is not in accordance with another small study that showed that patients who receive Campath have a very slow recovery of the CD4+ T-cell subset (327). This can result in a long-lasting impairment of anti-viral immunity, which is in turn associated with significant morbidity and mortality (328, 329). Previous pharmacokinetic analysis in Campath-treated patients showed that the CD52 antibody is detectable in lympholytic serum concentrations for up to 56 days after transplantation (330). TMG may be found in serum up to 35 days after HSCT (331). Taking these results together, one could speculate that since Campath can be found in the bloodstream for a longer time after administration than TMG, this drug may have a longer immunosuppressive effect.

Our group found in an earlier study that a low dose of TMG (4 mg/kg in total) increased the risk of severe acute GVHD, whereas 10 mg/kg increased the risk of death from infection. Medium doses of TMG (6–8 mg/kg) gave the lowest TRM and the best survival (332). Taking this observation into consideration, an additional factor that may have an influence on both GVHD and infections is the dose given to the patients. In this study, we found a dose effect of both preparations on acute and chronic GVHD.

When using anti-T-cell antibodies, the increased incidence of PTLD is of great concern. Surprisingly, in study II we found a low incidence of PTLD; and we could not detect a difference in development of PTLD between the two drugs. Earlier studies have shown a statistcally significantly increased risk of PTLD when using anti-T-cell antibodies (333). The low incidence of PTLD in our study might be explained by one of the limiting factors: that is, the small number of patients included.

4.3 PAPER III — VITAMIN D SUPPLEMENTATION IN PATIENTS WITH INCREASED SUSCEPTIBILITY TO INFECTIONS

In paper III, 140 patients were included in a double-blind, randomized controlled trial. Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections, i.e. more than 42 days with symptoms from the respiratory tract during a 12-month period prior to study start. 85 of the patients included had an antibody deficiency (selective IgA deficiency, common variable immune disorder, or IgG subclass deficiency) (see Table 2 in Materials and Methods). The aim of the study was to investigate whether supplementation with vitamin D could reduce infectious symptoms and antibiotic consumption in patients with frequent RTI. Vitamin D (4,000 IU) or placebo was given daily for 1 year. The primary endpoint was an infectious score based on five parameters: symptoms from the respiratory tract, ears, and sinuses, malaise, and antibiotic consumption. Secondary endpoints were serum levels of 25(OH)D, microbiological findings, and levels of AMPs in nasal fluid.

One year of treatment with vitamin D was significantly associated with a reduced total infectious score. In the temporal analysis, we concluded that the effect of vitamin D supplementation increases with time. When the individual items of the infectious score were analyzed separately, all point estimates indicated a reduction in the treatment group, although only the total score and antibiotic consumption reached statistical significance. The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D group, i.e. a reduction of 17 days/patient/year in the intervention group. **Figure 7A, B.**

The mean 25(OH)D level at study start in the intervention group was 51.5 nmol/l and in the placebo group it was 46.9 nmol/l, which means that there was no significant difference between the two groups at baseline. After 3 months, the vitamin D group had a significantly higher mean vitamin D level: 133 nmol/l as compared to 67 nmol/l. This increase remained throughout the study.

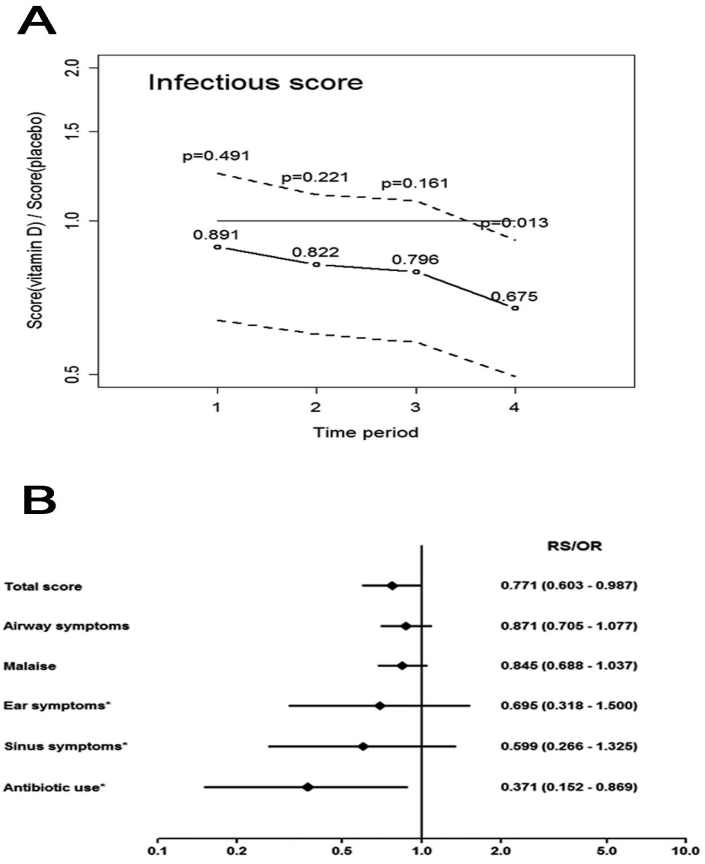

Figure 7. Primary endpoint. The adjusted total relative infectious score (A) is expressed ´per quarter`(3-month periods). The adjusted 1-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forest-plot (B) together with 95% CI. Effects are presented as relative scores (total score, airway and malaise) or OR (ear, sinus, antibiotics and indicated with asterisks).

During the study, 173 microbiological samples were obtained in the vitamin D group and 301 in the placebo group. The number of samples with at least one positive finding was higher in the placebo group ($p = 0.052$). The fraction of positive samples was the same for both groups.

There were no differences between the groups for respiratory pathogens such as *Haemophilus influenza, Moraxella catharralis*, and *Streptococcus pneumonia,* but there were significantly fewer findings of *Staphylococcos aureus*, *Candida spp.* and *Aspergillus spp*. in the treatment group. A trend was seen—although not significant—in that patients with asthma in the intervention group produced fewer bacterial cultures and fewer positive cultures than placebo-treated asthmatics ($p = 0.080$ and $p = 0.052$, respectively).

No statistically significant differences were noted between the vitamin D group and the placebo group when LL-37 and HNP1-3 were analyzed in nasal fluids. After 1 year, there was a non-significant trend for placebo-treated patients to have higher levels of AMPs than patients treated with vitamin D. We also found that after 12 months, no primary pathogens could be detected in nasal swabs from patients in the vitamin D group.

There was not reported more adverse events in the intervention group compared to the placebo group.

Discussion

All five components of the primary endpoints favoured the vitamin D group, and a statistically significant effect was seen both on the probability of treatment with antibiotics and on the total score. The overall infectious score was significantly reduced, mainly as a result of the large effect on the antibiotic parameter. These findings suggest that vitamin D supplementation may prevent respiratory tract infections and reduce antibiotic consumption, especially in patients with an increased susceptibility to infections.

One could speculate that the dose of vitamin D, the dosing interval, and the study length could be of importance with regard to prevention of infections. Other trials using lower doses of vitamin D (400–2,000 IU/day) have mainly shown no beneficial effects on infections (223, 225). Interestingly, a recent meta-analysis showed that the dosing interval also appears to be a key factor since the intervention studies using daily doses of vitamin D showed a better therapeutic effect than studies where participants were given large bolus doses of vitamin D at intervals of between 1 and 3 months (220). In addition, earlier studies that have shown no preventive effect of vitamin D supplementation on infections have been performed only during winter season or during shorter periods (of 6–12 weeks) (224, 334-336). One strength of our study was that a relatively large dose of vitamin D (4,000 IU) was administered daily for one year. Our study was the first to cover all four seasons, which is probably important in Sweden where there is known variation in 25(OH)D levels during a one-year period. Another strength was that the patients included had deficiency in vitamin D at the start of the study (mean 50 nmol/l). One study conducted by Murdoch *et al.*, showing no effect of vitamin D supplementation on respiratory tract infections, had healthy

participants with a normal mean vitamin D level at baseline (72 nmol/l) (221). Taking the earlier findings together, it seems plausible that only individuals with a deficiency in vitamin D and symptoms of infection benefit from supplementation.

The mechanisms of the effects observed in study III are not fully understood. Earlier studies have suggested that vitamin D modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs, and general anti-inflammatory effects (337).

It has already been shown that $1,25(OH)_2D$ induces AMPs in immune cells (338). However, an unexpected finding in our study was that we noted a trend of higher AMPs in the placebo group. To explain this, one could argue that this may be due to an effect of microbes on the mucosal production of AMP, which was recently shown in a study from our unit (339). Vitamin D may have the capacity to eliminate pathogens and thus reduce the trigger for AMP production. Given that vitamin D induces AMPs in epithelial cells, we expected a reduction in bacterial pathogens such as *H. influenza, M. catharralis* and *S. pneumoniae* in the participants treated with vitamin D. Notably, the frequency of these bacteria was not reduced; instead, we found a reduction in *S. aureus* and fungal species. A possible explanation could be that vitamin D reduces antibiotic consumption, and thereby also decreases the risk of growth of antibiotic-related colonization bacteria such as *S. aureus* and fungal species. Furthermore, vitamin D has been shown to modulate cytokine production induced by *C. albicans* (340). Alternatively, it is possible that vitamin D can prevent mainly viral infections. This is supported in earlier studies that put forward both mechanistic and clinical evidence for this proposition (210, 242, 243).

Interestingly, we observed a trend that adverse events were being reported more often in the placebo group, suggesting that vitamin D could possibly be efficient against other diseases. This was evident for cardiovascular diseases in particular.

A potential problem with study III was that the primary endpoint might be questioned, since it relied solely on patient-reported information. Another limitation was that the patient population was heterogeneous with regard to immune deficiency and concomitant diseases. We tried to adjust for these factors in the multivariate analysis of the primary endpoint, but unfortunately the sample sizes in each subgroup were too small to be able to draw any conclusions. Thus, the results from our study cannot be applied directly to the general population.

4.4 PAPER IV — VITAMIN D IN PAEDIATRIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

The main objective of study IV was to determine the clinical importance of vitamin D deficiency in children after HSCT. We wanted to investigate whether vitamin D is associated with short- and long-term outcome parameters in paediatric SCT.

123 patients who underwent HSCT between 2004 and 2011 were included in this retrospective study, and were followed for up to eight years with regard to their vitamin D status. The patients were divided into two groups based on serum 25(OH)D level at

baseline: low level (< 50 nmol/l) and sufficient level (≥ 50 nmol/l). The median vitamin D level at baseline was 33 nmol/l in the low-level group and 63 nmol/l in the sufficient-level group. 25(OH)D levels remained significantly higher in the sufficient-level group during follow-up of at least 6 months after transplantation.

In the group with sufficient vitamin D levels, moderate-to-severe acute GVHD occurred more frequently than in the low-level group (47 % vs. 29 %, p = 0.07). When comparing the patients with moderate-to-severe chronic GVHD to those without chronic GVHD, there was a significantly increased risk in the low-level group. Neutrophilic granulocytes and lymphocyte numbers rose significantly faster during the first 3 months post-transplantation in patients with sufficient vitamin D levels at baseline. When comparing patients with the highest baseline levels of vitamin D (> 75 nmol/l) to the others, significantly lower IgG levels were observed in the group with high levels. The number of clinical infections observed was not significantly different, except for a finding of lower incidence of HSV and VZV 3 months after transplantation in the sufficient-level group (p = 0.05 and p = 0.04, respectively). Overall survival (OS) in all patients was not significantly different between the groups. When focusing only on patients with malignant disease, sufficient levels were associated with better OS (p = 0.01). Relapse was found to be more common in patients in the low-level group (p = 0.03).

Discussion

Based on previous reports, we chose to define hypovitaminosis as levels of 25(OH)D below 50 nmol/l (203). Currently, there is no consensus regarding sufficient levels, but several authors claim that 75 nmol/l or higher is sufficient in both children and adults. Levels for optimal influence on immunological processes may be even higher. Patients who undergo HSCT are more prone to develop vitamin D deficiency due to lack of UV exposure, hospitalization, corticosteroid treatment, diminished uptake from the intestines affected by GVHD, and bacterial overgrowth (281, 282, 285). Duncan *et al.* followed 67 paediatric HSCT patients and found that 37% were deficient at baseline, but in the subgroup of patients ≥ 11 years of age 67% were deficient (282). This is in accordance with our findings (69%). The lowest levels in our study were found in patients who were transplanted during winter and early summer, which is the time of year before exposure to sun in Sweden is sufficient to raise vitamin D levels in serum.

In study IV, we found that the frequency of acute GVHD was higher and that of chronic GVHD was lower in patients with vitamin D levels > 50 nmol/l at baseline. These findings may highlight the different pathogenetic processes in the two forms of GVHD, but they may also indicate that vitamin D has dual roles—both immune-inhibitory and stimulatory. The link between vitamin D deficiency and increased risk of chronic GVHD is supported by earlier studies on adult haematopoietic stem cell-transplanted patients (296, 297). In our material, we found that the frequency of acute GVHD was higher in the patient group with sufficient levels of vitamin D. In contrast, Pakkala *et al.* have shown that a vitamin D analogue reduced signs of acute GVHD in mice, probably due to down-regulation of both T-lymphocyte activation and inflammatory effector mechanisms (288). Since T-cells have a main role in development of GVHD, it is interesting that an earlier study has shown that active vitamin D inhibits mixed lymphocyte cultures in a dose-dependent manner and affects

DC maturation, resulting in a Th2-polarized T-lymphocyte population (287). This finding may indicate that administration of high doses of vitamin D to transplanted patients can induce an immune-inhibitory effect and therefore reduce the risk of GVHD. Nevertheless, it is important to remember that it is crucial to maintain a fully activated Th1 type of response to maintain control of viral infections.

The effect of vitamin D on immune reconstitution is not well understood. In our material, neutrophil and lymphocyte counts were significantly higher first 3 months after HSCT in the sufficient-level group. One can speculate that in this case vitamin D may have an immune-stimulatory effect in the early phases of immune recovery. This is certainly an interesting observation since, as described above, acute GVHD was seen more frequently in the sufficient-level group in our study. A faster normalization of immune cell counts might have implications for susceptibility to infections, but no significant effect was seen in our material. One reason for us not being able to see any effect on infections could be that the baseline levels of vitamin D were generally low, and that any influence on infections are not apparent unless the levels of 25(OH)D are above 75 nmol/l.

In study IV, we could also conclude that patients with the highest vitamin D levels at baseline had the lowest IgG levels and more often received IgG replacement. This is in accordance with other studies in which vitamin D has been shown to suppress B-lymphocyte proliferation, plasma cell differentiation, and IgG secretion (299, 300). We concluded that in all patients overall survival (OS) did not differ in the two groups, probably because of the good survival rate in patients with non-malignant diseases. In patients transplanted due to malignancies, however, we noted that OS was significantly higher in patients with sufficient vitamin D levels. We could also conclude that in the patient group with vitamin D levels > 50 nmol/l, relapse was significantly less common.

It should be noted that there may be a “healthy patient bias” here, where a high vitamin D level constitutes a surrogate marker for well-being at the start of HSCT. Moreover, since study IV was a retrospective and observational study, no causuality could be inferred from these associations. However, our findings may suggest that vitamin D has a beneficial role. The triad of more acute GVHD, better overall survival, and less relapse may indicate that vitamin D has immune-stimulatory effects mediating expansion of alloreactive cells, including those with an ability to kill malignant cells (the graft-versus-leukaemia effect). Additional studies where a randomized, blind approach is taken are highly warranted in this field.

5 CONCLUSION AND FUTURE PLANS

The results presented in this thesis show that a better understanding of the panorama of infections in immunocompromised patients gives new views on treatment strategies in this patient group.

In study I, we concluded that a very strong predicting factor for death after HSCT is a low IgG level (< 4 g/L), measured twice during the first year after transplantation. In our view, our results provide evidence enough for us to continue examining IVIG treatment after HSCT. To further evaluate the importance of IgG levels after transplantation regarding survival, a prospective study in patients with low IgG levels after SCT should be conducted where the benefit of IVIG treatment in that specific patient group is examined.

One conclusion that can be drawn from study II, after comparing Campath and Thymoglobulin as part of a conditioning treatment before HSCT, is that both drugs must be taken into consideration to reduce the risk of GVHD and rejection after transplantation with alternative donors. There was no difference in IgG levels between the two groups. Campath was associated with an increased risk of fungal infections, which may have been due to a more immunosuppressive effect compared to TMG. This study was small (a comparison of 36 patients given Campath with a matched cohort of 72 patients receiving TMG), and we believe that larger prospective randomized studies will be needed to evaluate the clinical value of the drugs.

Study III showed that treatment with vitamin D reduced symptoms and antibiotic consumption in patients with an increased frequency of respiratory tract infections. Thus, vitamin D supplementation could provide an alternative strategy to reduce antibiotic use in high consumers, and it may—indirectly—also have an impact on emerging problems with bacterial resistance.

In study IV, we concluded that vitamin D status affected the clinical course in children undergoing HSCT. Vitamin D deficiency at baseline was associated with an increased risk of death, relapse, and chronic GVHD over an 8-year follow-up period. Patients with the highest levels of vitamin D at baseline had the lowest IgG levels after transplantation. Further studies are needed to evaluate our findings and to establish whether vitamin D and treatment with IVIG affect immunological processes in HSCT, and to investigate the subsequent outcome.

6 SAMMANFATTNING PÅ SVENSKA

Människan har under evolutionen utvecklat ett immunologiskt försvar i syfte att skydda oss mot mikroorganismer som skulle kunna vara skadliga. Immunförsvaret består av många olika komponenter, exempelvis:
• Hud och slemhinnor utgör en första barriär mot främmande smittämnen.
• Granulocyter är en typ av vita blodkroppar som oskadliggör mikroorganismer.
• Komplementsystemet utgörs av olika proteiner som hjälper till i vårt immunförsvar.
• T- och B-celler är vita blodkroppar som försvarar oss mot virus-, bakterie- och svampinfektioner.
• Antikroppar (immunglobuliner, t ex IgG), som bildas av B-cellerna, deltar i försvaret mot infektioner.

Det är mycket som kan gå fel i människans komplicerade infektionsförsvar. Vissa personer har en medfödd defekt i immunförsvaret, till exempel en oförmåga att bilda antikroppar, vilket ofta innebär en ökad benägenhet för luftvägsinfektioner. Ibland moduleras immunförsvaret medvetet, exempelvis i samband med stamcellstransplantation. I de fyra studierna, beskrivna i den här avhandlingen, har vi studerat infektionspanoramat hos patienter som genomgått stamcellstransplantation eller haft en ökad benägenhet att insjukna i infektioner. Ett särskilt fokus har lagts på vitamin D och dess effekter på immunförvaret.

Vid hematopoetisk stamcellstransplantation (HSCT), transplanteras de blodbildande stamcellerna (röda- och vita blodkroppar samt blodplättar) som finns i benmärgen. HSCT är idag en etablerad behandlingsmetod vid flera olika sjukdomar såsom leukemier och svåra immunbristtillstånd. Själva transplantationen innebär inte någon operation utan genomförs som en blodtransfusion. HSCT brukar indelas i två olika typer: *autolog HSCT* som innebär transplantation med egna benmärgsceller och *allogen HSCT* som innebär transplantation med benmärgsceller från en donator. De mest fruktade komplikationerna efter transplantation är återfall i grundsjukdomen, GVHD (graft-versus-host disease), infektion samt avstötning av de transplanterade cellerna. GVHD är en reaktion där de transplanterade cellerna reagerar mot patientens egen vävnad. Alla patienter som genomgår HSCT har på grund av avsaknad av vita blodkroppar, innan de transplanterade immunförsvarscellerna börjar fungera, en ökad risk att drabbas av infektioner. I samband med transplantation föreligger alltid en risk för avstötning d.v.s. att patientens eget immunförsvar dödar de transplanterade cellerna.

Alla patienter som genomgår HSCT behandlas innan själva transplantationen med cellgifter och/eller strålning i syfte att förgöra de sjuka cellerna i kroppen samt för att undvika att patientens immunförsvar stöter bort transplantatet. De nya benmärgscellerna har i sig en kraftfull effekt mot till exempel en leukemi-sjukdom. Patienter som genomgår allogen HSCT får ofta behandling med ett immunsupprimerande läkemedel som kalls för ATG (anti thymocyt globulin). Detta läkemedel ges ofta i syfte att förebygga GVHD och minska risken för avstötning.

Vitamin D bildas i huden under inverkan av solljus. I Sverige sker detta bara under sommarhalvåret eftersom UVB-strålningen är för svag under den mörka årstiden för att någon produktion skall kunna ske i huden. Globalt sett är vitamin D-syntesen från UVB viktigare än det vitamin D vi får i oss från kosten. De flesta svenskar har brist på vitamin D, särkilt under vinterhalvåret. Det mest kända tillståndet som orsakas av

allvarlig vitamin D-brist är rakit (Engelska sjukan) som ger skelettabnormiteter. Kroppens slemhinnor är utrustade med ett antimikrobiellt system som bygger på kontinuerlig produktion av antimikrobiella peptider (AMP). AMP kan frisättas av vitamin D och har en förmåga att döda invaderande mikroorganismer. Vitamin D har, förutom att påverka produktionen av AMP, också visat sig kunna både stimulera och hämma immunförsvaret i stort. Forskning har visat att det finns receptorer, mottagarmolekyler, för vitamin D i många av kroppens celler och att brist på vitamin D skulle kunna ge upphov till en rad sjukdomstillstånd. Tidigare studier har visat ett samband mellan låga koncentrationer av vitamin D och flertalet ”vällevnadssjukdomar”, såsom cancer och hjärt- och kärlsjukdomar men också en ökad benägenhet för infektioner.

I studie I undersökte vi retrospektivt (en studieform där man tittar tillbaka på det som skett) vuxna patienter, som genomgått HSCT, i syfte att utvärdera IgG-nivåer efter transplantation. Vi fann att IgG successivt ökade efter transplantationstillfället samt att låga nivåer av IgG var en riskfaktor för att dö efter transplantation.

I studie II jämförde vi retrospektivt två olika typer av ATG: Campath och TMG (thymoglobulin). Med hjälp av journalanteckningar och laboratorielistor jämförde vi hur patienterna mådde efter transplantationstillfället. Det visade sig inte vara någon stor skillnad mellan de två olika läkemedlen förutom att Campath troligen ger en något ökad risk för svåra infektioner jämfört med de patienter som behandlas med TMG.

I studie III undersökte vi om vitamin D-behandling kan minska infektionssymtom och antibiotikakonsumtion hos patienter med antikroppsbrist eller ökad benägenhet för luftvägsinfektion. 140 patienter, med problem med återkommande luftvägsinfektioner, inkluderades i vår studie som varade i ett år. 70 patienter fick placebo och 70 patienter fick 4000 IU vitamin D dagligen. Vi fann att både infektionssymtom från luftvägarna samt antibiotikakonsumtionen minskade hos de patienter som fick vitamin D.

I studie IV undersökte vi retrospektivt barn, som genomgått HSCT, i syfte att se om det fanns något samband mellan vilket vitamin D-värde patienterna hade vid transplantationen och hur de sedan mådde efteråt. Vi upptäckte att vitamin D-brist hos de studerade barnen gav en ökad risk att dö efter transplantation. Vi noterade också en ökad risk för GVHD och återinsjuknande i grundsjukdomen hos patienter med låga vitamin D-nivåer i blodet vid transplantationstillfället.

Förhoppningsvis har de genomförda studierna, presenterade i min avhandling, gett en bättre förståelse för vilka typer av infektioner som föreligger hos patienter med försvagat immunförsvar samt utvidgat kunskaperna runt betydelsen av immunglobulinbrist och vitamin D-brist hos denna patientkategori.

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8 REFERENCES

1. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007 Oct 18;449(7164):819-26. PubMed PMID: 17943118.
2. Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annual review of immunology. 2002;20:197-216. PubMed PMID: 11861602.
3. Singh IS, Hasday JD. Fever, hyperthermia and the heat shock response. International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group. 2013 Aug;29(5):423-35. PubMed PMID: 23863046.
4. Rice P, Martin E, He JR, Frank M, DeTolla L, Hester L, et al. Febrile-range hyperthermia augments neutrophil accumulation and enhances lung injury in experimental gram-negative bacterial pneumonia. Journal of immunology. 2005 Mar 15;174(6):3676-85. PubMed PMID: 15749906.
5. Bernheim HA, Bodel PT, Askenase PW, Atkins E. Effects of fever on host defense mechanisms after infection in the lizard Dipsosaurus dorsalis. British journal of experimental pathology. 1978 Feb;59(1):76-84. PubMed PMID: 638032. Pubmed Central PMCID: 2041320.
6. Jiang Q, Cross AS, Singh IS, Chen TT, Viscardi RM, Hasday JD. Febrile core temperature is essential for optimal host defense in bacterial peritonitis. Infection and immunity. 2000 Mar;68(3):1265-70. PubMed PMID: 10678936. Pubmed Central PMCID: 97277.
7. Zasloff M. Antibiotic peptides as mediators of innate immunity. Current opinion in immunology. 1992 Feb;4(1):3-7. PubMed PMID: 1596366.
8. Boman HG. Peptide antibiotics and their role in innate immunity. Annual review of immunology. 1995;13:61-92. PubMed PMID: 7612236.
9. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. Journal of immunology. 2004 Sep 1;173(5):2909-12. PubMed PMID: 15322146.
10. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002 Jan 24;415(6870):389-95. PubMed PMID: 11807545.
11. Ganz T. Microbiology: Gut defence. Nature. 2003 Apr 3;422(6931):478-9. PubMed PMID: 12673233.
12. Gennaro R, Zanetti M. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. Biopolymers. 2000;55(1):31-49. PubMed PMID: 10931440.
13. Chertov O, Yang D, Howard OM, Oppenheim JJ. Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. Immunological reviews. 2000 Oct;177:68-78. PubMed PMID: 11138786.
14. De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, et al. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. The Journal of experimental medicine. 2000 Oct 2;192(7):1069-74. PubMed PMID: 11015447. Pubmed Central PMCID: 2193321.
15. Walport MJ. Complement. First of two parts. The New England journal of medicine. 2001 Apr 5;344(14):1058-66. PubMed PMID: 11287977.
16. Mir MA, Agrewala JN. Signaling through CD80: an approach for treating lymphomas. Expert opinion on therapeutic targets. 2008 Aug;12(8):969-79. PubMed PMID: 18620519.

17. Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature. 2007 Sep 27;449(7161):419-26. PubMed PMID: 17898760.
18. Quah BJ, O'Neill HC. Maturation of function in dendritic cells for tolerance and immunity. Journal of cellular and molecular medicine. 2005 Jul-Sep;9(3):643-54. PubMed PMID: 16202211.
19. Stockinger B. Immunology: Cause of death matters. Nature. 2009 Mar 5;458(7234):44-5. PubMed PMID: 19262664.
20. Torchinsky MB, Garaude J, Martin AP, Blander JM. Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. Nature. 2009 Mar 5;458(7234):78-82. PubMed PMID: 19262671.
21. Trinchieri G. Biology of natural killer cells. Advances in immunology. 1989;47:187-376. PubMed PMID: 2683611.
22. Ljunggren HG, Karre K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. The Journal of experimental medicine. 1985 Dec 1;162(6):1745-59. PubMed PMID: 3877776. Pubmed Central PMCID: 2187973.
23. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SE, Yagita H, et al. Activation of NK cell cytotoxicity. Molecular immunology. 2005 Feb;42(4):501-10. PubMed PMID: 15607806.
24. Simmons D, Seed B. The Fc gamma receptor of natural killer cells is a phospholipid-linked membrane protein. Nature. 1988 Jun 9;333(6173):568-70. PubMed PMID: 2967436.
25. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature. 2004 Jul 8;430(6996):213-8. PubMed PMID: 15190255.
26. Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infection and immunity. 2005 Apr;73(4):1907-16. PubMed PMID: 15784530. Pubmed Central PMCID: 1087413.
27. Miller JF. Immunological function of the thymus. Lancet. 1961 Sep 30;2(7205):748-9. PubMed PMID: 14474038.
28. Jerne NK. The somatic generation of immune recognition. European journal of immunology. 1971 Jan;1(1):1-9. PubMed PMID: 14978855.
29. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology. 1989;7:145-73. PubMed PMID: 2523712.
30. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999 Oct 14;401(6754):708-12. PubMed PMID: 10537110.
31. O'Garra A, Murphy K. Role of cytokines in determining T-lymphocyte function. Current opinion in immunology. 1994 Jun;6(3):458-66. PubMed PMID: 7917115.
32. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. The Journal of experimental medicine. 2005 Jan 17;201(2):233-40. PubMed PMID: 15657292. Pubmed Central PMCID: 2212798.
33. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annual review of immunology. 2007;25:821-52. PubMed PMID: 17201677.
34. Qu N, Xu M, Mizoguchi I, Furusawa J, Kaneko K, Watanabe K, et al. Pivotal Roles of T-Helper 17-Related Cytokines, IL-17, IL-22, and IL-23, in Inflammatory Diseases. Clinical & developmental immunology. 2013;2013:968549. PubMed PMID: 23956763. Pubmed Central PMCID: 3728507.

35. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. The Journal of experimental medicine. 2006 Oct 2;203(10):2271-9. PubMed PMID: 16982811. Pubmed Central PMCID: 2118116.
36. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007 Jul 26;448(7152):484-7. PubMed PMID: 17581588.
37. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. Nature immunology. 2007 Sep;8(9):950-7. PubMed PMID: 17676044.
38. Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. Cell. 2010 Mar 19;140(6):845-58. PubMed PMID: 20303875.
39. Dong C. TH17 cells in development: an updated view of their molecular identity and genetic programming. Nature reviews Immunology. 2008 May;8(5):337-48. PubMed PMID: 18408735.
40. Gutcher I, Becher B. APC-derived cytokines and T cell polarization in autoimmune inflammation. The Journal of clinical investigation. 2007 May;117(5):1119-27. PubMed PMID: 17476341. Pubmed Central PMCID: 1857272.
41. Beissert S, Schwarz A, Schwarz T. Regulatory T cells. The Journal of investigative dermatology. 2006 Jan;126(1):15-24. PubMed PMID: 16417213.
42. Delves PJ, Roitt IM. The immune system. First of two parts. The New England journal of medicine. 2000 Jul 6;343(1):37-49. PubMed PMID: 10882768.
43. Ajitkumar P, Geier SS, Kesari KV, Borriello F, Nakagawa M, Bluestone JA, et al. Evidence that multiple residues on both the alpha-helices of the class I MHC molecule are simultaneously recognized by the T cell receptor. Cell. 1988 Jul 1;54(1):47-56. PubMed PMID: 3260136.
44. Villadangos JA, Galocha B, Lopez de Castro JA. Unusual topology of an HLA-B27 allospecific T cell epitope lacking peptide specificity. Journal of immunology. 1994 Mar 1;152(5):2317-23. PubMed PMID: 7510742.
45. Sherman LA, Chattopadhyay S. The molecular basis of allorecognition. Annual review of immunology. 1993;11:385-402. PubMed PMID: 8476567.
46. Matzinger P, Bevan MJ. Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens? Cellular immunology. 1977 Mar 1;29(1):1-5. PubMed PMID: 300293.
47. Steele DJ, Laufer TM, Smiley ST, Ando Y, Grusby MJ, Glimcher LH, et al. Two levels of help for B cell alloantibody production. The Journal of experimental medicine. 1996 Feb 1;183(2):699-703. PubMed PMID: 8627185. Pubmed Central PMCID: 2192460.
48. Shoskes DA, Wood KJ. Indirect presentation of MHC antigens in transplantation. Immunology today. 1994 Jan;15(1):32-8. PubMed PMID: 8136009.
49. Simpson E. Minor transplantation antigens: animal models for human host-versus-graft, graft-versus-host, and graft-versus-leukemia reactions. Transplantation. 1998 Mar 15;65(5):611-6. PubMed PMID: 9521192.
50. Ringden O, Witherspoon R, Storb R, Ekelund E, Thomas ED. B cell function in human marrow transplant recipients assessed by direct and indirect hemolysis-in-gel assays. Journal of immunology. 1979 Dec;123(6):2729-34. PubMed PMID: 41018.
51. Witherspoon RP, Kopecky K, Storb RF, Flournoy N, Sullivan KM, Sosa R, et al. Immunological recovery in 48 patients following syngeneic marrow

transplantation or hematological malignancy. Transplantation. 1982 Feb;33(2):143-9. PubMed PMID: 7036468.
52. Thomas ED, Lochte HL, Jr., Lu WC, Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. The New England journal of medicine. 1957 Sep 12;257(11):491-6. PubMed PMID: 13464965.
53. Bortin MM. A compendium of reported human bone marrow transplants. Transplantation. 1970 Jun;9(6):571-87. PubMed PMID: 4911417.
54. Dausset J. [Iso-leuko-antibodies]. Acta haematologica. 1958 Jul-Oct;20(1-4):156-66. PubMed PMID: 13582558. Iso-leuco-anticorps.
55. Van Rood JJ, Eernisse JG, Van Leeuwen A. Leucocyte antibodies in sera from pregnant women. Nature. 1958 Jun 21;181(4625):1735-6. PubMed PMID: 13566127.
56. Thomas ED, Buckner CD, Banaji M, Clift RA, Fefer A, Flournoy N, et al. One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood. 1977 Apr;49(4):511-33. PubMed PMID: 14751.
57. Weiden PL, Flournoy N, Thomas ED, Prentice R, Fefer A, Buckner CD, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. The New England journal of medicine. 1979 May 10;300(19):1068-73. PubMed PMID: 34792.
58. Ringden O, Labopin M, Gluckman E, Reiffers J, Vernant JP, Jouet JP, et al. Graft-versus-leukemia effect in allogeneic marrow transplant recipients with acute leukemia is maintained using cyclosporin A combined with methotrexate as prophylaxis. Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Bone marrow transplantation. 1996 Nov;18(5):921-9. PubMed PMID: 8932846.
59. Ljungman P, Bregni M, Brune M, Cornelissen J, de Witte T, Dini G, et al. Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe 2009. Bone marrow transplantation. 2010 Feb;45(2):219-34. PubMed PMID: 19584824.
60. Thomas ED, Buckner CD, Clift RA, Fefer A, Johnson FL, Neiman PE, et al. Marrow transplantation for acute nonlymphoblastic leukemia in first remission. The New England journal of medicine. 1979 Sep 13;301(11):597-9. PubMed PMID: 381925.
61. Santos GW, Sensenbrenner LL, Burke PJ, Mullins GM, Blas WB, Tutschka PJ, et al. The use of cyclophosphamide for clinical marrow transplantation. Transplantation proceedings. 1972 Dec;4(4):559-64. PubMed PMID: 4405147.
62. Thomas ED, Sanders JE, Flournoy N, Johnson FL, Buckner CD, Clift RA, et al. Marrow transplantation for patients with acute lymphoblastic leukemia: a long-term follow-up. Blood. 1983 Nov;62(5):1139-41. PubMed PMID: 6354306.
63. Santos GW, Tutschka PJ, Brookmeyer R, Saral R, Beschorner WE, Bias WB, et al. Marrow transplantation for acute nonlymphocytic leukemia after treatment with busulfan and cyclophosphamide. The New England journal of medicine. 1983 Dec 1;309(22):1347-53. PubMed PMID: 6355849.
64. Hassan M, Oberg G, Bekassy AN, Aschan J, Ehrsson H, Ljungman P, et al. Pharmacokinetics of high-dose busulphan in relation to age and chronopharmacology. Cancer chemotherapy and pharmacology. 1991;28(2):130-4. PubMed PMID: 2060084.
65. Clift RA, Buckner CD, Appelbaum FR, Bearman SI, Petersen FB, Fisher LD, et al. Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens. Blood. 1990 Nov 1;76(9):1867-71. PubMed PMID: 2224134.

66. Blume KG, Forman SJ, O'Donnell MR, Doroshow JH, Krance RA, Nademanee AP, et al. Total body irradiation and high-dose etoposide: a new preparatory regimen for bone marrow transplantation in patients with advanced hematologic malignancies. Blood. 1987 Apr;69(4):1015-20. PubMed PMID: 3548841.
67. Petersen FB, Appelbaum FR, Bigelow CL, Buckner CD, Clift RA, Sanders JE, et al. High-dose cytosine arabinoside, total body irradiation and marrow transplantation for advanced malignant lymphoma. Bone marrow transplantation. 1989 Sep;4(5):483-8. PubMed PMID: 2676043.
68. Weiden PL, Sullivan KM, Flournoy N, Storb R, Thomas ED. Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. The New England journal of medicine. 1981 Jun 18;304(25):1529-33. PubMed PMID: 7015133.
69. Barrett AJ. Mechanisms of the graft-versus-leukemia reaction. Stem cells. 1997;15(4):248-58. PubMed PMID: 9253108.
70. Antin JH. Stem cell transplantation-harnessing of graft-versus-malignancy. Current opinion in hematology. 2003 Nov;10(6):440-4. PubMed PMID: 14564175.
71. Slavin S, Nagler A, Naparstek E, Kapelushnik Y, Aker M, Cividalli G, et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. Blood. 1998 Feb 1;91(3):756-63. PubMed PMID: 9446633.
72. McSweeney PA, Storb R. Mixed chimerism: preclinical studies and clinical applications. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 1999;5(4):192-203. PubMed PMID: 10465099.
73. Giralt S, Estey E, Albitar M, van Besien K, Rondon G, Anderlini P, et al. Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. Blood. 1997 Jun 15;89(12):4531-6. PubMed PMID: 9192777.
74. Sandmaier BM, Mackinnon S, Childs RW. Reduced intensity conditioning for allogeneic hematopoietic cell transplantation: current perspectives. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2007 Jan;13(1 Suppl 1):87-97. PubMed PMID: 17222778. Pubmed Central PMCID: 1829153.
75. Beatty PG, Hansen JA, Longton GM, Thomas ED, Sanders JE, Martin PJ, et al. Marrow transplantation from HLA-matched unrelated donors for treatment of hematologic malignancies. Transplantation. 1991 Feb;51(2):443-7. PubMed PMID: 1994541.
76. Hows J, Bradley BA, Gore S, Downie T, Howard M, Gluckman E. Prospective evaluation of unrelated donor bone marrow transplantation. The International Marrow Unrelated Search and Transplant (IMUST) Study. Bone marrow transplantation. 1993 Oct;12(4):371-80. PubMed PMID: 8275037.
77. Ringden O, Remberger M, Carlens S, Hagglund H, Mattsson J, Aschan J, et al. Low incidence of acute graft-versus-host disease, using unrelated HLA-A-, HLA-B-, and HLA-DR-compatible donors and conditioning, including anti-T-cell antibodies. Transplantation. 1998 Sep 15;66(5):620-5. PubMed PMID: 9753343.
78. Kiehl MG, Kraut L, Schwerdtfeger R, Hertenstein B, Remberger M, Kroeger N, et al. Outcome of allogeneic hematopoietic stem-cell transplantation in adult patients with acute lymphoblastic leukemia: no difference in related compared with unrelated transplant in first complete remission. Journal of clinical oncology :

official journal of the American Society of Clinical Oncology. 2004 Jul 15;22(14):2816-25. PubMed PMID: 15254049.
79. Remberger M, Svahn BM, Hentschke P, Lofgren C, Ringden O. Effect on cytokine release and graft-versus-host disease of different anti-T cell antibodies during conditioning for unrelated haematopoietic stem cell transplantation. Bone marrow transplantation. 1999 Oct;24(8):823-30. PubMed PMID: 10516691.
80. Bonnefoy-Berard N, Vincent C, Revillard JP. Antibodies against functional leukocyte surface molecules in polyclonal antilymphocyte and antithymocyte globulins. Transplantation. 1991 Mar;51(3):669-73. PubMed PMID: 2006524.
81. Rebellato LM, Gross U, Verbanac KM, Thomas JM. A comprehensive definition of the major antibody specificities in polyclonal rabbit antithymocyte globulin. Transplantation. 1994 Mar 15;57(5):685-94. PubMed PMID: 7908150.
82. Hale G, Cobbold S, Novitzky N, Bunjes D, Willemze R, Prentice HG, et al. CAMPATH-1 antibodies in stem-cell transplantation. Cytotherapy. 2001;3(3):145-64. PubMed PMID: 12171722.
83. Popow I, Leitner J, Majdic O, Kovarik JJ, Saemann MD, Zlabinger GJ, et al. Assessment of batch to batch variation in polyclonal antithymocyte globulin preparations. Transplantation. 2012 Jan 15;93(1):32-40. PubMed PMID: 22186936.
84. Krenger W, Ferrara JL. Graft-versus-host disease and the Th1/Th2 paradigm. Immunologic research. 1996;15(1):50-73. PubMed PMID: 8739565.
85. Billingham RE. The biology of graft-versus-host reactions. Harvey lectures. 1966;62:21-78. PubMed PMID: 4875305.
86. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation. 1974 Oct;18(4):295-304. PubMed PMID: 4153799.
87. Thomas E, Storb R, Clift RA, Fefer A, Johnson FL, Neiman PE, et al. Bone-marrow transplantation (first of two parts). The New England journal of medicine. 1975 Apr 17;292(16):832-43. PubMed PMID: 234595.
88. Passweg JR, Halter J, Bucher C, Gerull S, Heim D, Rovo A, et al. Hematopoietic stem cell transplantation: a review and recommendations for follow-up care for the general practitioner. Swiss medical weekly. 2012;142:w13696. PubMed PMID: 23135685.
89. Bradley BA, Hows JM, Gore SM, Bidwell JL, Clay T, Downie TR, et al. Current status of unrelated-donor bone marrow transplantation. The International Marrow Unrelated Search and Transplant (IMUST) Study. Clinical transplants. 1992:91-107. PubMed PMID: 1306726.
90. Ferrara JL, Levy R, Chao NJ. Pathophysiologic mechanisms of acute graft-vs.-host disease. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 1999;5(6):347-56. PubMed PMID: 10595812.
91. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science. 1999 Jul 16;285(5426):412-5. PubMed PMID: 10411505.
92. Duffner UA, Maeda Y, Cooke KR, Reddy P, Ordemann R, Liu C, et al. Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. Journal of immunology. 2004 Jun 15;172(12):7393-8. PubMed PMID: 15187116.
93. Goker H, Haznedaroglu IC, Chao NJ. Acute graft-vs-host disease: pathobiology and management. Experimental hematology. 2001 Mar;29(3):259-77. PubMed PMID: 11274753.

94. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. Nature reviews Immunology. 2012 Jun;12(6):443-58. PubMed PMID: 22576252. Pubmed Central PMCID: 3552454.
95. Broady R, Yu J, Chow V, Tantiworawit A, Kang C, Berg K, et al. Cutaneous GVHD is associated with the expansion of tissue-localized Th1 and not Th17 cells. Blood. 2010 Dec 16;116(25):5748-51. PubMed PMID: 20864580.
96. Nikolic B, Lee S, Bronson RT, Grusby MJ, Sykes M. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. The Journal of clinical investigation. 2000 May;105(9):1289-98. PubMed PMID: 10792004. Pubmed Central PMCID: 315439.
97. Via CS. Kinetics of T cell activation in acute and chronic forms of murine graft-versus-host disease. Journal of immunology. 1991 Apr 15;146(8):2603-9. PubMed PMID: 2016521.
98. Theobald M, Nierle T, Bunjes D, Arnold R, Heimpel H. Host-specific interleukin-2-secreting donor T-cell precursors as predictors of acute graft-versus-host disease in bone marrow transplantation between HLA-identical siblings. The New England journal of medicine. 1992 Dec 3;327(23):1613-7. PubMed PMID: 1435898.
99. Rowbottom AW, Riches PG, Downie C, Hobbs JR. Monitoring cytokine production in peripheral blood during acute graft-versus-host disease following allogeneic bone marrow transplantation. Bone marrow transplantation. 1993 Dec;12(6):635-41. PubMed PMID: 8136747.
100. Atkinson K, Matias C, Guiffre A, Seymour R, Cooley M, Biggs J, et al. In vivo administration of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF, interleukin-1 (IL-1), and IL-4, alone and in combination, after allogeneic murine hematopoietic stem cell transplantation. Blood. 1991 Mar 15;77(6):1376-82. PubMed PMID: 1705839.
101. Piguet PF, Grau GE, Allet B, Vassalli P. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. The Journal of experimental medicine. 1987 Nov 1;166(5):1280-9. PubMed PMID: 3316469. Pubmed Central PMCID: 2189667.
102. Nestel FP, Price KS, Seemayer TA, Lapp WS. Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease. The Journal of experimental medicine. 1992 Feb 1;175(2):405-13. PubMed PMID: 1732411. Pubmed Central PMCID: 2119110.
103. Troutt AB, Kelso A. Enumeration of lymphokine mRNA-containing cells in vivo in a murine graft-versus-host reaction using the PCR. Proceedings of the National Academy of Sciences of the United States of America. 1992 Jun 15;89(12):5276-80. PubMed PMID: 1608934. Pubmed Central PMCID: 49274.
104. Allen RD, Staley TA, Sidman CL. Differential cytokine expression in acute and chronic murine graft-versus-host-disease. European journal of immunology. 1993 Feb;23(2):333-7. PubMed PMID: 8436168.
105. Velardi A, Varese P, Terenzi A, Dembech C, Albi N, Grossi CE, et al. Lymphokine production by T-cell clones after human bone marrow transplantation. Blood. 1989 Oct;74(5):1665-72. PubMed PMID: 2506949.
106. Blazar BR, Taylor PA, Smith S, Vallera DA. Interleukin-10 administration decreases survival in murine recipients of major histocompatibility complex disparate donor bone marrow grafts. Blood. 1995 Feb 1;85(3):842-51. PubMed PMID: 7833486.
107. Emmanouilides CE, Luo J, Baldwin G, Buckley D, Lau P, Lopez E, et al. Murine IL-10 fails to reduce GVHD despite inhibition of alloreactivity in vitro. Bone marrow transplantation. 1996 Aug;18(2):369-75. PubMed PMID: 8864448.

108. Yabe M, Yabe H, Hattori K, Shimizu T, Matsumoto M, Morimoto T, et al. Role of interleukin-12 in the development of acute graft-versus-host disease in bone marrow transplant patients. Bone marrow transplantation. 1999 Jul;24(1):29-34. PubMed PMID: 10435731.
109. Sykes M, Szot GL, Nguyen PL, Pearson DA. Interleukin-12 inhibits murine graft-versus-host disease. Blood. 1995 Sep 15;86(6):2429-38. PubMed PMID: 7662991.
110. Schultz KR, Paquet J, Bader S, HayGlass KT. Requirement for B cells in T cell priming to minor histocompatibility antigens and development of graft-versus-host disease. Bone marrow transplantation. 1995 Aug;16(2):289-95. PubMed PMID: 7581150.
111. Perez L, Anasetti C, Pidala J. Have we improved in preventing and treating acute graft-versus-host disease? Current opinion in hematology. 2011 Nov;18(6):408-13. PubMed PMID: 21912253. Pubmed Central PMCID: 3432261.
112. Gatza E, Rogers CE, Clouthier SG, Lowler KP, Tawara I, Liu C, et al. Extracorporeal photopheresis reverses experimental graft-versus-host disease through regulatory T cells. Blood. 2008 Aug 15;112(4):1515-21. PubMed PMID: 18411417. Pubmed Central PMCID: 2515148.
113. Appelbaum FR. Hematopoietic-cell transplantation at 50. The New England journal of medicine. 2007 Oct 11;357(15):1472-5. PubMed PMID: 17928594.
114. Ringden O. Immunotherapy by allogeneic stem cell transplantation. Advances in cancer research. 2007;97:25-60. PubMed PMID: 17419940.
115. Remberger M, Ackefors M, Berglund S, Blennow O, Dahllof G, Dlugosz A, et al. Improved survival after allogeneic hematopoietic stem cell transplantation in recent years. A single-center study. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2011 Nov;17(11):1688-97. PubMed PMID: 21620989.
116. Bortin MM, Horowitz MM, Rimm AA. Increasing utilization of allogeneic bone marrow transplantation. Results of the 1988-1990 survey. Annals of internal medicine. 1992 Mar 15;116(6):505-12. PubMed PMID: 1739242.
117. Giralt SA, Champlin RE. Leukemia relapse after allogeneic bone marrow transplantation: a review. Blood. 1994 Dec 1;84(11):3603-12. PubMed PMID: 7949117.
118. Koreth J, Schlenk R, Kopecky KJ, Honda S, Sierra J, Djulbegovic BJ, et al. Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. JAMA : the journal of the American Medical Association. 2009 Jun 10;301(22):2349-61. PubMed PMID: 19509382. Pubmed Central PMCID: 3163846.
119. Bassan R, Hoelzer D. Modern therapy of acute lymphoblastic leukemia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011 Feb 10;29(5):532-43. PubMed PMID: 21220592.
120. Marmont A, Frassoni F, Bacigalupo A, Podesta M, Piaggio G, Van Lint MT, et al. Recurrence of Ph'-positive leukemia in donor cells after marrow transplantation for chronic granulocytic leukemia. The New England journal of medicine. 1984 Apr 5;310(14):903-6. PubMed PMID: 6366558.
121. Goh K, Klemperer MR. In vivo leukemic transformation: cytogenetic evidence of in vivo leukemic transformation of engrafted marrow cells. American journal of hematology. 1977;2(3):283-90. PubMed PMID: 339712.
122. Elfenbein GJ, Brogaonkar DS, Bias WB, Burns WH, Saral R, Sensenbrenner LL, et al. Cytogenetic evidence for recurrence of acute myelogenous leukemia after allogeneic bone marrow transplantation in donor hematopoietic cells. Blood. 1978 Sep;52(3):627-36. PubMed PMID: 354703.

123. Newburger PE, Latt SA, Pesando JM, Gustashaw K, Powers M, Chaganti RS, et al. Leukemia relapse in donor cells after allogeneic bone-marrow transplantation. The New England journal of medicine. 1981 Mar 19;304(12):712-4. PubMed PMID: 7007882.
124. Thomas ED, Bryant JI, Buckner CD, Clift RA, Fefer A, Johnson FL, et al. Leukaemic transformation of engrafted human marrow cells in vivo. Lancet. 1972 Jun 17;1(7764):1310-3. PubMed PMID: 4113402.
125. Murata M, Ishikawa Y, Ohashi H, Terakura S, Ozeki K, Kiyoi H, et al. Donor cell leukemia after allogeneic peripheral blood stem cell transplantation: a case report and literature review. International journal of hematology. 2008 Jul;88(1):111-5. PubMed PMID: 18470599.
126. Ruiz-Arguelles GJ, Ruiz-Arguelles A, Garces-Eisele J. Donor cell leukemia: a critical review. Leukemia & lymphoma. 2007 Jan;48(1):25-38. PubMed PMID: 17325845.
127. Barrett AJ, Battiwalla M. Relapse after allogeneic stem cell transplantation. Expert review of hematology. 2010 Aug;3(4):429-41. PubMed PMID: 21083034. Pubmed Central PMCID: 3426446.
128. Barrett AJ, Horowitz MM, Gale RP, Biggs JC, Camitta BM, Dicke KA, et al. Marrow transplantation for acute lymphoblastic leukemia: factors affecting relapse and survival. Blood. 1989 Aug 1;74(2):862-71. PubMed PMID: 2665858.
129. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990 Feb 1;75(3):555-62. PubMed PMID: 2297567.
130. Sullivan KM, Weiden PL, Storb R, Witherspoon RP, Fefer A, Fisher L, et al. Influence of acute and chronic graft-versus-host disease on relapse and survival after bone marrow transplantation from HLA-identical siblings as treatment of acute and chronic leukemia. Blood. 1989 May 1;73(6):1720-8. PubMed PMID: 2653460.
131. Marmont AM, Horowitz MM, Gale RP, Sobocinski K, Ash RC, van Bekkum DW, et al. T-cell depletion of HLA-identical transplants in leukemia. Blood. 1991 Oct 15;78(8):2120-30. PubMed PMID: 1912589.
132. Hermans A, Heisterkamp N, von Linden M, van Baal S, Meijer D, van der Plas D, et al. Unique fusion of bcr and c-abl genes in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell. 1987 Oct 9;51(1):33-40. PubMed PMID: 2820585.
133. de Klein A, van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, et al. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature. 1982 Dec 23;300(5894):765-7. PubMed PMID: 6960256.
134. van der Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia. 2003 Jun;17(6):1013-34. PubMed PMID: 12764363.
135. van der Velden VH, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia. 2007 Apr;21(4):604-11. PubMed PMID: 17287850.
136. van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia. 1999 Dec;13(12):1901-28. PubMed PMID: 10602411.

137. Mattsson J, Uzunel M, Tammik L, Aschan J, Ringden O. Leukemia lineage-specific chimerism analysis is a sensitive predictor of relapse in patients with acute myeloid leukemia and myelodysplastic syndrome after allogeneic stem cell transplantation. Leukemia. 2001 Dec;15(12):1976-85. PubMed PMID: 11753621.
138. Zetterquist H, Mattsson J, Uzunel M, Nasman-Bjork I, Svenberg P, Tammik L, et al. Mixed chimerism in the B cell lineage is a rapid and sensitive indicator of minimal residual disease in bone marrow transplant recipients with pre-B cell acute lymphoblastic leukemia. Bone marrow transplantation. 2000 Apr;25(8):843-51. PubMed PMID: 10808205.
139. Lion T, Daxberger H, Dubovsky J, Filipcik P, Fritsch G, Printz D, et al. Analysis of chimerism within specific leukocyte subsets for detection of residual or recurrent leukemia in pediatric patients after allogeneic stem cell transplantation. Leukemia. 2001 Feb;15(2):307-10. PubMed PMID: 11236951.
140. Sairafi D, Remberger M, Uhlin M, Ljungman P, Ringden O, Mattsson J. Leukemia lineage-specific chimerism analysis and molecular monitoring improve outcome of donor lymphocyte infusions. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2010 Dec;16(12):1728-37. PubMed PMID: 20542125.
141. Meyers JD, Flournoy N, Thomas ED. Nonbacterial pneumonia after allogeneic marrow transplantation: a review of ten years' experience. Reviews of infectious diseases. 1982 Nov-Dec;4(6):1119-32. PubMed PMID: 6296961.
142. Wingard JR. Infections in allogeneic bone marrow transplant recipients. Seminars in oncology. 1993 Oct;20(5 Suppl 6):80-7. PubMed PMID: 8211220.
143. Lundgren G, Wilczek H, Lonnqvist B, Lindholm A, Wahren B, Ringden O. Acyclovir prophylaxis in bone marrow transplant recipients. Scandinavian journal of infectious diseases Supplementum. 1985;47:137-44. PubMed PMID: 3006228.
144. Wade JC, Newton B, Flournoy N, Meyers JD. Oral acyclovir for prevention of herpes simplex virus reactivation after marrow transplantation. Annals of internal medicine. 1984 Jun;100(6):823-8. PubMed PMID: 6326632.
145. Ljungman P. Prophylaxis against herpesvirus infections in transplant recipients. Drugs. 2001;61(2):187-96. PubMed PMID: 11270937.
146. Myers JD, Spencer HC, Jr., Watts JC, Gregg MB, Stewart JA, Troupin RH, et al. Cytomegalovirus pneumonia after human marrow transplantation. Annals of internal medicine. 1975 Feb;82(2):181-8. PubMed PMID: 234706.
147. Ljungman P, Aschan J, Lewensohn-Fuchs I, Carlens S, Larsson K, Lonnqvist B, et al. Results of different strategies for reducing cytomegalovirus-associated mortality in allogeneic stem cell transplant recipients. Transplantation. 1998 Nov 27;66(10):1330-4. PubMed PMID: 9846518.
148. Ljungman P. Risk assessment in haematopoietic stem cell transplantation: viral status. Best practice & research Clinical haematology. 2007 Jun;20(2):209-17. PubMed PMID: 17448957.
149. Einsele H, Hebart H, Kauffmann-Schneider C, Sinzger C, Jahn G, Bader P, et al. Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. Bone marrow transplantation. 2000 Apr;25(7):757-63. PubMed PMID: 10745262.
150. Andrei G, De Clercq E, Snoeck R. Novel inhibitors of human CMV. Current opinion in investigational drugs. 2008 Feb;9(2):132-45. PubMed PMID: 18246516.
151. Li CR, Greenberg PD, Gilbert MJ, Goodrich JM, Riddell SR. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood. 1994 Apr 1;83(7):1971-9. PubMed PMID: 8142663.

152. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science. 1992 Jul 10;257(5067):238-41. PubMed PMID: 1352912.
153. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. The New England journal of medicine. 1995 Oct 19;333(16):1038-44. PubMed PMID: 7675046.
154. Uhlin M, Wikell H, Sundin M, Blennow O, Maeurer M, Ringden O, et al. Risk factors for Epstein Barr virus related post-transplant lymphoproliferative disease after allogeneic hematopoietic stem cell transplantation. Haematologica. 2013 Sep 20. PubMed PMID: 24056821.
155. Witherspoon RP, Fisher LD, Schoch G, Martin P, Sullivan KM, Sanders J, et al. Secondary cancers after bone marrow transplantation for leukemia or aplastic anemia. The New England journal of medicine. 1989 Sep 21;321(12):784-9. PubMed PMID: 2671734.
156. Shapiro RS, McClain K, Frizzera G, Gajl-Peczalska KJ, Kersey JH, Blazar BR, et al. Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. Blood. 1988 May;71(5):1234-43. PubMed PMID: 2833957.
157. Locksley RM, Flournoy N, Sullivan KM, Meyers JD. Infection with varicella-zoster virus after marrow transplantation. The Journal of infectious diseases. 1985 Dec;152(6):1172-81. PubMed PMID: 3905982.
158. Martin MA, Bock MJ, Pfaller MA, Wenzel RP. Respiratory syncytial virus infections in adult bone marrow transplant recipients. Lancet. 1988 Jun 18;1(8599):1396-7. PubMed PMID: 2898070.
159. Ljungman P, Ward KN, Crooks BN, Parker A, Martino R, Shaw PJ, et al. Respiratory virus infections after stem cell transplantation: a prospective study from the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Bone marrow transplantation. 2001 Sep;28(5):479-84. PubMed PMID: 11593321.
160. Harrington RD, Hooton TM, Hackman RC, Storch GA, Osborne B, Gleaves CA, et al. An outbreak of respiratory syncytial virus in a bone marrow transplant center. The Journal of infectious diseases. 1992 Jun;165(6):987-93. PubMed PMID: 1583345.
161. Blennow O, Mattsson J, Remberger M. Pre-engraftment blood stream infection is a risk factor for acute GVHD grades II-IV. Bone marrow transplantation. 2013 Jul 22. PubMed PMID: 23872739.
162. Bodey GP, Buckley M, Sathe YS, Freireich EJ. Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. Annals of internal medicine. 1966 Feb;64(2):328-40. PubMed PMID: 5216294.
163. Sparrelid E, Hagglund H, Remberger M, Ringden O, Lonnqvist B, Ljungman P, et al. Bacteraemia during the aplastic phase after allogeneic bone marrow transplantation is associated with early death from invasive fungal infection. Bone marrow transplantation. 1998 Oct;22(8):795-800. PubMed PMID: 9827978.
164. Marty FM, Rubin RH. The prevention of infection post-transplant: the role of prophylaxis, preemptive and empiric therapy. Transplant international : official journal of the European Society for Organ Transplantation. 2006 Jan;19(1):2-11. PubMed PMID: 16359371.
165. Pizzo PA. Approach to the patient with prolonged granulocytopenia. Recent results in cancer research Fortschritte der Krebsforschung Progres dans les recherches sur le cancer. 1993;132:57-65. PubMed PMID: 8265871.

166. Koll BS, Brown AE. The changing epidemiology of infections at cancer hospitals. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1993 Nov;17 Suppl 2:S322-8. PubMed PMID: 8274595.
167. Bochud PY, Calandra T, Francioli P. Bacteremia due to viridans streptococci in neutropenic patients: a review. The American journal of medicine. 1994 Sep;97(3):256-64. PubMed PMID: 8092175.
168. Wald A, Leisenring W, van Burik JA, Bowden RA. Epidemiology of Aspergillus infections in a large cohort of patients undergoing bone marrow transplantation. The Journal of infectious diseases. 1997 Jun;175(6):1459-66. PubMed PMID: 9180187.
169. Blennow O, Remberger M, Klingspor L, Omazic B, Fransson K, Ljungman P, et al. Randomized PCR-based therapy and risk factors for invasive fungal infection following reduced-intensity conditioning and hematopoietic SCT. Bone marrow transplantation. 2010 Dec;45(12):1710-8. PubMed PMID: 20190840.
170. Meyers JD. Fungal infections in bone marrow transplant patients. Seminars in oncology. 1990 Jun;17(3 Suppl 6):10-3. PubMed PMID: 2353204.
171. Tollemar J, Ringden O, Bostrom L, Nilsson B, Sundberg B. Variables predicting deep fungal infections in bone marrow transplant recipients. Bone marrow transplantation. 1989 Nov;4(6):635-41. PubMed PMID: 2555004.
172. Denning DW, Marinus A, Cohen J, Spence D, Herbrecht R, Pagano L, et al. An EORTC multicentre prospective survey of invasive aspergillosis in haematological patients: diagnosis and therapeutic outcome. EORTC Invasive Fungal Infections Cooperative Group. The Journal of infection. 1998 Sep;37(2):173-80. PubMed PMID: 9821093.
173. Gotzsche PC, Johansen HK. Routine versus selective antifungal administration for control of fungal infections in patients with cancer. Cochrane database of systematic reviews. 2000 (4):CD000026. PubMed PMID: 11034669.
174. Martin MV, Yates J, Hitchcock CA. Comparison of voriconazole (UK-109,496) and itraconazole in prevention and treatment of Aspergillus fumigatus endocarditis in guinea pigs. Antimicrobial agents and chemotherapy. 1997 Jan;41(1):13-6. PubMed PMID: 8980747. Pubmed Central PMCID: 163652.
175. Pfaller MA, Yu WL. Antifungal susceptibility testing. New technology and clinical applications. Infectious disease clinics of North America. 2001 Dec;15(4):1227-61. PubMed PMID: 11780273.
176. Espinel-Ingroff A, Boyle K, Sheehan DJ. In vitro antifungal activities of voriconazole and reference agents as determined by NCCLS methods: review of the literature. Mycopathologia. 2001;150(3):101-15. PubMed PMID: 11469757.
177. Arikan S, Lozano-Chiu M, Paetznick V, Rex JH. In vitro susceptibility testing methods for caspofungin against Aspergillus and Fusarium isolates. Antimicrobial agents and chemotherapy. 2001 Jan;45(1):327-30. PubMed PMID: 11120990. Pubmed Central PMCID: 90285.
178. Holick MF. Vitamin D: A millenium perspective. Journal of cellular biochemistry. 2003 Feb 1;88(2):296-307. PubMed PMID: 12520530.
179. Holick MF. Resurrection of vitamin D deficiency and rickets. The Journal of clinical investigation. 2006 Aug;116(8):2062-72. PubMed PMID: 16886050. Pubmed Central PMCID: 1523417.
180. Hess AF, Unger LJ, Pappenheimer AM. Experimental rickets in rats. III. The prevention of rickets in rats by exposure to sunlight. 1922. J. Biol. Chem. 50, 77-81. The Journal of biological chemistry. 2002 Jun 7;277(23):e1-2. PubMed PMID: 12061339.

181. Hess AF. The Prevention and Cure of Rickets by Sunlight. Am J Public Health (N Y). 1922 Feb;12(2):104-7. PubMed PMID: 18010639. Pubmed Central PMCID: 1354036.
182. Hess AF. The Contribution of Biology, Chemistry and Physics to the Newer Knowledge of Rickets. Science. 1928 Mar 30;67(1735):333-5. PubMed PMID: 17735125.
183. Hess AF, Unger LJ, Pappenheimer AM. Experimental Rickets in Rats : Vii. The Prevention of Rickets by Sunlight, by the Rays of the Mercury Vapor Lamp, and by the Carbon Arc Lamp. The Journal of experimental medicine. 1922 Sep 30;36(4):427-46. PubMed PMID: 19868685. Pubmed Central PMCID: 2128316.
184. Reichert TA, Simonsen L, Sharma A, Pardo SA, Fedson DS, Miller MA. Influenza and the winter increase in mortality in the United States, 1959-1999. American journal of epidemiology. 2004 Sep 1;160(5):492-502. PubMed PMID: 15321847.
185. Gwaltney JM, Jr., Hendley JO, Simon G, Jordan WS, Jr. Rhinovirus infections in an industrial population. I. The occurrence of illness. The New England journal of medicine. 1966 Dec 8;275(23):1261-8. PubMed PMID: 4288780.
186. Khoo AL, Chai L, Koenen H, Joosten I, Netea M, van der Ven A. Translating the role of vitamin D3 in infectious diseases. Critical reviews in microbiology. 2012 May;38(2):122-35. PubMed PMID: 22304022.
187. Chan TY. Vitamin D deficiency and susceptibility to tuberculosis. Calcified tissue international. 2000 Jun;66(6):476-8. PubMed PMID: 10821887.
188. Ginde AA, Mansbach JM, Camargo CA, Jr. Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. Archives of internal medicine. 2009 Feb 23;169(4):384-90. PubMed PMID: 19237723. Pubmed Central PMCID: 3447082.
189. Karatekin G, Kaya A, Salihoglu O, Balci H, Nuhoglu A. Association of subclinical vitamin D deficiency in newborns with acute lower respiratory infection and their mothers. European journal of clinical nutrition. 2009 Apr;63(4):473-7. PubMed PMID: 18030309.
190. DeLuca HF. Overview of general physiologic features and functions of vitamin D. The American journal of clinical nutrition. 2004 Dec;80(6 Suppl):1689S-96S. PubMed PMID: 15585789.
191. Holick MF. Vitamin D deficiency. The New England journal of medicine. 2007 Jul 19;357(3):266-81. PubMed PMID: 17634462.
192. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. The American journal of clinical nutrition. 2000 Sep;72(3):690-3. PubMed PMID: 10966885.
193. Holick MF, MacLaughlin JA, Doppelt SH. Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator. Science. 1981 Feb 6;211(4482):590-3. PubMed PMID: 6256855.
194. Holick MF. High prevalence of vitamin D inadequacy and implications for health. Mayo Clinic proceedings Mayo Clinic. 2006 Mar;81(3):353-73. PubMed PMID: 16529140.
195. DeLuca HF. Vitamin D and the parenteral nutrition patient. Gastroenterology. 2009 Nov;137(5 Suppl):S79-91. PubMed PMID: 19874954.
196. Boullata JI. Vitamin D supplementation: a pharmacologic perspective. Current opinion in clinical nutrition and metabolic care. 2010 Nov;13(6):677-84. PubMed PMID: 20975351.
197. Holick MF. Vitamin D: a D-Lightful health perspective. Nutrition reviews. 2008 Oct;66(10 Suppl 2):S182-94. PubMed PMID: 18844847.

198. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. The American journal of clinical nutrition. 2008 Apr;87(4):1080S-6S. PubMed PMID: 18400738.
199. Holick MF, Chen TC, Lu Z, Sauter E. Vitamin D and skin physiology: a D-lightful story. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2007 Dec;22 Suppl 2:V28-33. PubMed PMID: 18290718.
200. Moan J, Porojnicu AC, Dahlback A, Setlow RB. Addressing the health benefits and risks, involving vitamin D or skin cancer, of increased sun exposure. Proceedings of the National Academy of Sciences of the United States of America. 2008 Jan 15;105(2):668-73. PubMed PMID: 18180454. Pubmed Central PMCID: 2206594.
201. Looker AC, Pfeiffer CM, Lacher DA, Schleicher RL, Picciano MF, Yetley EA. Serum 25-hydroxyvitamin D status of the US population: 1988-1994 compared with 2000-2004. The American journal of clinical nutrition. 2008 Dec;88(6):1519-27. PubMed PMID: 19064511. Pubmed Central PMCID: 2745830.
202. Matsuoka LY, Ide L, Wortsman J, MacLaughlin JA, Holick MF. Sunscreens suppress cutaneous vitamin D3 synthesis. The Journal of clinical endocrinology and metabolism. 1987 Jun;64(6):1165-8. PubMed PMID: 3033008.
203. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism. 2011 Jul;96(7):1911-30. PubMed PMID: 21646368.
204. Heaney RP, Dowell MS, Hale CA, Bendich A. Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. Journal of the American College of Nutrition. 2003 Apr;22(2):142-6. PubMed PMID: 12672710.
205. Chapuy MC, Preziosi P, Maamer M, Arnaud S, Galan P, Hercberg S, et al. Prevalence of vitamin D insufficiency in an adult normal population. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 1997;7(5):439-43. PubMed PMID: 9425501.
206. Holick MF, Siris ES, Binkley N, Beard MK, Khan A, Katzer JT, et al. Prevalence of Vitamin D inadequacy among postmenopausal North American women receiving osteoporosis therapy. The Journal of clinical endocrinology and metabolism. 2005 Jun;90(6):3215-24. PubMed PMID: 15797954.
207. Rovner AJ, Stallings VA, Schall JI, Leonard MB, Zemel BS. Vitamin D insufficiency in children, adolescents, and young adults with cystic fibrosis despite routine oral supplementation. The American journal of clinical nutrition. 2007 Dec;86(6):1694-9. PubMed PMID: 18065588.
208. Stephenson A, Brotherwood M, Robert R, Atenafu E, Corey M, Tullis E. Cholecalciferol significantly increases 25-hydroxyvitamin D concentrations in adults with cystic fibrosis. The American journal of clinical nutrition. 2007 May;85(5):1307-11. PubMed PMID: 17490967.
209. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. The American journal of clinical nutrition. 2003 Jan;77(1):204-10. PubMed PMID: 12499343.
210. Sabetta JR, DePetrillo P, Cipriani RJ, Smardin J, Burns LA, Landry ML. Serum 25-hydroxyvitamin d and the incidence of acute viral respiratory tract infections in healthy adults. PloS one. 2010;5(6):e11088. PubMed PMID: 20559424. Pubmed Central PMCID: 2885414.

211. Jacobus CH, Holick MF, Shao Q, Chen TC, Holm IA, Kolodny JM, et al. Hypervitaminosis D associated with drinking milk. The New England journal of medicine. 1992 Apr 30;326(18):1173-7. PubMed PMID: 1313547.
212. Koutkia P, Chen TC, Holick MF. Vitamin D intoxication associated with an over-the-counter supplement. The New England journal of medicine. 2001 Jul 5;345(1):66-7. PubMed PMID: 11439958.
213. Vieth R. Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. The American journal of clinical nutrition. 1999 May;69(5):842-56. PubMed PMID: 10232622.
214. Hollis BW, Wagner CL. Assessment of dietary vitamin D requirements during pregnancy and lactation. The American journal of clinical nutrition. 2004 May;79(5):717-26. PubMed PMID: 15113709.
215. McGrath J. Does 'imprinting' with low prenatal vitamin D contribute to the risk of various adult disorders? Medical hypotheses. 2001 Mar;56(3):367-71. PubMed PMID: 11359362.
216. Huh SY, Gordon CM. Vitamin D deficiency in children and adolescents: epidemiology, impact and treatment. Reviews in endocrine & metabolic disorders. 2008 Jun;9(2):161-70. PubMed PMID: 18175220.
217. Hewison M. Antibacterial effects of vitamin D. Nature reviews Endocrinology. 2011 Jun;7(6):337-45. PubMed PMID: 21263449.
218. Nagpal S, Na S, Rathnachalam R. Noncalcemic actions of vitamin D receptor ligands. Endocrine reviews. 2005 Aug;26(5):662-87. PubMed PMID: 15798098.
219. Jolliffe DA, Griffiths CJ, Martineau AR. Vitamin D in the prevention of acute respiratory infection: systematic review of clinical studies. The Journal of steroid biochemistry and molecular biology. 2013 Jul;136:321-9. PubMed PMID: 23220552.
220. Bergman P, Lindh AU, Bjorkhem-Bergman L, Lindh JD. Vitamin D and Respiratory Tract Infections: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. PloS one. 2013;8(6):e65835. PubMed PMID: 23840373. Pubmed Central PMCID: 3686844.
221. Murdoch DR, Slow S, Chambers ST, Jennings LC, Stewart AW, Priest PC, et al. Effect of vitamin D3 supplementation on upper respiratory tract infections in healthy adults: the VIDARIS randomized controlled trial. JAMA : the journal of the American Medical Association. 2012 Oct 3;308(13):1333-9. PubMed PMID: 23032549.
222. Rees JR, Hendricks K, Barry EL, Peacock JL, Mott LA, Sandler RS, et al. Vitamin D3 Supplementation and Upper Respiratory Tract Infections in a Randomized, Controlled Trial. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2013 Sep 6. PubMed PMID: 24014734.
223. Li-Ng M, Aloia JF, Pollack S, Cunha BA, Mikhail M, Yeh J, et al. A randomized controlled trial of vitamin D3 supplementation for the prevention of symptomatic upper respiratory tract infections. Epidemiology and infection. 2009 Oct;137(10):1396-404. PubMed PMID: 19296870.
224. Laaksi I, Ruohola JP, Mattila V, Auvinen A, Ylikomi T, Pihlajamaki H. Vitamin D supplementation for the prevention of acute respiratory tract infection: a randomized, double-blinded trial among young Finnish men. The Journal of infectious diseases. 2010 Sep 1;202(5):809-14. PubMed PMID: 20632889.
225. Laaksi IT, Ruohola JP, Ylikomi TJ, Auvinen A, Haataja RI, Pihlajamaki HK, et al. Vitamin D fortification as public health policy: significant improvement in vitamin D status in young Finnish men. European journal of clinical nutrition. 2006 Aug;60(8):1035-8. PubMed PMID: 16482069.

226. Lehouck A, Mathieu C, Carremans C, Baeke F, Verhaegen J, Van Eldere J, et al. High doses of vitamin D to reduce exacerbations in chronic obstructive pulmonary disease: a randomized trial. Annals of internal medicine. 2012 Jan 17;156(2):105-14. PubMed PMID: 22250141.
227. Camargo CA, Jr., Ganmaa D, Frazier AL, Kirchberg FF, Stuart JJ, Kleinman K, et al. Randomized trial of vitamin D supplementation and risk of acute respiratory infection in Mongolia. Pediatrics. 2012 Sep;130(3):e561-7. PubMed PMID: 22908115.
228. Manaseki-Holland S, Maroof Z, Bruce J, Mughal MZ, Masher MI, Bhutta ZA, et al. Effect on the incidence of pneumonia of vitamin D supplementation by quarterly bolus dose to infants in Kabul: a randomised controlled superiority trial. Lancet. 2012 Apr 14;379(9824):1419-27. PubMed PMID: 22494826. Pubmed Central PMCID: 3348565.
229. Martineau AR. Bolus-dose vitamin D and prevention of childhood pneumonia. Lancet. 2012 Apr 14;379(9824):1373-5. PubMed PMID: 22494825.
230. Nielsen NO, Skifte T, Andersson M, Wohlfahrt J, Soborg B, Koch A, et al. Both high and low serum vitamin D concentrations are associated with tuberculosis: a case-control study in Greenland. The British journal of nutrition. 2010 Nov;104(10):1487-91. PubMed PMID: 20553638.
231. Henry HL. Regulation of vitamin D metabolism. Best practice & research Clinical endocrinology & metabolism. 2011 Aug;25(4):531-41. PubMed PMID: 21872796.
232. Hollis BW, Conrad HR, Hibbs JW. Changes in plasma 25-hydroxycholecalciferol and selected blood parameters after injection of massive doses of cholecalciferol or 25-hydroxycholecalciferol in non-lactating dairy cows. The Journal of nutrition. 1977 Apr;107(4):606-13. PubMed PMID: 191582.
233. Vieth R. How to optimize vitamin D supplementation to prevent cancer, based on cellular adaptation and hydroxylase enzymology. Anticancer research. 2009 Sep;29(9):3675-84. PubMed PMID: 19667164.
234. Gibney KB, MacGregor L, Leder K, Torresi J, Marshall C, Ebeling PR, et al. Vitamin D deficiency is associated with tuberculosis and latent tuberculosis infection in immigrants from sub-Saharan Africa. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2008 Feb 1;46(3):443-6. PubMed PMID: 18173355.
235. Martineau AR, Nhamoyebonde S, Oni T, Rangaka MX, Marais S, Bangani N, et al. Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa. Proceedings of the National Academy of Sciences of the United States of America. 2011 Nov 22;108(47):19013-7. PubMed PMID: 22025704. Pubmed Central PMCID: 3223428.
236. Wilkinson RJ, Llewelyn M, Toossi Z, Patel P, Pasvol G, Lalvani A, et al. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet. 2000 Feb 19;355(9204):618-21. PubMed PMID: 10696983.
237. Adams JS, Ren S, Liu PT, Chun RF, Lagishetty V, Gombart AF, et al. Vitamin d-directed rheostatic regulation of monocyte antibacterial responses. Journal of immunology. 2009 Apr 1;182(7):4289-95. PubMed PMID: 19299728. Pubmed Central PMCID: 2683618.
238. Adams JS, Hewison M. Update in vitamin D. The Journal of clinical endocrinology and metabolism. 2010 Feb;95(2):471-8. PubMed PMID: 20133466. Pubmed Central PMCID: 2840860.
239. Olsen K, Falch BM, Danielsen K, Johannessen M, Ericson Sollid JU, Thune I, et al. Staphylococcus aureus nasal carriage is associated with serum 25-

hydroxyvitamin D levels, gender and smoking status. The Tromso Staph and Skin Study. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 2012 Apr;31(4):465-73. PubMed PMID: 21811869. Pubmed Central PMCID: 3303067.
240. Gropp R, Frye M, Wagner TO, Bargon J. Epithelial defensins impair adenoviral infection: implication for adenovirus-mediated gene therapy. Human gene therapy. 1999 Apr 10;10(6):957-64. PubMed PMID: 10223729.
241. Daher KA, Selsted ME, Lehrer RI. Direct inactivation of viruses by human granulocyte defensins. Journal of virology. 1986 Dec;60(3):1068-74. PubMed PMID: 3023659. Pubmed Central PMCID: 253347.
242. Beard JA, Bearden A, Striker R. Vitamin D and the anti-viral state. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2011 Mar;50(3):194-200. PubMed PMID: 21242105. Pubmed Central PMCID: 3308600.
243. Hansdottir S, Monick MM, Lovan N, Powers L, Gerke A, Hunninghake GW. Vitamin D decreases respiratory syncytial virus induction of NF-kappaB-linked chemokines and cytokines in airway epithelium while maintaining the antiviral state. Journal of immunology. 2010 Jan 15;184(2):965-74. PubMed PMID: 20008294. Pubmed Central PMCID: 3035054.
244. Cantorna MT, Yu S, Bruce D. The paradoxical effects of vitamin D on type 1 mediated immunity. Molecular aspects of medicine. 2008 Dec;29(6):369-75. PubMed PMID: 18561994. Pubmed Central PMCID: 2633636.
245. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet. 2002 Dec 7;360(9348):1831-7. PubMed PMID: 12480361.
246. Bischoff-Ferrari HA, Giovannucci E, Willett WC, Dietrich T, Dawson-Hughes B. Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. The American journal of clinical nutrition. 2006 Jul;84(1):18-28. PubMed PMID: 16825677.
247. Broe KE, Chen TC, Weinberg J, Bischoff-Ferrari HA, Holick MF, Kiel DP. A higher dose of vitamin d reduces the risk of falls in nursing home residents: a randomized, multiple-dose study. Journal of the American Geriatrics Society. 2007 Feb;55(2):234-9. PubMed PMID: 17302660.
248. Pettifor JM. Rickets and vitamin D deficiency in children and adolescents. Endocrinology and metabolism clinics of North America. 2005 Sep;34(3):537-53, vii. PubMed PMID: 16085158.
249. Bischoff-Ferrari HA, Dietrich T, Orav EJ, Hu FB, Zhang Y, Karlson EW, et al. Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged > or =60 y. The American journal of clinical nutrition. 2004 Sep;80(3):752-8. PubMed PMID: 15321818.
250. Boland R. Role of vitamin D in skeletal muscle function. Endocrine reviews. 1986 Nov;7(4):434-48. PubMed PMID: 3536463.
251. Dukas L, Bischoff HA, Lindpaintner LS, Schacht E, Birkner-Binder D, Damm TN, et al. Alfacalcidol reduces the number of fallers in a community-dwelling elderly population with a minimum calcium intake of more than 500 mg daily. Journal of the American Geriatrics Society. 2004 Feb;52(2):230-6. PubMed PMID: 14728632.
252. Sambrook PN, Chen JS, March LM, Cameron ID, Cumming RG, Lord SR, et al. Serum parathyroid hormone predicts time to fall independent of vitamin D status in a frail elderly population. The Journal of clinical endocrinology and metabolism. 2004 Apr;89(4):1572-6. PubMed PMID: 15070914.

253. Bischoff-Ferrari HA, Dawson-Hughes B, Willett WC, Staehelin HB, Bazemore MG, Zee RY, et al. Effect of Vitamin D on falls: a meta-analysis. JAMA : the journal of the American Medical Association. 2004 Apr 28;291(16):1999-2006. PubMed PMID: 15113819.
254. Gorham ED, Garland CF, Garland FC, Grant WB, Mohr SB, Lipkin M, et al. Vitamin D and prevention of colorectal cancer. The Journal of steroid biochemistry and molecular biology. 2005 Oct;97(1-2):179-94. PubMed PMID: 16236494.
255. Hanchette CL, Schwartz GG. Geographic patterns of prostate cancer mortality. Evidence for a protective effect of ultraviolet radiation. Cancer. 1992 Dec 15;70(12):2861-9. PubMed PMID: 1451068.
256. Grant WB. An estimate of premature cancer mortality in the US due to inadequate doses of solar ultraviolet-B radiation. Cancer. 2002 Mar 15;94(6):1867-75. PubMed PMID: WOS:000174478000031. English.
257. Giovannucci E, Liu Y, Rimm EB, Hollis BW, Fuchs CS, Stampfer MJ, et al. Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. Journal of the National Cancer Institute. 2006 Apr 5;98(7):451-9. PubMed PMID: 16595781.
258. Feskanich D, Ma J, Fuchs CS, Kirkner GJ, Hankinson SE, Hollis BW, et al. Plasma vitamin D metabolites and risk of colorectal cancer in women. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2004 Sep;13(9):1502-8. PubMed PMID: 15342452.
259. Chang ET, Smedby KE, Hjalgrim H, Porwit-MacDonald A, Roos G, Glimelius B, et al. Family history of hematopoietic malignancy and risk of lymphoma. Journal of the National Cancer Institute. 2005 Oct 5;97(19):1466-74. PubMed PMID: 16204696.
260. Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP. Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. The American journal of clinical nutrition. 2007 Jun;85(6):1586-91. PubMed PMID: 17556697.
261. Ahonen MH, Tenkanen L, Teppo L, Hakama M, Tuohimaa P. Prostate cancer risk and prediagnostic serum 25-hydroxyvitamin D levels (Finland). Cancer causes & control : CCC. 2000 Oct;11(9):847-52. PubMed PMID: 11075874.
262. Garland CF, Garland FC, Gorham ED, Lipkin M, Newmark H, Mohr SB, et al. The role of vitamin D in cancer prevention. American journal of public health. 2006 Feb;96(2):252-61. PubMed PMID: 16380576. Pubmed Central PMCID: 1470481.
263. Mantell DJ, Owens PE, Bundred NJ, Mawer EB, Canfield AE. 1 alpha,25-dihydroxyvitamin D-3 inhibits angiogenesis in vitro and in vivo. Circulation research. 2000 Aug 4;87(3):214-20. PubMed PMID: WOS:000088862600010. English.
264. Cantorna MT, Zhu Y, Froicu M, Wittke A. Vitamin D status, 1,25-dihydroxyvitamin D3, and the immune system. The American journal of clinical nutrition. 2004 Dec;80(6 Suppl):1717S-20S. PubMed PMID: 15585793.
265. Ponsonby AL, McMichael A, van der Mei I. Ultraviolet radiation and autoimmune disease: insights from epidemiological research. Toxicology. 2002 Dec 27;181-182:71-8. PubMed PMID: 12505287.
266. Merlino LA, Curtis J, Mikuls TR, Cerhan JR, Criswell LA, Saag KG, et al. Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. Arthritis and rheumatism. 2004 Jan;50(1):72-7. PubMed PMID: 14730601.
267. McAlindon TE, Felson DT, Zhang Y, Hannan MT, Aliabadi P, Weissman B, et al. Relation of dietary intake and serum levels of vitamin D to

progression of osteoarthritis of the knee among participants in the Framingham Study. Annals of internal medicine. 1996 Sep 1;125(5):353-9. PubMed PMID: 8702085.
268. Hypponen E, Laara E, Reunanen A, Jarvelin MR, Virtanen SM. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. Lancet. 2001 Nov 3;358(9292):1500-3. PubMed PMID: 11705562.
269. Kreindler JL, Steele C, Nguyen N, Chan YR, Pilewski JM, Alcorn JF, et al. Vitamin D3 attenuates Th2 responses to Aspergillus fumigatus mounted by CD4+ T cells from cystic fibrosis patients with allergic bronchopulmonary aspergillosis. The Journal of clinical investigation. 2010 Sep;120(9):3242-54. PubMed PMID: 20714107. Pubmed Central PMCID: 2929900.
270. Daniel C, Sartory NA, Zahn N, Radeke HH, Stein JM. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. The Journal of pharmacology and experimental therapeutics. 2008 Jan;324(1):23-33. PubMed PMID: 17911375.
271. Lemire JM, Archer DC, Beck L, Spiegelberg HL. Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. The Journal of nutrition. 1995 Jun;125(6 Suppl):1704S-8S. PubMed PMID: 7782931.
272. Zittermann A. Vitamin D and disease prevention with special reference to cardiovascular disease. Progress in biophysics and molecular biology. 2006 Sep;92(1):39-48. PubMed PMID: 16600341.
273. Rostand SG. Ultraviolet light may contribute to geographic and racial blood pressure differences. Hypertension. 1997 Aug;30(2 Pt 1):150-6. PubMed PMID: 9260973.
274. Krause R, Buhring M, Hopfenmuller W, Holick MF, Sharma AM. Ultraviolet B and blood pressure. Lancet. 1998 Aug 29;352(9129):709-10. PubMed PMID: 9728997.
275. Eyles DW, Smith S, Kinobe R, Hewison M, McGrath JJ. Distribution of the vitamin D receptor and 1 alpha-hydroxylase in human brain. Journal of chemical neuroanatomy. 2005 Jan;29(1):21-30. PubMed PMID: 15589699.
276. Gloth FM, 3rd, Alam W, Hollis B. Vitamin D vs broad spectrum phototherapy in the treatment of seasonal affective disorder. The journal of nutrition, health & aging. 1999;3(1):5-7. PubMed PMID: 10888476.
277. McGrath J, Selten JP, Chant D. Long-term trends in sunshine duration and its association with schizophrenia birth rates and age at first registration--data from Australia and the Netherlands. Schizophrenia research. 2002 Apr 1;54(3):199-212. PubMed PMID: 11950544.
278. Cannell JJ. Autism and vitamin D. Medical hypotheses. 2008;70(4):750-9. PubMed PMID: 17920208.
279. Joseph RW, Alousi A, Konda B, Komanduri K, Neumann J, Trevino C, et al. High incidence of vitamin D deficiency in patients undergoing allogeneic stem cell transplantation. American journal of hematology. 2011 Nov;86(11):954-6. PubMed PMID: 21948087.
280. Urbain P, Ihorst G, Biesalski HK, Bertz H. Course of serum 25-hydroxyvitamin D(3) status and its influencing factors in adults undergoing allogeneic hematopoietic cell transplantation. Annals of hematology. 2012 May;91(5):759-66. PubMed PMID: 22080146.
281. Sproat L, Bolwell B, Rybicki L, Dean R, Sobecks R, Pohlman B, et al. Vitamin D level after allogeneic hematopoietic stem cell transplant. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2011 Jul;17(7):1079-83. PubMed PMID: 21193053.

282. Duncan CN, Vrooman L, Apfelbaum EM, Whitley K, Bechard L, Lehmann LE. 25-hydroxy vitamin D deficiency following pediatric hematopoietic stem cell transplant. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2011 May;17(5):749-53. PubMed PMID: 20951818.
283. Sato T, Fukagawa M, Uchida K, Katayama A, Nagasaka T, Matsuoka S, et al. 1,25-dihydroxyvitamin D synthesis after renal transplantation: the role of fibroblast growth factor 23 and cyclosporine. Clinical transplantation. 2009 Jun-Jul;23(3):368-74. PubMed PMID: 19519602.
284. Lo CW, Paris PW, Clemens TL, Nolan J, Holick MF. Vitamin D absorption in healthy subjects and in patients with intestinal malabsorption syndromes. The American journal of clinical nutrition. 1985 Oct;42(4):644-9. PubMed PMID: 4050723.
285. Robien K, Strayer LG, Majhail N, Lazovich D, Baker KS, Smith AR, et al. Vitamin D status among long-term survivors of hematopoietic cell transplantation. Bone marrow transplantation. 2011 Nov;46(11):1472-9. PubMed PMID: 21243026. Pubmed Central PMCID: 3131431.
286. Monkawa T, Yoshida T, Hayashi M, Saruta T. Identification of 25-hydroxyvitamin D3 1alpha-hydroxylase gene expression in macrophages. Kidney international. 2000 Aug;58(2):559-68. PubMed PMID: 10916079.
287. Rosenblatt J, Bissonnette A, Ahmad R, Wu Z, Vasir B, Stevenson K, et al. Immunomodulatory effects of vitamin D: implications for GVHD. Bone marrow transplantation. 2010 Sep;45(9):1463-8. PubMed PMID: 20081878.
288. Pakkala I, Taskinen E, Pakkala S, Raisanen-Sokolowski A. MC1288, a vitamin D analog, prevents acute graft-versus-host disease in rat bone marrow transplantation. Bone marrow transplantation. 2001 Apr;27(8):863-7. PubMed PMID: 11477445.
289. Vanham G, Van Baelen H, Tan BK, Bouillon R. The effect of vitamin D analogs and of vitamin D-binding protein on lymphocyte proliferation. Journal of steroid biochemistry. 1988 Apr;29(4):381-6. PubMed PMID: 3374126.
290. Ferreira GB, Overbergh L, Verstuyf A, Mathieu C. 1alpha,25-Dihydroxyvitamin D(3) and its analogs as modulators of human dendritic cells: A comparison dose-titration study. The Journal of steroid biochemistry and molecular biology. 2012 Oct 23. PubMed PMID: 23098690.
291. Griffin MD, Lutz WH, Phan VA, Bachman LA, McKean DJ, Kumar R. Potent inhibition of dendritic cell differentiation and maturation by vitamin D analogs. Biochemical and biophysical research communications. 2000 Apr 21;270(3):701-8. PubMed PMID: 10772887.
292. Piemonti L, Monti P, Sironi M, Fraticelli P, Leone BE, Dal Cin E, et al. Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. Journal of immunology. 2000 May 1;164(9):4443-51. PubMed PMID: 10779743.
293. D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. The Journal of clinical investigation. 1998 Jan 1;101(1):252-62. PubMed PMID: 9421488. Pubmed Central PMCID: 508562.
294. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. Journal of immunology. 2000 Mar 1;164(5):2405-11. PubMed PMID: 10679076.

295. van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. The Journal of steroid biochemistry and molecular biology. 2005 Oct;97(1-2):93-101. PubMed PMID: 16046118.
296. Silva F, Perez-Simon JA, Caballero-Velazquez T, Sanchez-Guijo FM, Villanueva-Gomez F, Vazquez L, et al. Effect of vitamin D treatment in chronic GVHD. Bone marrow transplantation. 2011 Oct;46(10):1395-7. PubMed PMID: 21243033.
297. Glotzbecker B, Ho VT, Aldridge J, Kim HT, Horowitz G, Ritz J, et al. Low levels of 25-hydroxyvitamin D before allogeneic hematopoietic SCT correlate with the development of chronic GVHD. Bone marrow transplantation. 2013 Apr;48(4):593-7. PubMed PMID: 23000654.
298. Lemire JM, Adams JS, Sakai R, Jordan SC. 1 alpha,25-dihydroxyvitamin D3 suppresses proliferation and immunoglobulin production by normal human peripheral blood mononuclear cells. The Journal of clinical investigation. 1984 Aug;74(2):657-61. PubMed PMID: 6611355. Pubmed Central PMCID: 370520.
299. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nature reviews Immunology. 2008 Sep;8(9):685-98. PubMed PMID: 19172691. Pubmed Central PMCID: 2906676.
300. Di Rosa M, Malaguarnera M, Nicoletti F, Malaguarnera L. Vitamin D3: a helpful immuno-modulator. Immunology. 2011 Oct;134(2):123-39. PubMed PMID: 21896008. Pubmed Central PMCID: 3194221.
301. Ringden O, Ruutu T, Remberger M, Nikoskelainen J, Volin L, Vindelov L, et al. A randomized trial comparing busulfan with total body irradiation as conditioning in allogeneic marrow transplant recipients with leukemia: a report from the Nordic Bone Marrow Transplantation Group. Blood. 1994 May 1;83(9):2723-30. PubMed PMID: 8167351.
302. Svenberg P, Remberger M, Svennilson J, Mattsson J, Leblanc K, Gustafsson B, et al. Allogenic stem cell transplantation for nonmalignant disorders using matched unrelated donors. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2004 Dec;10(12):877-82. PubMed PMID: 15570256.
303. Hentschke P, Barkholt L, Uzunel M, Mattsson J, Wersall P, Pisa P, et al. Low-intensity conditioning and hematopoietic stem cell transplantation in patients with renal and colon carcinoma. Bone marrow transplantation. 2003 Feb;31(4):253-61. PubMed PMID: 12621459.
304. Mattsson J, Uzunel M, Brune M, Hentschke P, Barkholt L, Stierner U, et al. Mixed chimaerism is common at the time of acute graft-versus-host disease and disease response in patients receiving non-myeloablative conditioning and allogeneic stem cell transplantation. British journal of haematology. 2001 Dec;115(4):935-44. PubMed PMID: 11843830.
305. Hale G, Waldmann H. Control of graft-versus-host disease and graft rejection by T cell depletion of donor and recipient with Campath-1 antibodies. Results of matched sibling transplants for malignant diseases. Bone marrow transplantation. 1994 May;13(5):597-611. PubMed PMID: 8054913.
306. Ringden O, Pihlstedt P, Markling L, Aschan J, Baryd I, Ljungman P, et al. Prevention of graft-versus-host disease with T cell depletion or cyclosporin and methotrexate. A randomized trial in adult leukemic marrow recipients. Bone marrow transplantation. 1991 Mar;7(3):221-6. PubMed PMID: 2059758.
307. Storb R, Deeg HJ, Whitehead J, Appelbaum F, Beatty P, Bensinger W, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. The New England journal of medicine. 1986 Mar 20;314(12):729-35. PubMed PMID: 3513012.

308. Ljungman P, Lore K, Aschan J, Klaesson S, Lewensohn-Fuchs I, Lonnqvist B, et al. Use of a semi-quantitative PCR for cytomegalovirus DNA as a basis for pre-emptive antiviral therapy in allogeneic bone marrow transplant patients. Bone marrow transplantation. 1996 Apr;17(4):583-7. PubMed PMID: 8722359.
309. Ringden O, Remberger M, Persson U, Ljungman P, Aldener A, Andstrom E, et al. Similar incidence of graft-versus-host disease using HLA-A, -B and -DR identical unrelated bone marrow donors as with HLA-identical siblings. Bone marrow transplantation. 1995 Apr;15(4):619-25. PubMed PMID: 7655390.
310. Svahn BM, Remberger M, Myrback KE, Holmberg K, Eriksson B, Hentschke P, et al. Home care during the pancytopenic phase after allogeneic hematopoietic stem cell transplantation is advantageous compared with hospital care. Blood. 2002 Dec 15;100(13):4317-24. PubMed PMID: 12393737.
311. Sundin M, Nordin K, Jostemyr Y, Winiarski J. Subcutaneous IgG replacement after pediatric SCT. Pediatric transplantation. 2012 Dec;16(8):866-71. PubMed PMID: 22931490.
312. Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. The American journal of medicine. 1980 Aug;69(2):204-17. PubMed PMID: 6996481.
313. Pettit SJ, Bourne H, Spickett GP. Survey of infection in patients receiving antibody replacement treatment for immune deficiency. Journal of clinical pathology. 2002 Aug;55(8):577-80. PubMed PMID: 12147649. Pubmed Central PMCID: 1769738.
314. Roifman CM, Levison H, Gelfand EW. High-dose versus low-dose intravenous immunoglobulin in hypogammaglobulinaemia and chronic lung disease. Lancet. 1987 May 9;1(8541):1075-7. PubMed PMID: 2883406.
315. Omazic B, Hentschke P, Nasman-Bjork I, Mattsson J, Oxelius VA, Ringden O, et al. Reconstitution of the Ig heavy chain CDR3 repertoire after allogeneic haematopoietic stem cell transplantation with myeloablative or reduced-intensity conditioning regimens. Scandinavian journal of immunology. 2005 Jan;61(1):72-81. PubMed PMID: 15644125.
316. Atkinson K. Reconstruction of the haemopoietic and immune systems after marrow transplantation. Bone marrow transplantation. 1990 Apr;5(4):209-26. PubMed PMID: 2186835.
317. Storek J, Wells D, Dawson MA, Storer B, Maloney DG. Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation. Blood. 2001 Jul 15;98(2):489-91. PubMed PMID: 11435323.
318. Sheridan JF, Tutschka PJ, Sedmak DD, Copelan EA. Immunoglobulin G subclass deficiency and pneumococcal infection after allogeneic bone marrow transplantation. Blood. 1990 Apr 1;75(7):1583-6. PubMed PMID: 2317564.
319. Paulin T, Ringden O, Nilsson B. Immunological recovery after bone marrow transplantation: role of age, graft-versus-host disease, prednisolone treatment and infections. Bone marrow transplantation. 1987 Feb;1(3):317-28. PubMed PMID: 3332141.
320. Lum LG. Immune recovery after bone marrow transplantation. Hematology/oncology clinics of North America. 1990 Jun;4(3):659-75. PubMed PMID: 2193017.
321. Sullivan KM, Kopecky KJ, Jocom J, Fisher L, Buckner CD, Meyers JD, et al. Immunomodulatory and antimicrobial efficacy of intravenous immunoglobulin in bone marrow transplantation. The New England journal of medicine. 1990 Sep 13;323(11):705-12. PubMed PMID: 2167452.

322. Witherspoon RP, Storb R, Ochs HD, Fluornoy N, Kopecky KJ, Sullivan KM, et al. Recovery of antibody production in human allogeneic marrow graft recipients: influence of time posttransplantation, the presence or absence of chronic graft-versus-host disease, and antithymocyte globulin treatment. Blood. 1981 Aug;58(2):360-8. PubMed PMID: 6454452.
323. Winston DJ, Ho WG, Lin CH, Bartoni K, Budinger MD, Gale RP, et al. Intravenous immune globulin for prevention of cytomegalovirus infection and interstitial pneumonia after bone marrow transplantation. Annals of internal medicine. 1987 Jan;106(1):12-8. PubMed PMID: 3024542.
324. Phillips NE, Parker DC. Cross-linking of B lymphocyte Fc gamma receptors and membrane immunoglobulin inhibits anti-immunoglobulin-induced blastogenesis. Journal of immunology. 1984 Feb;132(2):627-32. PubMed PMID: 6228594.
325. Nasman Bjork I, Fesel C, Brissac C, Lundkvist I. Prophylactic intravenous immunoglobulin treatment influences serum immunoglobulin M repertoire development after allogeneic bone marrow transplantation. Scandinavian journal of immunology. 1999 Jul;50(1):73-82. PubMed PMID: 10404055.
326. Hale G, Jacobs P, Wood L, Fibbe WE, Barge R, Novitzky N, et al. CD52 antibodies for prevention of graft-versus-host disease and graft rejection following transplantation of allogeneic peripheral blood stem cells. Bone marrow transplantation. 2000 Jul;26(1):69-76. PubMed PMID: 10918407.
327. D'Sa S, Peggs K, Pizzey A, Verfuerth S, Thuraisundaram D, Watts M, et al. T- and B-cell immune reconstitution and clinical outcome in patients with multiple myeloma receiving T-cell-depleted, reduced-intensity allogeneic stem cell transplantation with an alemtuzumab-containing conditioning regimen followed by escalated donor lymphocyte infusions. British journal of haematology. 2003 Oct;123(2):309-22. PubMed PMID: 14531914.
328. Chakrabarti S, Mackinnon S, Chopra R, Kottaridis PD, Peggs K, O'Gorman P, et al. High incidence of cytomegalovirus infection after nonmyeloablative stem cell transplantation: potential role of Campath-1H in delaying immune reconstitution. Blood. 2002 Jun 15;99(12):4357-63. PubMed PMID: 12036862.
329. Chakrabarti S, Mautner V, Osman H, Collingham KE, Fegan CD, Klapper PE, et al. Adenovirus infections following allogeneic stem cell transplantation: incidence and outcome in relation to graft manipulation, immunosuppression, and immune recovery. Blood. 2002 Sep 1;100(5):1619-27. PubMed PMID: 12176880.
330. Morris EC, Rebello P, Thomson KJ, Peggs KS, Kyriakou C, Goldstone AH, et al. Pharmacokinetics of alemtuzumab used for in vivo and in vitro T-cell depletion in allogeneic transplantations: relevance for early adoptive immunotherapy and infectious complications. Blood. 2003 Jul 1;102(1):404-6. PubMed PMID: 12623851.
331. Remberger M, Sundberg B. Rabbit-immunoglobulin G levels in patients receiving thymoglobulin as part of conditioning before unrelated donor stem cell transplantation. Haematologica. 2005 Jul;90(7):931-8. PubMed PMID: 15996931.
332. Remberger M, Svahn BM, Mattsson J, Ringden O. Dose study of thymoglobulin during conditioning for unrelated donor allogeneic stem-cell transplantation. Transplantation. 2004 Jul 15;78(1):122-7. PubMed PMID: 15257050.
333. Lynch BA, Vasef MA, Comito M, Gilman AL, Lee N, Ritchie J, et al. Effect of in vivo lymphocyte-depleting strategies on development of lymphoproliferative disorders in children post allogeneic bone marrow transplantation. Bone marrow transplantation. 2003 Sep;32(5):527-33. PubMed PMID: 12942101.
334. Urashima M, Segawa T, Okazaki M, Kurihara M, Wada Y, Ida H. Randomized trial of vitamin D supplementation to prevent seasonal influenza A in

schoolchildren. The American journal of clinical nutrition. 2010 May;91(5):1255-60. PubMed PMID: 20219962.
335. Martineau AR, Timms PM, Bothamley GH, Hanifa Y, Islam K, Claxton AP, et al. High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. Lancet. 2011 Jan 15;377(9761):242-50. PubMed PMID: 21215445.
336. Nursyam EW, Amin Z, Rumende CM. The effect of vitamin D as supplementary treatment in patients with moderately advanced pulmonary tuberculous lesion. Acta medica Indonesiana. 2006 Jan-Mar;38(1):3-5. PubMed PMID: 16479024.
337. Hewison M. Vitamin D and immune function: an overview. The Proceedings of the Nutrition Society. 2012 Feb;71(1):50-61. PubMed PMID: 21849106.
338. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science. 2006 Mar 24;311(5768):1770-3. PubMed PMID: 16497887.
339. Cederlund A, Olliver M, Rekha RS, Lindh M, Lindbom L, Normark S, et al. Impaired release of antimicrobial peptides into nasal fluid of hyper-IgE and CVID patients. PloS one. 2011;6(12):e29316. PubMed PMID: 22216247. Pubmed Central PMCID: 3246483.
340. Khoo AL, Chai LY, Koenen HJ, Kullberg BJ, Joosten I, van der Ven AJ, et al. 1,25-dihydroxyvitamin D3 modulates cytokine production induced by Candida albicans: impact of seasonal variation of immune responses. The Journal of infectious diseases. 2011 Jan 1;203(1):122-30. PubMed PMID: 21148505. Pubmed Central PMCID: 3086448.

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ORIGINAL ARTICLE

Allogeneic stem cell transplantation: low immunoglobulin levels associated with decreased survival

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The aim of this study was to evaluate the effects and kinetics of IgG levels after allogeneic stem cell transplantation (SCT). This study retrospectively examines 179 consecutive patients undergoing SCT between 1995 and 2002. Diagnoses included acute and chronic leukemia ($n=136$), solid tumors ($n=11$), other malignancies ($n=16$) and non-malignant diseases ($n=16$). Standard myeloablative conditioning was given to 146 patients, and 33 patients received reduced intensity conditioning. Serum samples for measurement of IgG levels were collected 3, 6 and 12 months after SCT, and then yearly. IgG levels increased after SCT throughout the study period. Factors that were associated with low IgG levels after SCT were acute graft-versus-host disease (GVHD), patient age $\leqslant 30$ years, female donor-to-male recipient, not receiving anti-thymocyte globulin and type of GVHD prophylaxis. Compared to patients with moderately low or normal levels as measured twice during the first year after transplantation, patients with low IgG levels (<4 g/l) showed a decreased survival rate (54 vs 71%, $P=0.04$) and an increased incidence of transplant-related mortality (27 vs 9%, $P<0.01$). IgG levels generally increase after SCT. Persistent low levels of IgG are a risk factor for death after SCT.

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Introduction

Reconstitution of the immune system after allogeneic stem cell transplantation (SCT) depends on the conditioning regimen before SCT, graft-versus-host disease (GVHD) and time after SCT.[1,2] Parts of the innate immune system, such as granulocytes, monocytes and NK cells, recover within 2–6 months after SCT.[3] Patients who develop chronic GVHD after SCT may never recover normal immune function.[4] After SCT, the reconstitution of the B-cell repertoire is often delayed because of complications associated with GVHD, age and infections.[5,6] Normal B-cell counts are reached between 6 and 9 months after SCT and after more than 12 months if GVHD has occurred.[7] Antibody production is also impaired after SCT. Serum IgM levels return to normal usually within 3–6 months, whereas recovery of serum IgG levels may be delayed up to one year or longer.[4,7] Impairment of antibody immunity correlates with an increased risk of infections after SCT primarily due to encapsulated bacteria.[8,9] In a randomized trial of long-term administration of intravenous immunoglobulin (IVIG), no differences in chronic GVHD or infections were shown between patients receiving IVIG and controls;[9] however, in this study patients with IgG <4 g/l received IVIG even if randomized to the control arm. It has also been suggested that immunization of the recipient and the donor before SCT may improve antibody immunity after SCT.[10,11]

In this study, we evaluated the clinical influence of IgG levels after SCT in 179 patients who received transplants between 1995 and 2002. This study evaluates the influence of IgG levels on incidence of infections, transplant-related mortality (TRM), and survival.

Patients and methods

Patients

This study retrospectively examines 179 consecutive patients who had undergone allogeneic SCT at Karolinska University Hospital Huddinge between 1995 and 2002. The ethical committee at the Karolinska Institute (Karolinska University Hospital Huddinge) approved all aspects of this study. Only patients with at least two IgG levels measured during the first 12 months were included in the study. This study excluded patients with myeloma, patients treated with IVIG and children younger than 10 years. Our experience is that the ability to produce IgG in children younger than 10 years can be very diverse and therefore difficult to evaluate. The indications for

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transplantation included acute leukemia ($n=83$), chronic leukemia ($n=53$), solid tumor ($n=11$), other malignancies ($n=16$) and non-malignant disease ($n=16$). Table 1 summarizes the patients' demographics.

Donors and tissue typing

Unstimulated bone marrow (BM) and peripheral blood stem cells (PBSC's) were obtained from 85 and 94 donors respectively. The donors of PBSC's were treated with granulocyte-colony-stimulating-factor (10 μg/kg/day) before the donation.[12] Pre-transplant histo-compatibility testing of donors and patients consisted of HLA class I and II typing by allele level PCR single-stranded polymorphism.[13] The majority of patients had an HLA-identical sibling donor or a HLA-A, -B and -DRβ1 identical unrelated donor, but 12 with mismatched donors were also included.

Table 1 Characteristics of patients included in the study of IgG levels after allogeneic hematopoietic stem cell transplantation

	All patients (n = *179*)	*Two low IgG* (n = *26*)	*Normal IgG* (n = *153*)
Sex (M/F)	102/77	16/10	86/67
Age	35 (10–65)	29 (10–60)	37 (10–65)
Children (10–18 years)	35 (20%)	5 (19%)	30 (20%)
Donor sex (M/F)	108/71	14/12	94/59
Donor age	35 (5–62)	34 (14–62)	35 (5–61)
Diagnose			
Non-malignant	16 (9%)	2 (8%)	14 (9%)
Acute leukemia	83 (46%)	13 (50%)	70 (46%)
Chronic leukemia	53 (30%)	7 (27%)	46 (30%)
Other malignancies[a]	16 (9%)	3 (12%)	13 (8%)
Solid tumor	11 (6%)	1 (4%)	10 (7%)
Disease stage (early/late)[b]	111/57	17/8	94/49
Donor			
Sibling	91 (51%)	12 (46%)	79 (52%)
MUD	76 (42%)	13 (50%)	63 (41%)
Mismatch	12 (7%)	1 (4%)	11 (7%)
Fem D to Male R	32 (18%)	6 (23%)	26 (17%)
Stem-cell source (BM/PBSC)	85/94	11/15	74/79
NC dose ($\times 10^8$/kg)	4.5 (0.5–27.6)	6.2 (1.1–25.6)	4.1 (0.5–27.6)
CD34+ dose ($\times 10^6$/kg)	5.3 (0.3–25.5)	6.2 (0.7–9.5)	5.2 (0.3–25.5)
G-CSF after SCT	129 (72%)	20 (77%)	109 (71%)
Conditioning			
TBI based	96 (54%)	16 (61%)	80 (52%)
Non-TBI based	50 (28%)	7 (27%)	43 (28%)
RIC	33 (18%)	3 (12%)	30 (20%)
ATG	110 (61%)	14 (54%)	96 (63%)
GVHD prophylaxis			
CsA + MTX	157 (88%)	25 (96%)	132 (86%)
Other combinations	22	1 (4%)	21 (14%)
Acute GVHD			
No	55 (31%)	5 (19%)	50 (33%)
I	85 (47%)	14 (54%)	71 (46%)
II	33 (18%)	4 (15%)	29 (19%)
III–IV	6 (3%)	3 (12%)	3 (2%)
Chronic GVHD	99 (55%)	20 (77%)	79 (52%)

Abbreviations: ATG = anti-thymocyte globuline; BM = bone marrow; CsA + MTX = cyclosporine + methotrexate; Fem D to Male R = female donor-to-male recipient; G-CSF = granulocyte colony-stimulating factor; GVHD = graft-versus-host disease; MUD = HLA-A, -B and -DR identical unrelated donor; NC = nucleated cell; PBSC = peripheral blood stem cells; RIC = reduced intensity conditioning; TBI = total-body irradiation.
[a]Including lymphoma 9, MDS 5 and myelofibrosis 2.
[b]Early, CR/CP1 and non-malignant diseases, late; later stages.

Conditioning

Myeloablative conditioning regimens consisted of cyclophosphamide (60 mg/kg daily) for 2 days in combination with either 7.5–10 Gy single dose total body irradiation (TBI) ($n=65$), 12 Gy (4×3 Gy) of fractionated TBI ($n=31$), or busulfan (1 mg/kg daily) for 4 days ($n=45$).[14,15] Six patients with aplastic anemia received 200 mg/kg of cyclophosphamide and anti-thymocyte globulin (ATG). All patients with unrelated or mismatched donors were treated with ATG (2–5 mg/kg daily) for 2–5 days before transplantation.[16] The reduced intensity conditioning (RIC) was fludarabine (30 mg/m^2 daily) for 3–6 days, combined with 2 Gy TBI ($n=5$), 60 mg/kg cyclophosphamide ($n=9$) or busulfan (4 mg/kg daily) for 2 days and ATG (2 mg/kg daily) for 4 days ($n=18$).[17–19]

GVHD prophylaxis

The majority of patients (88%) received cyclosporine (CsA) combined with four doses of methotrexate (MTX) as prophylaxis against GVHD.[20,21] If GVHD did not occur, CsA was discontinued after 6 months for patients who received a matched unrelated donor (MUD) or mismatched grafts, and 3 months in the case of sibling transplants. No GVHD prophylaxis was used following the four syngeneic transplants. Other protocols included CsA ($n=2$), T-cell depletion ($n=3$) and CsA combined with either prednisolone ($n=1$) or mycofenolate mofetil ($n=12$).[18] Four patients received mycofenolate mofetil and tacrolimus.

Supportive care

During the pancytopenic phase, all patients received prophylactic treatment with oral ciprofloxacin (500 mg twice daily), fluconazole (100 mg daily) and nystatin (200 000 IU 4 times daily) until the absolute neutrophil count exceeded 0.5×10^9/l. During the first six months after engraftment, trimethoprim–sulfametoxazol was administered as prophylaxis against *Pneumocystis carinii* infection. Patients with a herpes simplex virus IgG that exceeded 10 000 (determined by enzyme-linked immunosorbent assay; ELISA) received oral or intravenous acyclovir prophylaxis during the pancytopenic period.

If patients had a fever, empirical antibiotic therapy was initiated immediately after samples for culture had been obtained. The primary antibiotic regimen used was trimethoprim–sulfamethoxazole combined with an amino glycoside (amikacin) except in patients with known hypersensitivity to sulfonamides. Treatment was adjusted according to bacterial susceptibility as soon as the etiology had been established in cultures. If a patient had an

antibiotic-resistant fever lasting for more than 4–5 days, empirical antifungal therapy was initiated with i.v. amphotericin-B (0.3–0.8 mg/kg daily) or i.v. liposomal amphotericin-B (1–3 mg/kg daily).

Blood samples were tested weekly for cytomegalovirus (CMV) reactivation until 14 weeks after SCT. This test was done using a semi-quantitative PCR or quantitative real-time PCR for detection of CMV-DNA in peripheral blood lymphocytes.[22,23] Definition and treatment of CMV disease after hemopoietic stem cell transplantation have been previously described.[24,25]

To achieve faster engraftment after transplantation, granulocyte colony-stimulating factor was administered to 129 patients until 2001. This treatment ended when an association with increased risk for acute GVHD was found.[26]

Definitions

Diagnosis and grading of acute and chronic GVHD were performed based on clinical symptoms and/or biopsies according to established criteria.[27,28] For the diagnosis of bacteremia, at least one positive blood culture was required. Invasive fungal infection was defined as positive blood culture and/or positive cultures from at least two organs for *Candida* or *Aspergillus* species. *Aspergillus pneumonia* was defined as pulmonary infiltrates and positive cultures of bronchoalveolar lavage fluid, sputum or autopsy samples. Patients with serum IgG values below 4 g/l were defined as significantly deficient according to the existing guidelines for antibody-replacement therapy.[29,30]

Measurement of IgG Levels

Serum samples for measurement of IgG levels were collected 3, 6, 9 and 12 months following hemopoietic stem cell transplantation and then at yearly intervals until 5 years posttransplantation. Serum IgG was analyzed routinely by nephelometry at Karolinska University Laboratory, Department of Clinical Chemistry. Reference levels were 6–15 g/l.

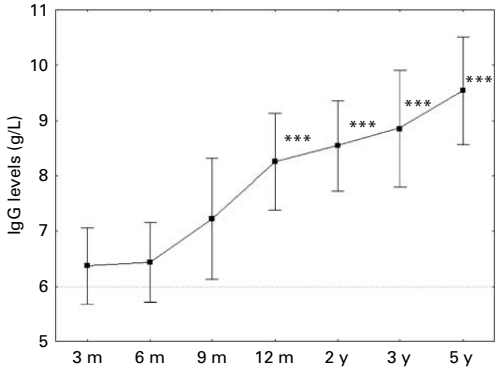

Figure 1 IgG levels at different time points after SCT in 179 patients. Mean±95% CI. Dotted line indicates lower reference level for the lab. ***$P \leqslant 0.001$ vs IgG levels at 3 months.

Statistics

Variables related to the patients, donors, disease and transplant were analyzed for their potential prognostic value on overall survival and TRM. Univariate and multivariate Cox proportional hazard regression models were used to identify independent risk factors for death. Relapse and TRM were competing events. For this reason, assessment of factors predicting TRM were based on the proportional hazard model for sub-distribution of competing risk. Univariate and multivariate analyses were performed using Gray's test and the proportional sub-distribution hazard regression model developed by Fine and Gray. A stepwise backward procedure was used to construct a set of independent predictors. All predictors with a *P*-value below 0.10 in the univariate analysis were included in the multivariate analysis. All tests were two-sided.

The multiple linear regression method was used in the analysis of factors affecting IgG levels at different time points . Factors tested by univariate analysis were: patient and donor sex and age, sex mismatch, type of donor,

Table 2 Results from the multivariate analysis of factors associated with IgG levels at different time points after SCT in 179 patients

Factor	*RH*	*95% CI*	P-*value*
3 months			
Acute GVHD			
No	1		
Grades I–IV	0.79	0.66–0.94	<0.01
Age (years)			
⩽30	1		
>30	1.25	1.04–1.49	0.015
6 months			
Acute GVHD			
No	1		
Grades I–IV	0.67	0.58–0.70	<0.001
ATG			
No	1		
Yes	1.22	1.06–1.40	<0.01
Age (years)			
⩽30	1		
>30	1.23	1.07–1.42	<0.01
12 months			
Acute GVHD			
No	1		
Grades I–IV	0.79	0.68–0.93	<0.01
Sex match			
Other	1		
Fem D to Male R	0.82	0.70–0.97	0.017
GVHD prophylaxis			
CsA + MTX	1		
Other	1.20	1.02–1.40	0.026

Abbreviations: ATG = anti-thymocyte globuline; CI = confidence interval; CsA + MTX = cyclosporine and methotrexate; Fem D to Male R = female donor-to male-recipient; GVHD = graft-versus-host disease; RH = relative hazard.
(If RH < 1 indicates an association to low IgG levels and if RH > 1 indicates an association to high IgG levels).

GVHD prophylaxis, disease stage, nucleated cell-dose, type of conditioning (TBI/non-TBI, RIC/conventional), ATG in the conditioning, GVHD and type of graft (BM/PBSC). Chronic GVHD were analyzed in a time-dependent manner, where only patients who developed GVHD were assessed as having GVHD from the time point when they developed GVHD. All analyses were carried out using the cmprsk package (developed by Gray, June 2001) on Splus 2000 software and Statistica software.

Results

Reconstitution of IgG levels after SCT

IgG levels were not available for every patient at each time point. After SCT, IgG levels increased throughout the study period, especially from 6 to 12 months (Figure 1). In 37 patients, only 2 samples were available; 64 patients had 3 samples; 50 patients had 4 samples; and 28 patients had more than 4 samples.

Factors influencing IgG levels after SCT

Multivariate analysis was performed to further evaluate the factors associated with low antibody levels after transplantation. The following variables were included in the model: acute and chronic GVHD, patient age, donor age, HLA mismatch, treatment with ATG and conditioning therapy. Two factors were associated with low IgG levels three months after SCT: acute GVHD grades I–IV ($P<0.01$) and patient age $\leqslant 30$ years ($P=0.015$). Six months after SCT acute GVHD grades I–IV ($P<0.001$), patient age $\leqslant 30$ years ($P<0.01$) and no treatment with ATG ($P<0.01$) were associated with low IgG levels. One year after SCT, three variables were associated with low IgG levels: acute GVHD grades I–IV ($P<0.01$); female donor-to-male recipient ($P=0.017$); and GVHD prophylaxis with CsA + MTX ($P=0.026$) (Table 2, Figures 2a–e).

IgG levels and its relation to survival and TRM

Figure 3 shows IgG levels after SCT and its correlation to survival and TRM. Patients with low IgG levels (<4 g/l),

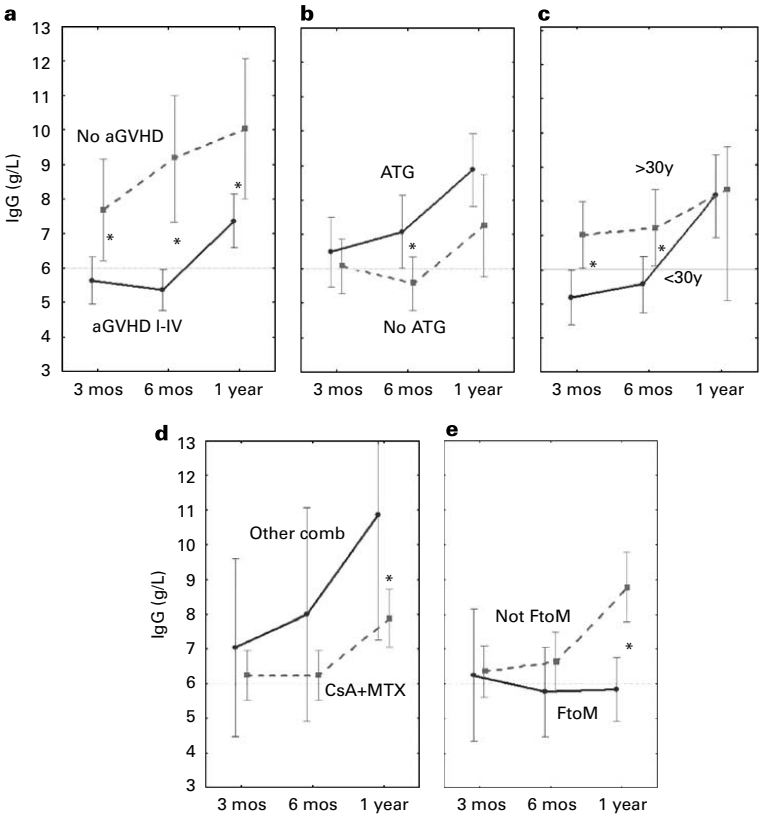

Figure 2 IgG levels at 3, 6 and 12 months after SCT in patients (**a**) with or without acute GVHD, (**b**) with or without ATG as part of conditioning therapy, (**c**) more or less than 30 years of age, (**d**) with different GVHD prophylaxis, and (**e**) with female donor-to-male recipient compared to other sex combinations. Mean ± 95% CI. Dotted line indicates lower reference level for the lab. *Indicates time points where the factor was significant in the multivariate analysis.

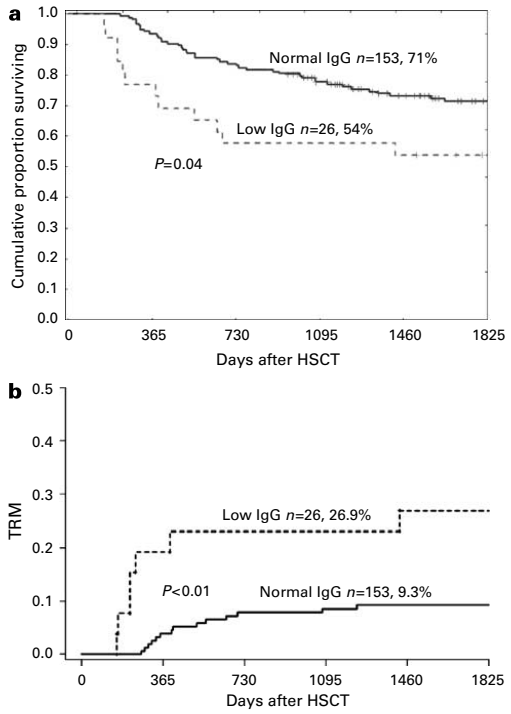

Figure 3 Different outcome variables in patients depending on IgG levels. 'Low IgG' indicates patients with at least two IgG levels <4 g/l at two occasions during the first year after SCT. 'Normal' indicates all other patients with at least two measured IgG levels during the first year after hemopoietic stem cell transplantation. (**a**) Overall survival and (**b**) transplant-related mortality (TRM).

measured twice during the first year after transplantation, exhibited a decreased cumulative proportion of survival and an increased risk of TRM compared to patients with moderately low or normal levels. Multivariate analysis showed that low IgG levels, TBI-based conditioning, acute GVHD grades II–IV and CMV infection were significantly correlated with higher TRM (Table 3). Factors associated with a lower overall survival were low IgG levels, higher patient age and acute GVHD grades II–IV (Table 3). Causes of death in the group with low IgG levels were relapse 5(19%), GVHD 1(4%), infection 4(15%) and others 2(8%). Causes of death in the group with normal IgG levels were relapse 26(17%), GVHD 5(3%), infection 7(5%) and others 7(5%). Patients with low IgG levels had a borderline ($P=0.056$) significant increased incidence of infections when they died.

Discussion

Following allogenic SCT, the patient undergoes a period of pronounced cellular and humoral immunodeficiency. Although immunological function gradually increases, some patients, particularly those who suffer from GVHD, do not exhibit reconstitution of the immune system. Most of the patients were immunologically competent within 2 years.[2,4,31–33]

In this study, IgG levels after SCT increased throughout the study period. Five factors had a negative effect on IgG levels after SCT: acute GVHD, patient age $\leqslant 30$ years at the time of transplantation, lack of treatment with ATG, a female donor-to-a male recipient and treatment with CsA and MTX as GVHD prophylaxis. We hypothesize that some of these factors increase GVHD and thereby decrease immunological reconstitution. On multivariate analysis, low IgG levels significantly correlated with higher TRM and inferior survival. These results suggest that a reasonably high level of IgG in peripheral blood after SCT is one important factor for a good outcome post-SCT.

Several serious problems—GVHD, relapse and infections—need to be considered after SCT. Patients who have undergone SCT are very susceptible to viral, bacterial and fungal infections. A way of overcoming the problem of severe infections is to give prophylactic treatment with high doses of IVIG. There are contradictory findings in the literature regarding the beneficial effects of IVIG treatment.[34] IVIG has been shown to have a positive effect on bacterial infections.[35] Some studies suggest that IVIG could have immunomodulatory effects *in vivo*, a treatment that decreases the risk of acute GVHD post-SCT.[32,33] Other studies, however, conclude that IVIG treatment after SCT does not significantly affect GVHD or survival.[7,36]

Table 3 Results from the multivariate analysis of factors associated with various outcome variables in 179 SCT patients

Transplant Related Mortality			
Factor	*RH*	*95% CI*	P-*value*
IgG-levels			
Normal	1		
Two low levels	3.32	1.25–8.85	0.017
Conditioning			
Non-TBI	1		
TBI	4.62	1.30–16.4	0.019
Acute GVHD			
Grades 0–I	1		
Grades II–IV	2.72	1.13–6.55	0.028
CMV infection			
No	1		
Yes	3.10	1.07–8.94	0.035
Death			
IgG-levels			
Normal	1		
Two low levels	2.47	1.30–4.76	0.006
Age			
Continuous	1.03	1.01–1.05	0.003
Acute GVHD			
Grades 0–I	1		
Grades II–IV	2.08	1.17–3.67	0.012

Abbreviations: CI = confidence interval; GVHD = graft-versus-host disease; RH = relative hazard; TBI = total-body irradiation.

A retrospective clinical evaluation of prophylactic IVIG treatment post-SCT showed that the overall survival was unaffected, but the incidence of death due to infections was lower in the IVIG-treated group.[36] Although fewer patients treated with IVIG compared to non-IVIG-treated patients died due to infections, more IVIG-treated patients died of veno-occlusive disease of the liver.[37] One study concludes that IVIG reduces proliferation of B-lymphocytes and immunoglobulin production by co-ligation of the B-cell antigen receptor and the Fc-γ RIIb receptor mediating the Fc receptor off signal.[38] In addition, one study notes that both IgG and IgM profiles become less heterogeneous in patients who have been treated with IVIG after SCT and that this effect persists months to years after the IVIG treatment has been completed.[33] In summary, we think that these earlier studies show both advantages and disadvantages of IVIG treatment.

We believe that our results, showing a strong correlation between persistent low levels of IgG and death after SCT, provide enough evidence to continue examining IVIG treatment after SCT. Do patients with low IgG levels post transplant benefit from IVIG treatment? In previous studies, IVIG was given regardless of IgG levels post-transplant. Our results raise one main question: is it possible that treatment with immunoglobulin during the first year post-SCT can increase survival? The major indication for IVIG treatment is primary immune deficiency, but there are also indications for IVIG treatment in various autoimmune disorders and infections in immunocompromised patients. Our present results suggest an evaluation of IVIG treatment post-SCT for patients with low IgG levels. We have found no evidence that a low IgG level in itself increases mortality. A low IgG level may be a surrogate marker for other adverse prognostic indicators. Nevertheless, our data show that a very strong predicting factor for death after SCT is a low IgG level (<4 g/l) measured twice during the first year after transplantation. To further evaluate the importance of IgG levels after SCT with respect to survival, researchers should conduct a prospective study in patients with low IgG levels post-SCT and examine the benefit of IVIG treatment in that specific patient group.

Acknowledgements

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References

1 Raaphorst FM. Reconstitution of the B cell repertoire after bone marrow transplantation does not recapitulate human fetal development. *Bone Marrow Transplant* 1999; **24**: 1267–1272.
2 Paulin T, Ringdén O, Nilsson B. Immunological recovery after bone marrow transplantation: role of age, graft-versus-host disease, prednisolone treatment and infections. *Bone Marrow Transplant* 1987; **1**: 317–328.
3 Storek J, Saxon A. Reconstitution of B cell immunity following bone marrow transplantation. *Bone Marrow Transplant* 1992; **9**: 395–408.
4 Atkinson K. Reconstruction of the haemopoietic and immune systems after marrow transplantation. *Bone Marrow Transplant* 1990; **5**: 209–226.
5 Ringdén O, Witherspoon R, Storb R, Ekelund E, Thomas ED. B cell function in human marrow transplant recipients assessed by direct and indirect hemolysis-in-gel assays. *J Immunol* 1979; **123**: 2729–2734.
6 Omazic B, Hentschke P, Nasman-Bjork I, Mattsson J, Oxelius V-A, Ringdén O *et al*. Reconstitution of the Ig heavy chain CDR3 repertoire after allogeneic haematopoietic stem cell transplantation with myeloablative or reduced-intensity conditioning regimens. *Scand J Immunol* 2005; **61**: 72–81.
7 Storek J, Wells D, Dawson MA, Storer B, Maloney DG. Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation. *Blood* 2001; **98**: 489–491.
8 Sheridan JF, Tutschka PJ, Sedmak DD, Copelan EA. Immunoglobulin G subclass deficiency and pneumococcal infection after allogeneic bone marrow transplantation. *Blood* 1990; **75**: 1583–1586.
9 Sullivan KM, Storek J, Kopecky KJ, Jocom J, Longton G, Flowers M *et al*. A controlled trial of long-term administration of intravenous immunoglobulin to prevent late infection and chronic graft-vs-host disease after marrow transplantation: clinical outcome and effect on subsequent immune recovery. *Biol Blood Marrow Transplant* 1996; **2**: 44–53.
10 Wimperis JZ, Brenner MK, Prentice HG, Reittie JE, Karayiannis P, Griffiths PD *et al*. Transfer of a functioning humoral immune system in transplantation of T-lymphocyte-depleted bone marrow. *Lancet* 1986; **1**: 339–343.
11 Storek J, Viganego F, Dawson MA, Herremans MMPT, Boeckh M, Flowers MED *et al*. Factors affecting antibody levels after allogeneic hematopoietic cell transplantation. *Blood* 2003; **101**: 3319–3324.
12 Ringden O, Remberger M, Runde V, Bornhauser M, Blau IW, Basara N *et al*. Peripheral blood stem cell transplantation from unrelated donors: a comparison with marrow transplantation. *Blood* 1999; **94**: 455–464.
13 Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 h: an alternative to serological DR typing in clinical practice including donor–recipient matching in cadaveric transplantation. *Tissue Antigens* 1992; **39**: 225–235.
14 Ringden O, Ruutu T, Remberger M, Nikoskelainen J, Volin L, Vindelöv L *et al*. A randomized trial comparing busulfan with total body irradiation as conditioning in allogeneic marrow transplant recipients with leukemia: a report from the Nordic Bone Marrow Transplantation Group. *Blood* 1994; **83**: 2723–2730.
15 Svenberg P, Remberger M, Svennilson J, Mattsson J, Leblanc K, Gustafsson B *et al*. Allogenic stem cell transplantation for nonmalignant disorders using matched unrelated donors. *Biol Blood Marrow Transplant* 2004; **10**: 877–882.
16 Remberger M, Svahn BM, Hentschke P, Lofgren C, Ringden O. Effect on cytokine release and graft-versus-host disease of different anti-T cell antibodies during conditioning for unrelated haematopoietic stem cell transplantation. *Bone Marrow Transplant* 1999; **24**: 823–830.
17 Slavin S, Nagler A, Naparstek E, Kapelushnik Y, Aker M, Cividalli G *et al*. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood* 1998; **91**: 756–763.

18 Hentschke P, Barkholt L, Uzunel M, Mattsson J, Wersall P, Pisa P *et al*. Low-intensity conditioning and hematopoietic stem cell transplantation in patients with renal and colon carcinoma. *Bone Marrow Transplant* 2003; **31**: 253–261.
19 Mattsson J, Uzunel M, Brune M, Hentschke P, Barkholt L, Stierna U *et al*. Mixed chimaerism is common at the time of acute graft-versus-host disease and disease response in patients receiving non-myeloablative conditioning and allogeneic stem cell transplantation. *Br J Haematol* 2001; **115**: 935–944.
20 Ringdén O, Pihlstedt P, Markling L, Aschan J, Båryd I, Ljungman P *et al*. Prevention of graft-versus-host disease with T cell depletion or cyclosporin and methotrexate. A randomized trial in adult leukemic marrow recipients. *Bone Marrow Transplant* 1991; **7**: 221–226.
21 Storb R, Deeg H, Whitehead J, Appelbaum F, Beatty P, Bensinger W *et al*. Pharm G. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *N Engl J Med* 1986; **314**: 729–735.
22 Einsele H, Ehninger G, Hebart H, Wittkowski KM, Schuler U, Jahn G *et al*. Polymerase chain reaction monitoring reduces the incidence of cytomegalovirus disease and the duration and side effects of antiviral therapy after bone marrow transplantation. *Blood* 1995; **86**: 2815–2820.
23 Yun Z, Lewensohn-Fuchs I, Ljungman P, Ringholm L, Jonsson J, Albert J. A real-time TaqMan PCR for routine quantitation of cytomegalovirus DNA in crude leukocyte lysates from stem cell transplant patients. *J Virol Methods* 2003; **110**: 73–79.
24 Ljungman P, Griffiths P, Paya C. Definitions of cytomegalovirus infection and disease in transplant recipients. *Clin Infect Dis* 2002; **34**: 1094–1097.
25 Ljungman P, Perez-Bercoff L, Jonsson J, Avetisyan G, Sparrelid E, Aschan J *et al*. Risk factors for the development of cytomegalovirus disease after allogeneic stem cell transplantation. *Haematologica* 2006; **91**: 78–83.
26 Remberger M, Naseh N, Aschan J, Barkholt L, LeBlanc K, Svennberg P *et al*. G-CSF given after haematopoietic stem cell transplantation using HLA-identical sibling donors is associated to a higher incidence of acute GVHD II-IV. *Bone Marrow Transplant* 2003; **32**: 217–223.
27 Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA *et al*. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation* 1974; **18**: 295–304.
28 Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE *et al*. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *Am J Med* 1980; **69**: 204–217.
29 Pettit SJ, Bourne H, Spickett GP. Survey of infection in patients receiving antibody replacement treatment for immune deficiency. *J Clin Pathol* 2002; **55**: 577–580.
30 Roifman CM, Levison H, Gelfand EW. High-dose versus low-dose intravenous immunoglobulin in hypogammaglobulinaemia and chronic lung disease. *Lancet* 1987; **1**: 1075–1077.
31 Lum LG. Immune recovery after bone marrow transplantation. *Hematol Oncol Clin North Am* 1990; **4**: 659–675.
32 Witherspoon RP, Storb R, Ochs HD, Fluornoy N, Kopecky KJ, Sull Deeg KM *et al*. Recovery of antibody production in human allogenic marrow graft recipients: influence of time posttransplantation, the presence or absence of chronic graft-versus-host disease, and antithymocyte globulin treatment. *Blood* 1981; **58**: 360–368.
33 Näsman Björk I, Fesel C, Brissac C, Lundkvist I. Prophylactic intravenous immunoglobulin treatment influences serum immunoglobulin M repertoire development after allogenic bone marrow transplantation. *Scand J Immunol* 1999; **50**: 73–82.
34 Saral R. The role of immunoglobulin in bone marrow transplantation. *Transplant Proc* 1991; **23**: 2128–2132.
35 Sullivan KM, Kopecky KJ, Jocom J, Fisher L, Buckner CD, Meyers JD *et al*. Immunomodulatory and antimicrobial efficacy of intravenous immunoglobulin in bone marrow transplantation? *N Engl J Med* 1990; **323**: 705–712.
36 Winston DJ, Ho WG, Lin CH, Bartoni K, Budinger MD, Gale RP *et al*. Intravenous immune globulin for prevention of cytomegalovirus infection and interstitial pneumonia after bone marrow transplantation. *Ann Intern Med* 1987; **106**: 12–18.
37 Klaesson S, Ringden O, Ljungman P, Aschan J, Hagglund H, Winiarski J. Does high-dose intravenous immune globulin treatment after bone marrow transplantation increase mortality in veno-occlusive disease of the liver? *Transplantation* 1995; **60**: 1225–1230.
38 Phillips NE, Parker DC. Cross-linking of B lymphocyte Fc gamma receptors and membrane immunoglobulin inhibits anti-immunoglobulin-induced blastogenesis. *J Immunol* 1984; **132**: 627–632.

II

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ORIGINAL ARTICLE

A comparison of Campath and Thymoglobulin as part of the conditioning before allogeneic hematopoietic stem cell transplantation

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Abstract

Background: In vivo T-cell depletion with anti-thymocyte globulin is a commonly used strategy for the prevention of graft-versus-host disease (GVHD) and to avoid rejection after hematopoietic stem cell transplantation (HSCT). *Methods:* We compared 36 patients given Campath (alemtuzumab) as part of the conditioning with a matched cohort of 72 patients receiving Thymoglobulin (TMG). Most patients had a hematologic malignancy beyond first remission. Median age was 55 (1–67). The majority of patients had an unrelated donor (70%) and 82% were given peripheral blood stem cells. Most patients received reduced-intensity conditioning. *Results:* Graft failure occurred in 8% of the patients in each group. No difference in time-to-engraftment of neutrophils and platelets was found. The cumulative incidence of acute GVHD of any grade was 34% and 53% ($P = 0.07$), and the incidence of chronic GVHD was 46% and 25% in the Campath and TMG groups, respectively. In multivariate analysis, a low antibody dose was associated with acute and chronic GVHD and Campath was correlated with chronic GVHD. No differences in transplant-related mortality (28% vs. 18%), overall survival (54% vs. 58%), and relapse-free survival (39% vs. 43%) were found between the two groups. No difference in the proportion of T and B lymphocytes during the first year after HSCT was found. *Conclusions:* TMG and Campath as part of the conditioning result in similar outcome. Campath was associated with less acute but more chronic GVHD.

Key words ATG; Campath; HSCT

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Hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for patients with hematologic malignancies, bone marrow (BM) failure syndrome, and some inherited metabolic disorders. However, graft-versus-host disease (GVHD) is still a major cause of morbidity and mortality (1, 2). The risk of rejection is higher in unrelated donor (URD) HSCT and when a less toxic, reduced-intensity conditioning (RIC) is used. To reduce the risk of GVHD and rejection after HSCT, many centers add anti-thymocyte globulin (ATG) to the conditioning protocol when using URDs (3–7). Some RIC protocols also include ATG independently of donor (8). By adding ATG to the pretransplant conditioning protocol, acute GVHD and early mortality of URD HSCT have been reduced to levels similar to those of matched related HSCT (5, 9). The mechanism of action is depletion of alloreactive T cells in both the recipient and the graft, so-called *in vivo* T-cell depletion. Many different doses and types of ATG are used today.

In this retrospective study, we compared patients who were given Campath as part of the conditioning protocol with a matched cohort of patients given Thymoglobulin (TMG).

Patients and methods

Patients

This study included 36 consecutive patients receiving Campath (Genzyme, Cambridge, MA, USA) as part of

conditioning before HSCT between November 2002 and October 2009 at Karolinska University Hospital. Median age was 52 yr (range 2–67). Most patients had a hematologic malignancy, while three had a metabolic disorder. Twenty-six patients (72%) were beyond CR1 (complete remission)/CP1 (chronic phase) and were considered to be at high risk.

As controls, we selected 72 patients receiving TMG (Genzyme) instead of Campath. The controls were selected out of 289 patients given TMG during the same time period and matched according to age, diagnosis, stage of disease, donor, conditioning, and GVHD prophylaxis. Patient and donor demographics are listed in Table 1.

The study was approved by the local ethical committee and was performed in accordance with the declaration of Helsinki. Informed consent was obtained from all patients included in the study.

Table 1 Patient and donor characteristics for patients treated with two different anti-T-cell antibodies after hematopoietic stem cell transplantation

	Campath	Thymoglobulin
N	36	72
Diagnosis		
Acute leukemia	6	19
CML/CLL	1/6	10/4
MDS/MPS	16	24
Other hematologic malignancy	4	10
Metabolic disorder	3	5
High-risk disease	26 (72%)	52 (72%)
Sex (M/F)	14/22	40/32
Age	52 (2–67)	55 (1–67)
Donor		
Sibling	13	19
MUD	20	47
Allele mmURD	3	6
Donor sex (M/F)	20/16	45/27
Donor age	35 (21–65)	34 (21–71)
Stem cell source (BM/PBSC)	4/32	15/57
NC dose	12.2 (2.5–31.0)	10.2 (1.0–54.5)
CD34 dose	9.4 (0.6–21.9)	7.1 (0.8–26.0)**
Conditioning		
Busulfan + Cy	5	12
RIC	31	60
GVHD prophylaxis		
CsA + MTX	35	60
Tacrolimus + rapamycin	1	12
G-CSF	4 (11%)	12 (17%)
Acute GVHD		
No	24 (67%)	34 (47%)
I	3 (8%)	18 (25%)
II	6 (17%)	16 (22%)
III–IV	3 (8%)	4 (6%)
Chronic GVHD	14/33	17/66§
Follow-up (yr)	3.3 (0.7–7.7)	4.0 (0.7–9.8)

§$P = 0.09$, **$P = 0.01$.

URD, unrelated donor; CML, chronic myeloid leukemia; CLL, chronic lymphoid leukemia; MDS, myelodysplastic syndrome; MPS, myeloproliferative syndrome; High-risk disease, beyond CR1/CP1 or in MDS not RA; MUD, matched URD; mmURD, mismatched; BM, bone marrow; PBSC, peripheral blood stem cells; NC, nucleated cell; Cy, cyclophosphamide; RIC, reduced-intensity conditioning; CsA, cyclosporine; MTX, methotrexate; GVHD, graft-versus-host disease; G-CSF, granulocyte colony-stimulating factor.

Donors

Among the Campath-treated patients, 13 patients had an HLA-identical sibling donor while 20 had an HLA-A, HLA-B, and HLA-DR matched URD (MUD) and three had an allele-mismatched URD (mmURD) (10). There were 14 male donors and 22 female donors with a median age of 35 yr (range 21–71).

In the control group, 19 patients had an HLA-identical sibling donor while 47 had an MUD and 6 had an mmURD (10). There were 45 male donors and 27 female donors with a median age of 34 yr (21–71).

HLA typing

All patients and donors were typed using PCR-sequence-specific primers high-resolution typing for both HLA class I and II antigens (11, 12).

Conditioning

In the Campath group, most patients received reduced-intensity conditioning (RIC) with fludarabine (Flu) (30 mg/m^2 for 3–5 d) in combination with oral busulfan (Bu, 4 mg/kg/d for 2 d) (n = 19) (8), 2x3 Gy fractionated total-body irradiation (fTBI) and cyclophosphamide (Cy, 30 mg/kg/d for 2 d) (n = 7) (13), Cy alone at 30 mg/kg/d for 2 d (n = 4) (14), or Treosulfan at 14 g/m^2/d for 3 d (n = 1) (15). Five patients received full-dose conditioning with busulfan (4 mg/kg/d for 4 d) and cyclophosphamide (60 mg/kg/d for 2 d) (16). Campath was given at a dose of 30 mg/d for 1 (n = 25), 2 (n = 3), or 3 (n = 8) days (17).

In the control group, 41 patients received RIC consisting of Flu/Bu, 12 received Flu/fTBI/Cy, two received Flu/Cy and five patients received Flu/Treosulfan. Conventional conditioning with Bu/Cy was given to 12 patients. TMG was given at a dose of 2 mg/kg/d for 3 (n = 31) or 4 (n = 41) days. Both antibodies were given with the last dose on the day before infusion of the cells (6).

GVHD prophylaxis

Most patients received GVHD prophylaxis with cyclosporine (CsA) and four doses of methotrexate (MTX) (18, 19). During the first month, blood CsA levels were kept at 100–150 ng/mL in patients with sibling donors

and at 200–300 ng/mL in those with URDs. In the absence of GVHD, CsA was discontinued after 3 months using HLA-identical sibling donors and after 6 months using URDs (5, 20).

Supportive care

As prophylactic gut decontamination, the patients received ciprofloxacin 500 mg twice daily in combination with oral amphotericin-B. Granulocyte colony-stimulating factor (G-CSF) was given to all patients after HSCT until neutrophil engraftment ($>0.5 \times 10^9$/L) until August 2001 (21). Thereafter, prophylactic G-CSF was not given because we found that there was an increased risk of acute GVHD of grades II–IV with this drug (22).

Stem cell source

Stem cells from peripheral blood (PBSCs) were given to 32 and 57 patients in the Campath and control group, respectively, while four and 15 patients received BM (23). Before aphaeresis, all donors of PBSCs were treated subcutaneously once a day with G-CSF (Rhône-Poulenc Rorer, Lyon, France or Amgen-Roche Inc., Thousand Oaks, CA, USA), 10 μg/kg/d for 4–6 d.

Diagnosis and treatment of GVHD

Acute and chronic GVHD was diagnosed on the basis of clinical symptoms and/or biopsies (skin, liver, gastrointestinal tract, or oral mucosa) according to standard criteria (24). The patients were treated for grade-I acute GVHD with prednisolone, starting at 2 mg/kg/d, which was tapered after the initial response (25). In more severe cases, ATG, methylprednisolone, MTX, psoralene, and UV light (PUVA) or mesenchymal stem cells were used (26, 27). Chronic GVHD was initially treated with CsA and steroids.

Cell-surface markers and immunoglobulin

Relative numbers of T ($CD3^+$) and B lymphocytes ($CD19^+$) and NK cells ($CD56^+$) were determined by flow cytometry (FACS) at 3, 6, and 12 months after HSCT. Serum samples for measurement of IgG levels were collected 3, 6, and 12 months following HSCT. Serum IgG was analyzed routinely by nephelometry at the Department of Clinical Chemistry, Karolinska University Laboratory. Reference levels were 6–15 g/L.

Definitions

Engraftment was established by chimerism analysis using PCR amplification of variable numbers of tandem repeats (until 2003) and short tandem repeats (28).

Leukemia relapse was defined as >20% blasts in BM. Cytogenetic relapse was reappearance of cells with the Philadelphia chromosome seen by conventional karyotyping analysis.

Bacteraemia was defined as the first positive blood culture related to a febrile episode during the first 30 d after transplantation (29).

Cytomegalovirus (CMV) reactivation was determined weekly by PCR of leukocyte DNA. CMV disease was defined according to previous definitions (30, 31). Herpes simplex virus (HSV) and varicella zoster virus (VZV) infections were defined as a positive PCR finding from the affected area. Invasive fungal infection was defined as positive blood culture and/or positive cultures from at least two organs for *Candida* or *Aspergillus* species. *Aspergillus* pneumonia was defined as pulmonary infiltrates and positive cultures of bronchoalveolar lavage fluid, sputum, or autopsy samples.

Engraftment was defined as stable absolute neutrophil counts of $>0.5 \times 10^9$/L for three consecutive days, and platelet engraftment was defined as platelet counts of $>30 \times 10^9$/L for seven consecutive days without transfusions.

Statistics

The analysis was performed in July 2010. The probabilities of overall survival and relapse-free survival (RFS) were estimated using the Kaplan–Meyer method and compared with the log-rank test (32). The incidence of GVHD, transplant-related mortality (TRM), and relapse was estimated non-parametrically by cumulative incidence curves (33, 34). Patients were censored at the time of death, relapse, or last follow-up. Predictive analyses for GVHD, TRM, and relapse were based on the proportional hazards model for subdistribution of competing risks. Univariate and multivariate analyses were then performed using Gray's test, and the proportional subdistribution hazard regression model was developed by Fine and Gray (34). A stepwise backward procedure was used to construct a set of independent predictors for each end-point. All predictors with a *P*-value below 0.10 were considered, and sequentially removed if the *P*-value in the multiple model was above 0.05. All tests were two-sided. The type-I error rate was fixed at 0.05 for factors potentially associated with time-to-event outcomes. Factors analyzed in the univariate analysis included patient and donor sex and age, sex-mismatch, disease stage, conditioning (TBI-based), G-CSF, CMV serology, nucleated and $CD34^+$ cell dose, type of antibody, antibody dose and GVHD. A Campath total dose of 30 mg and a TMG total dose of 6 mg/kg were considered as a low dose. Analyses were performed using the cmprsk software package (developed by Gray, June 2001), SPLUS 6.2

software (Insightful Corp., Seattle, WA, USA), and STATISTICA software (StatSoft, Tulsa, OK, USA). The Mann–Whitney *U* test was used to compare continuous variables, and Fisher's exact test was used to compare the distribution of categorical variables.

Results

Transfusions

No difference in erythrocyte and platelet transfusions between the two groups was seen. The median number of erythrocyte and platelet transfusions in patients with Campath and TMG was 4 (range 0–18), 2 (0–22), 2 (0–48), and 1 (0–28), respectively. Of the Campath-treated patients, six were given granulocyte transfusion when compared to three in the TMG group ($P = 0.024$).

Engraftment

All patients had engraftment initially, but three and six patients in the Campath and TMG group suffered from graft failure (GF) at a median of 137 d (range 40–211) after transplantation. GF tended to occur earlier in Campath-treated patients (days 40, 41 and 137) than in TMG patients (days 49, 125, 147, 182, 207 and 211) ($P = 0.09$). Time to neutrophil and platelet engraftment was similar in the two groups (Table 2).

Infections

The incidence of bacteraemia was 25% and 21% (ns), and the incidence of CMV reactivation was 61% in both groups. CMV reactivation occurred at a median of 37 d (24–242) and 34 d (9–410) after HSCT in patients treated with Campath and TMG, respectively. CMV disease occurred in three and four patients, respectively, in the two groups. No differences in HSV and VZV reactivations were seen between the two groups. However, there was a non-significant higher incidence of invasive fungal infections in the Campath-treated patients (16.7% vs. 4.2%, $P = 0.057$). Post-transplant lymphoproliferative disease (PTLD) occurred in one and three patient in the two groups, respectively.

Table 2 Time to engraftment, transfusions and graft failure (GF) in patients treated with two different anti-T-cell antibodies after hematopoietic stem cell transplantation

	Campath	Thymoglobulin	*P*-value
Time to ANC $>0.5 \times 10^9$/L	19 (10–34)	18 (11–50)	NS
Time to platelets $>30 \times 10^9$/L	14 (0–46)	13 (0–160)	NS
Time to platelets $>50 \times 10^9$/L	15 (9–76)	15 (0–170)	NS
No. of RBC transfusions	3.5 (0–18)	2 (0–48)	NS
No. of Platelet transfusions	1.5 (0–22)	1 (0–28)	NS
Granulocyte transfusions	6 patients	3 patients	0.024
GF	3	6	NS
Time to GF (d)	41 (40–137)	164 (49–211)	0.09
PTLD	1	3	NS

ANC, absolute neutrophil count; RBC, red blood cells; PTLD, post-transplant lymphoproliferative disease; NS, not significant.

Graft-versus-host disease

In the Campath-treated patients, the cumulative incidence of any grade of acute GVHD was 34% when compared to 53% in patients given TMG ($P = 0.07$) (Fig. 1A). The cumulative incidence of acute GVHD of grades II–IV was 25% and 28% (ns) and the cumulative incidence of chronic GVHD was 46% and 25% ($P = 0.09$) (Fig. 1B) in patients who received Campath and TMG, respectively. We found dose effect of ATG and Campath on the incidence of acute GVHD ($P = 0.057$) (Fig. 1C).

In the multivariate analysis, Campath (RH 1.31, 95% CI 1.03–1.67, $P = 0.02$) and low antibody dose (2.14, 1.05–4.35, $P = 0.03$) were associated with chronic GVHD.

Transplant-related mortality

There was no significant difference in TRM between the two groups: 8% and 7% at 100 d, and 28% and 18% at 1 yr in patients given Campath and TMG, respectively (Fig. 2A).

In multivariate analysis, higher patient age (in decades) (1.70, 1.06–2.72, $P = 0.028$) and TBI-based conditioning (2.59, 1.12–5.99, $P = 0.029$) were associated with TRM.

Relapse

Thirty patients (28%) had a leukemia relapse at a median of 285 d (60–2227) after the transplant. The 5-yr cumulative incidence of relapse was 26% in the Campath-treated patients and 34% in patients given TMG (ns) (Fig. 2B). If patients with chronic lymphoid leukemia were removed from the analysis, the incidences of relapse were 21% and 33%, respectively ($P = 0.24$).

In multivariate analysis, higher patient age (1.88, 1.22–2.89, $P = 0.004$) and G-CSF given after HSCT (2.59, 1.14–5.87, $P = 0.023$) were associated with relapse.

Donor lymphocyte infusions

Donor lymphocyte infusions (DLI) were given to 50 patients: 22 (41%) in the Campath group and 28 (39%) in the TMG group. Reasons for DLI treatment were relapse ($n = 4$), GF ($n = 1$) and increasing recipient

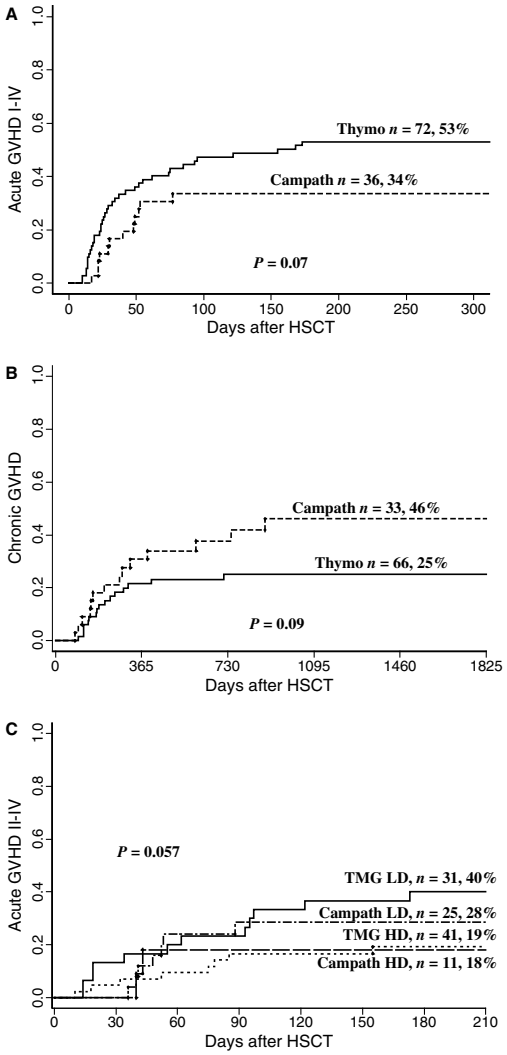

Figure 1 Cumulative incidence of acute GVHD grades I–IV (A), chronic GVHD (B) and (C) acute GVHD grades II–IV after allogeneic hematopoietic stem cell transplantation in patients treated with TMG or Campath. TMG LD, Thymoglobulin low dose, 6 mg/kg; TMG HD, Thymoglobulin high dose, 8 mg/kg; Camp LD, Campath low dose, 30 mg; Camp HD, Campath high dose, 60–90 mg; GVHD, graft-versus-host disease.

chimerism ($n = 17$) in Campath-treated patients, and GF ($n = 2$), relapse ($n = 6$), and chimerism ($n = 20$) in TMG patients. All patients received escalating doses of DLI with a median of two doses (range 1–5) given in each group. The escalations in doses were performed in steps of 0.5–1 log starting between 1×10^5 to 1×10^6 $CD3^+$ cells per kilo, depending on the type of donor, degree of HLA match, and history of GVHD in the recipient. DLI was given at a median of 138 d (35–504) and 132 d (35–1922) after HSCT in the two groups, respectively. DLI-induced GVHD occurred in 11 (50%) Campath patients and 13 (46%) TMG patients and was severe (grades III–IV) in three patients in each group.

Survival

The 5-yr overall survival was 53% and 58% in patients who received Campath and TMG, respectively (ns) (Fig. 2C). RFS at 5 yr was 39% and 43%, respectively (ns) (Fig. 2D).

Causes of death in patients who received Campath and TMG were relapse in 6 and 13 cases, respectively, infections in 6 and 11 cases, GVHD in four and one case, and other causes in one and three cases.

In multivariate analysis, higher patient age (2.09, 1.48–2.97, $P < 0.001$) and acute GVHD II–IV (1.81, 0.97–3.39, $P = 0.06$) were associated with inferior survival. Higher patient age (2.05, 1.48–2.86, $P < 0.001$) was the only factor affecting RFS in the multivariate analysis.

Cell-surface molecules and IgG

No differences in levels of $CD3^+$ T lymphocytes and $CD19^+$ B lymphocytes were seen (Fig. 3A–B), although levels of $CD3^+$ lymphocytes tended to be higher in TMG-treated patients compared to in Campath-treated patients. No significant difference in NK-cell ($CD56^+$) levels was seen between the two groups. IgG levels were determined in 83 patients. No significant difference between the two groups was seen. In 13 patients, low levels of IgG (<4 mg/L) were seen. Overall survival for these 13 patients was significantly inferior to that for patients with normal IgG levels (35% vs. 72%, $P < 0.01$).

Chimerism

Chimerism data for the $CD3^+$ cell line (T cells) were available in 106 of the 108 patients. Of the TMG-treated patients, 43 (60%) reached full donor chimerism (DC) within 90 d, 48 (67%) reached full DC within 6 months, and 52 (72%) within 1 yr. For the Campath-treated group, the corresponding figures were 15 (44%) at 90 d, 18 (53%) at 6 months, and 19 (56%) at 1 yr, $P = 0.12$. Median time to full DC in the $CD3^+$ cell line was 49 (14–530) days ($n = 54$) and 49 (15–925) days ($n = 21$) in the TMG and Campath groups, respectively (ns). Median time to full DC in $CD33^+$ cells was 28 (14–504)

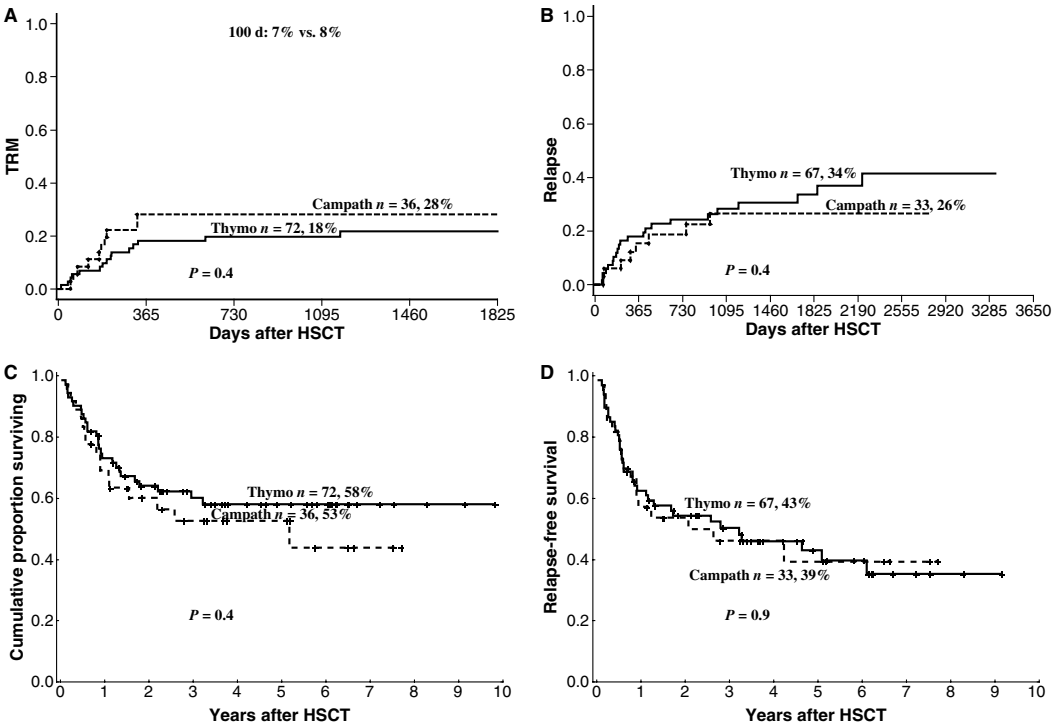

Figure 2 Cumulative incidence of transplant-related mortality (A), relapse (B), probability of survival (C), relapse-free survival (D) in patients receiving Thymoglobulin or Campath during conditioning before allogeneic hematopoietic stem cell transplantation.

days (n = 62) and 28 (14–400) days (n = 24) in patients who received TMG and Campath (ns). Median time to full DC in $CD19^+$ cells was 35 (14–504) days (n = 64) and 48 (17–365) days (n = 24) in patients who received TMG and Campath (P = 0.14).

Discussion

In this study, we evaluated the effect of two different drugs, Campath and TMG, which have both been developed to achieve a so-called *in vivo* T-cell depletion. We compared 36 patients given Campath as part of conditioning with a matched cohort of 72 patients who received TMG. *In vivo* T-cell depletion with ATG is a commonly used strategy for the prevention of GVHD and to avoid rejection after HSCT. Many protocols have been used, and comparisons between different drugs have been performed in some studies (6, 35–39). Campath is a humanized monoclonal antibody to human CD52, an antigen that is expressed on B, T, and natural killer (NK-) cells, but not on other hematopoietic cells (40). In contrast, TMG consists of rabbit immunoglobulins and is produced by immunizing rabbits with fresh human thymocytes. The immunoglobulin fraction contains polyclonal antibodies to multiple cell-surface antigens (41, 42). TMG binds to T cells and has a direct effect on T cells, resulting in T-cell depletion via opsonization and lysis following complement activation. TMG also binds to B cells, monocytes, macrophages, and neutrophils, but to a lesser extent (41, 42).

In the present study, we were able to show that Campath was associated with less overall acute GVHD (43) but more chronic GVHD. Whether this was attributed to a greater effect of Campath than of ATG, in terms of early depletion of alloreactive T cells of graft origin, remains unclear. Our result is supported by data from the studies of Hale *et al.* (44) in which GVHD was well controlled in the majority of patients treated with Campath. More chronic GVHD in Campath-treated patients were not translated into a lower relapse rate in this study (45). Probably the size of the study is the main reason for this finding. It may be a correlation between the cell dose of the graft and chronic GVHD, but this was not found in this study. However, Campath-treated

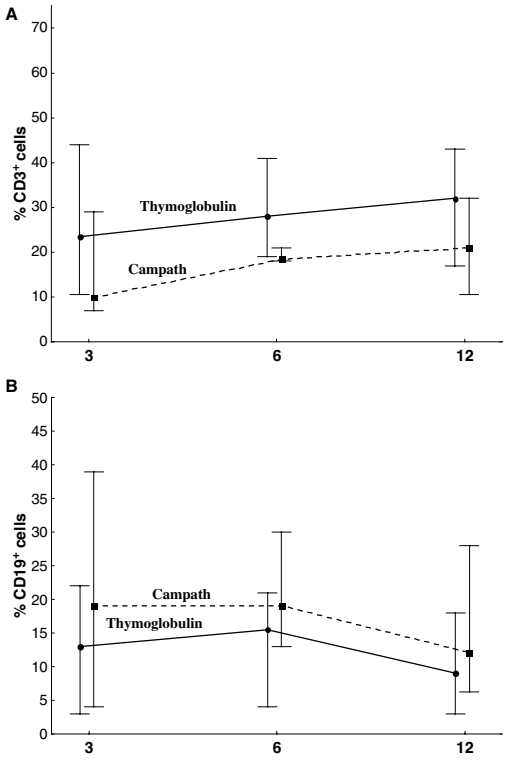

Figure 3 Levels of $CD3^+$ T cells (A) and $CD19^+$ B cells (B) in bone marrow at different time points after allogeneic hematopoietic stem cell transplantation in patients treated with Thymoglobulin or Campath.

patients received a higher CD34 cell dose and had more chronic GVHD.

Regarding bacterial and viral infections, we did not see any difference between the Campath and the TMG groups. There was a trend ($P = 0.057$) of more fungal infections in the patients treated with Campath. In an as yet unpublished study, we have shown that treatment with Campath increases the risk of invasive infection with *Aspergillus* after RIC HSCT (46). Furthermore, six (17%) Campath-treated patients compared to three (4%) TMG-treated patients were given granulocyte transfusions as treatment for infections that did not respond to conventional treatment. This may indicate that patients treated with Campath are more susceptible to severe infections. In our retrospective study, no differences in T- and B-lymphocyte levels were found during the first year after HSCT. Previous pharmacokinetic analysis, by Morris *et al.* (47), of patients treated with Campath revealed that the antibody is detectable in lympholytic serum concentrations for up to 56 d after transplantation. TMG may be detected in serum up to 5 wk after HSCT (48). Another study has shown that patients who receive Campath have a poor immune reconstitution, particularly with very slow recovery of the CD4 T-cell subset (49). This leads to a strong and long-lasting impairment of antiviral immunity, which is associated with significant morbidity and mortality (50, 51). Taking the results of these studies together, one could speculate that because Campath can be detected in blood for a longer time after administration compared to TMG, this drug may have a longer immunosuppressive effect.

No significant difference in IgG levels was seen between the two groups during the first year after transplantation. An important observation made earlier by our group (6) is that a low dose of TMG (4 mg/kg in total) increased the risk of severe acute GVHD, whereas 10 mg/kg increased the risk of death from infection. Medium doses of TMG (6–8 mg/kg) resulted in the lowest TRM and best survival. Considering this observation, an additional factor that could probably interfere with both GVHD and infections is the dose of Campath and TMG. In this study, we found a dose effect of both preparations on acute and chronic GVHD.

The increased incidence of PTLDs is a concern when using anti-T-cell antibodies. In this study, we could see no difference regarding the incidence of PTLDs. This is a surprisingly low incidence, since earlier studies by Lynch *et al.* (52) have shown a statistically significant increase in the incidence of PTLD in patients treated with anti-T-cell antibodies. While depletion of B cells by *ex vivo* treatment of the marrow with anti-B-cell antibodies may reduce the risk of PTLD, a protective effect of *in vivo* B-cell-depleting therapies following bone-marrow transplantation has not been well established (53–55).

One must remember that this is a small study, and the conclusions that we can draw are therefore somewhat limited. Even so, we believe that both TMG and Camath are drugs that must be taken into consideration to reduce the risk of GVHD and rejection after HSCT with alternative donors. Prospective randomized studies are needed to evaluate the positions of the drugs. A number of recent studies (1, 2) suggest that there may be a limited use of *in vivo* purging agents, in alternative donors with RIC. Therefore, we believe that this is a central issue to address in studies that are in the planning stage. We also believe that in further studies a larger number of patients have to be included. We suggest that studies in the future should focus partly on GVHD, because any attempt to reduce GVHD may be offset by a higher risk of relapse, and the results should be interpreted with caution.

Acknowledgements

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References

1. Hansen JA, Gooley T, Martin P, *et al*. Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia. *N Engl J Med* 1998;**338**:962–8.
2. Sierra J, Storer B, Hansen JA, *et al*. Unrelated donor marrow transplantation for acute myeloid leukemia: an update of the Seattle experience [In Process Citation]. *Bone Marrow Transplant* 2000;**26**:397–404.
3. Ringdén O, Remberger M, Carlens S, *et al*. Low incidence of acute graft-versus-host disease, using unrelated HLA-A, -B and -DR compatible donors and conditioning, including anti-T-cell antibodies. *Transplantation* 1998;**66**:620–5.
4. Byrne JL, Stainer C, Cull G, *et al*. The effect of the serotherapy regimen used and the marrow cell dose received on rejection, graft-versus-host disease and outcome following unrelated donor bone marrow transplantation for leukaemia. *Bone Marrow Transplant* 2000;**25**:411–7.
5. Ringdén O, Remberger M, Persson U, *et al*. Similar incidence of graft-versus-host disease using HLA-A, -B and -DR identical unrelated bone marrow donors as with HLA-identical siblings. *Bone Marrow Transplant* 1995;**15**:619–25.
6. Remberger M, Svahn BM, Mattsson J, Ringden O. Dose study of thymoglobulin during conditioning for unrelated donor allogeneic stem-cell transplantation. *Transplantation* 2004;**78**:122–7.
7. Zander A, Kröger N, Schleuning M, *et al*. ATG as part of the conditioning regimen reduces transplant-related mortality (TRM) and improves overall survival after unrelated stem cell transplantation in patients with chronic myelogenous leukemia (CML). *Bone Marrow Transplant* 2003;**32**:355–61.
8. Slavin S, Nagler A, Naparstek E, *et al*. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood* 1998;**91**:756–63.
9. Kiehl MG, Kraut L, Schwerdtfeger R, *et al*. Outcome of allogeneic hematopoietic stem-cell transplantation in adult patients with acute lymphoblastic leukemia: no difference in related compared with unrelated transplant in first complete remission. *J Clin Oncol* 2004;**22**:2816–25.
10. Hauzenberger D, Schaffer M, Ringden O, *et al*. Outcome of haematopoietic stem cell transplantation in patients transplanted with matched unrelated donors vs allele-mismatched donors: a single centre study. *Tissue Antigens* 2008;**72**:549–58.
11. Olerup O, Zetterqvist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992;**39**: 225–35.
12. Schaffer M, Aldener-Cannava A, Remberger M, Ringden O, Olerup O. Roles of HLA-B, HLA-C and HLA-DPA1 incompatibilities in the outcome of unrelated stem-cell transplantation. *Tissue Antigens* 2003;**62**:243–50.
13. Uzunel M, Remberger M, Sairafi D, *et al*. Unrelated versus related allogeneic stem cell transplantation after reduced intensity conditioning. *Transplantation* 2006;**82**:913–9.
14. Khouri IF, Keating M, Korbling M, *et al*. Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J Clin Oncol* 1998;**16**:2817–24.
15. Beelen DW, Trenschel R, Casper J, *et al*. Dose-escalated treosulphan in combination with cyclophosphamide as a new preparative regimen for allogeneic haematopoietic stem cell transplantation in patients with an increased risk for regimen-related complications. *Bone Marrow Transplant* 2005;**35**:233–41.
16. Ringdén O, Ruutu T, Remberger M, *et al*. A randomized trial comparing busulphan with total body irradiation as conditioning in allogenic marrow transplant recipient with leukemia. *Blood* 1994;**83**:2723–30.
17. Hale G, Waldmann H. Control of graft-versus-host disease and graft rejection by T cell depletion of donor and recipient with Campath-1 antibodies. Results of matched sibling transplants for malignant diseases [see comments]. *Bone Marrow Transplant* 1994;**13**:597–611.
18. Ringdén O, Horowitz M, Sondel P. Methotrexate, cyclosporine or both to prevent graft-versus-host disease after HLA-identical sibling bone marrow transplants for early leukemia. *Blood* 1993;**18**:1094–101.
19. Storb R, Deeg H, Pepe M, *et al*. Methotrexate and cyclosporine versus cyclosporine alone for prophylaxis of graft-versus-host disease in patients given HLA-identical marrow grafts for leukemia: long-term follow-up of a controled trial. *Blood* 1989;**73**:1729–34.
20. Carlens S, Aschan J, Remberger M, Dilber M, Ringdén O. Low dose cyclosporine of short duration increases the risk of mild and moderate GVHD and reduces the risk of relapse in HLA-identical sibling marrow transplant recipients with leukemia. *Bone Marrow Transplant* 1999;**24**:629–35.
21. Hägglund H, Ringdén O, Öhman S, Remberger M, Carlens S, Mattsson J. A prospective randomized trial of Filgrastim (r-metHuG-CSF) given at different times after unrelated bone marrow transplantation. *Bone Marrow Transplant* 1998;**24**:831–6.

22. Remberger M, Naseh N, Aschan J, *et al*. G-CSF given after haematopoietic stem cell transplantation using HLA-identical sibling donors is associated to a higher incidence of acute GVHD II–IV. *Bone Marrow Transplant* 2003;**32**:217–23.
23. Remberger M, Ringdén O, Blau I, *et al*. No difference in graft-versus-host disease, relapse and survival comparing peripheral stem cells with bone marrow using unrelated donors. *Blood* 2001;**98**:1739–45.
24. Przepiorka D, Weisdorf D, Martin P, *et al*. 1994 consensus conference on acute GVHD grading. (11Review) (11 refs). *Bone Marrow Transplant* 1995;**15**:825–8.
25. Ringdén O. Management of graft-versus-host disease. *Eur J Haematol* 1993;**51**:1–12.
26. Ringdén O, Båryd I, Gahrton G, *et al*. Early treatment and prophylaxis of graft-versus-host disease using prednisolone. *Excerpta Med Bone Marrow Transplant Eur* 1981;**2**:171–7.
27. Ringden O, Uzunel M, Rasmusson I, *et al*. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* 2006;**81**:1390–7.
28. Mattsson J, Uzunel M, Tammik L, Aschan J, Ringdén O. Leukemia lineage-specific chimerism analysis is a sensitive predictor of relapse in patients with acute myeloid leukemia and myelodysplastic syndrome after allogeneic stem cell transplantation. *Leukemia* 2001;**15**:1976–85.
29. Sparrelid E, Hagglund H, Remberger M, *et al*. Bacteraemia during the aplastic phase after allogeneic bone marrow transplantation is associated with early death from invasive fungal infection. *Bone Marrow Transplant* 1998;**22**:795–800.
30. Ljungman P, Griffiths P, Paya C. Definitions of cytomegalovirus infection and disease in transplant recipients. *Clin Infect Dis* 2002;**34**:1094–7.
31. Ljungman P, Lore K, Aschan J, *et al*. Use of a semi-quantitative PCR for cytomegalovirus DNA as a basis for pre-emptive antiviral therapy in allogeneic bone marrow transplant patients. *Bone Marrow Transplant* 1996;**17**:583–7.
32. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958;**53**:457–81.
33. Kalbfleisch J, Prentice R. *The Statistical Analysis of Failure Time Data*. New York, NY: Wiley, 1980.
34. Fine J, Gray R. Proportional hazard model for the subdistribution of competing risks. *J Am Stat Assoc* 1999;**94**:496–509.
35. Perez-Simon JA, Kottaridis PD, Martino R, *et al*. Nonmyeloablative transplantation with or without alemtuzumab: comparison between 2 prospective studies in patients with lymphoproliferative disorders. *Blood* 2002;**100**:3121–7.
36. Chakrabarti S, MacDonald D, Hale G, *et al*. T-cell depletion with Campath-1H "in the bag" for matched related allogeneic peripheral blood stem cell transplantation is associated with reduced graft-versus-host disease, rapid immune constitution and improved survival. *Br J Haematol* 2003;**121**:109–18.
37. Chakrabarti S, Milligan DW, Brown J, *et al*. Influence of cytomegalovirus (CMV) sero-positivity on CMV infection, lymphocyte recovery and non-CMV infections following T-cell-depleted allogeneic stem cell transplantation: a comparison between two T-cell depletion regimens. *Bone Marrow Transplant* 2004;**33**:197–204.
38. Kroger N, Shaw B, Iacobelli S, *et al*. Comparison between antithymocyte globulin and alemtuzumab and the possible impact of KIR-ligand mismatch after dose-reduced conditioning and unrelated stem cell transplantation in patients with multiple myeloma. *Br J Haematol* 2005;**129**:631–43.
39. Juliusson G, Theorin N, Karlsson K, Frodin U, Malm C. Subcutaneous alemtuzumab vs ATG in adjusted conditioning for allogeneic transplantation: influence of Campath dose on lymphoid recovery, mixed chimerism and survival. *Bone Marrow Transplant* 2006;**37**:503–10.
40. Hale G, Cobbold S, Novitzky N, *et al*. CAMPATH-1 antibodies in stem-cell transplantation. *Cytotherapy* 2001;**3**:145–64.
41. Bonnefoy-Berard N, Vincent C, Revillard JP. Antibodies against functional leukocyte surface molecules in polyclonal antilymphocyte and antithymocyte globulins. *Transplantation* 1991;**51**:669–73.
42. Rebellato LM, Gross U, Verbanac KM, Thomas JM. A comprehensive definition of the major antibody specificities in polyclonal rabbit antithymocyte globulin. *Transplantation* 1994;**57**:685–94.
43. Bolanos-Meade J, Vogelsang GB. Acute graft-versus-host disease. *Clin Adv Hematol Oncol* 2004;**2**:672–82.
44. Hale G, Jacobs P, Wood L, *et al*. CD52 antibodies for prevention of graft-versus-host disease and graft rejection following transplantation of allogeneic peripheral blood stem cells. *Bone Marrow Transplant* 2000;**26**:69–76.
45. Hillmen P, Skotnicki AB, Robak T, *et al*. Alemtuzumab compared with chlorambucil as first-line therapy for chronic lymphocytic leukemia. *J Clin Orthod* 2007;**25**:5616–23.
46. Blennow O, Remberger M, Klingspor L, Omazic B, Fransson K, Ljungman P, Mattsson J, Ringdén O. Randomized PCR-based therapy and risk factors for invasive fungal infection following reduced-intensity conditioning and hematopoietic SCT. *Bone Marrow Transplant* 2010; E-pub.
47. Morris EC, Rebello P, Thomson KJ, *et al*. Pharmacokinetics of alemtuzumab used for in vivo and in vitro T-cell depletion in allogeneic transplantations: relevance for early adoptive immunotherapy and infectious complications. *Blood* 2003;**102**:404–6.
48. Remberger M, Sundberg B. Rabbit-immunoglobulin G levels in patients receiving thymoglobulin as part of conditioning before unrelated donor stem cell transplantation. *Haematologica* 2005;**90**:931–8.

49. D'Sa S, Peggs K, Pizzey A, *et al*. T- and B-cell immune reconstitution and clinical outcome in patients with multiple myeloma receiving T-cell-depleted, reduced-intensity allogeneic stem cell transplantation with an alemtuzumab-containing conditioning regimen followed by escalated donor lymphocyte infusions. *Br J Haematol* 2003;**123**:309–22.
50. Chakrabarti S, Mackinnon S, Chopra R, *et al*. High incidence of cytomegalovirus infection after nonmyeloablative stem cell transplantation: potential role of Campath-1H in delaying immune reconstitution. *Blood* 2002;**99**:4357–63.
51. Chakrabarti S, Mautner V, Osman H, *et al*. Adenovirus infections following allogeneic stem cell transplantation: incidence and outcome in relation to graft manipulation, immunosuppression, and immune recovery. *Blood* 2002;**100**:1619–27.
52. Lynch BA, Vasef MA, Comito M, *et al*. Effect of in vivo lymphocyte-depleting strategies on development of lymphoproliferative disorders in children post allogeneic bone marrow transplantation. *Bone Marrow Transplant* 2003;**32**:527–33.
53. Micallef IN, Chhanabhai M, Gascoyne RD, *et al*. Lymphoproliferative disorders following allogeneic bone marrow transplantation: the Vancouver experience. *Bone Marrow Transplant* 1998;**22**:981–7.
54. Cavazzana-Calvo M, Bensoussan D, Jabado N, *et al*. Prevention of EBV-induced B-lymphoproliferative disorder by ex vivo marrow B-cell depletion in HLA-phenoidentical or non-identical T-depleted bone marrow transplantation. *Br J Haematol* 1998;**103**:543–51.
55. Gross TG, Hinrichs SH, Davis JR, Mitchell D, Bishop MR, Wagner JE. Depletion of EBV-infected cells in donor marrow by counterflow elutriation. *Exp Hematol* 1998;**26**:395–9.

III

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Vitamin D_3 supplementation in patients with frequent respiratory tract infections: a randomised and double-blind intervention study

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ABSTRACT

Background: Low serum levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections (RTIs). Clinical trials with vitamin D_3 against various infections have been carried out but data are so far not conclusive. Thus, there is a need for additional randomised controlled trials of effects of vitamin D_3 on infections.

Objective: To investigate if supplementation with vitamin D_3 could reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs.

Design: A double-blind randomised controlled trial.

Setting: Karolinska University Hospital, Huddinge.

Participants: 140 patients with antibody deficiency (selective IgA subclass deficiency, IgG subclass deficiency, common variable immune disorder) and patients with increased susceptibility to RTIs (>4 bacterial RTIs/year) but without immunological diagnosis.

Intervention: Vitamin D_3 (4000 IU) or placebo was given daily for 1 year.

Primary and secondary outcome measures: The primary endpoint was an infectious score based on five parameters: symptoms from respiratory tract, ears and sinuses, malaise and antibiotic consumption. Secondary endpoints were serum levels of 25-hydroxyvitamin D_3, microbiological findings and levels of antimicrobial peptides (LL-37, HNP1–3) in nasal fluid.

Results: The overall infectious score was significantly reduced for patients allocated to the vitamin D group (202 points) compared with the placebo group (249 points; adjusted relative score 0.771, 95% CI 0.604 to 0.985, p=0.04).

Limitations: A single study centre, small sample size and a selected group of patients. The sample size calculation was performed using p=0.02 as the significance level whereas the primary and secondary endpoints were analysed using the conventional p=0.05 as the significance level.

Conclusions: Supplementation with vitamin D_3 may reduce disease burden in patients with frequent RTIs.

ARTICLE SUMMARY

Article focus

- Recent evidence suggests that vitamin D_3 has potent extraskeletal effects, such as suppression of inflammation and strengthening of mucosal immunity by induction of antimicrobial peptides.
- Data from observational studies suggest that low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of respiratory tract infections.
- Results from a limited number of randomised controlled trials on the protective role of vitamin D_3 against respiratory tract infections are inconclusive and thus additional studies are warranted.

Key messages

- Therefore we designed and carried out a randomised controlled trial where a large dose (4000 IU) of vitamin D_3 was given to patients with an increased susceptibility to infections for 1 year.
- The main conclusion is that vitamin D_3 supplementation reduces symptoms and antibiotic consumption among patients with an increased frequency of respiratory tract infections. Thus, vitamin D_3 supplementation may be an alternative strategy to reduce antibiotic use among patients with recurrent respiratory tract infections.

Strengths and limitations of this study

- A high daily dose of vitamin D_3 was used, the study time was a full year covering all seasons and patients with an increased frequency of respiratory tract infections were studied.
- A single study centre, small sample size (n=140) and a selected group of patients.

INTRODUCTION

Vitamin D was discovered when it was noted that rachitic children were improved by exposure to sunlight.[1] It was later shown by Holick *et al*[2] that vitamin D_3 is synthesised in the skin under the influence of ultraviolet light. Vitamin D_3 is further hydroxylated in the liver

to 25-hydroxyvitamin D_3, which is considered to reflect the vitamin D status of an individual patient.[3] The final activation to the active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) requires 1-α-hydroxylase activity. This enzyme (also designated CYP27B1) is expressed in the kidney but also in many other cell-types, including epithelial and immune cells.[4] The active vitamin D_3 ($1,25(OH)D_3$) binds to the vitamin D receptor (VDR), which belongs to the nuclear receptor family. Active vitamin D_3 is only present in minute amounts in the circulation and local activation in target cells is crucial for vitamin D-mediated effects on the immune system.[5]

Low levels of 25-hydroxyvitamin D_3 are associated with an increased risk of tuberculosis[6–8] and respiratory tract infections.[9] The mechanism is not fully elucidated but vitamin D_3 has been shown to induce antimicrobial peptides in immune cells.[10] In addition, active vitamin D_3 ($1,25(OH)D_3$) has broad anti-inflammatory effects on the adaptive immune system by shifting the T helper cell pool from a Th1/Th17-response to a Th2/Treg-dominated response.[11 12] Vitamin D_3 has also been shown to suppress the Th2-response in allergic bronchopulmonary aspergillosis.[13] Thus, vitamin D_3 modulates both the adaptive and innate immune system.[14] The bulk of data on vitamin D_3 and infections stems from in vitro experiments and retrospective observational studies. Results from randomised controlled trials (RCTs) where the effects of vitamin D_3 on infections have been investigated (reviewed by Yamshchikov *et al*[15]) are not conclusive and larger clinical trials are therefore warranted.

We designed a study to test the hypothesis that 4000 IU of vitamin D_3 given daily to patients with antibody deficiency and frequent respiratory tract infections for 1 year could prevent or ameliorate infections. In addition, we investigated whether genetic polymorphisms in genes involved in the effect and/or metabolism of vitamin D_3 have an influence on the outcome of vitamin D_3 supplementation.

METHODS

Study design

A prospective, randomised, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections. The study was approved by the local Ethical Committee and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The study was registered at www.clinicaltrials.gov prior to inclusion of the first patient (NCT01131858). The EudraCT number is 2009-011758-16. The full protocol is available from the corresponding author upon request.

Sample size calculation

The sample size was based on the assumption that the intervention would reduce the number of days with symptoms from 42 (210 points) to 28 days (140 points), that is, a reduction of the infectious burden by 30%. Given this assumption, a sample size of 60 patients per study group was predicted to provide the study 90% power at a significance level of p=0.02 (Student's t test). To compensate for predicted exclusion of participants, the groups were increased to include 70 patients per treatment arm. Importantly, the significance level of p=0.02 was chosen in the power calculation to ensure that a sufficient number of patients were recruited in order to avoid a type II error in the primary analysis. However, the conventional and widely accepted significance level of p=0.05 was used for statistical analyses of the primary and secondary endpoints.

Participants

Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March and June 2010 by the study nurses (SH, ML and KJ). Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections; that is, >42 days with symptoms from the respiratory tract during a 12-month period prior to study inclusion. Patients registered at the Immunodeficiency Unit are closely followed up with a diary of symptoms and antibiotic consumption. Thus, the patients are trained and used to apply such an instrument to assess their infectious status. Data from patients' standard diary were used as an instrument in order to assess patients for eligibility, both via telephone and by the responsible physician (PB and ACN) prior to inclusion. Patients with selective IgA-deficiency (*D80.2*), IgG-subclass deficiency (*D80.3*) and common variable immune disorder (CVID, *D83.0*) as well as patients without a defined immunological diagnosis (*D89.9*) were included. Exclusion criteria were prophylactic treatment with antibiotics, history of hypercalcaemia or stones in the urinary tract, sarcoidosis, ongoing supplementation with vitamin D_3 exceeding 400 IU/day, HIV-infection and pregnancy.

Interventions

Patients were randomised to 12 months' treatment with vitamin D_3 (Vigantol, 4000 IU/day, Merck GmbH, Darmstadt, Germany) or placebo oil. One drop contained 500 IU vitamin D_3 or placebo oil (Miglyol oil, Merck GmbH, Darmstadt, Germany) and the participants were asked to take eight drops daily. The participants had to mark their daily symptoms of infection in a diary, which was sent via regular mail to the study site every month. The following data were recorded: symptoms from the respiratory tract, ears and sinuses, treatment with antibiotics, numbers of bacterial cultures, times and reasons of visits to hospitals, frequency of travelling abroad and adherence to study drug.

Outcomes

The primary outcome was a composite infectious score, based on a daily patient-reported questionnaire and

included five parameters: symptoms from the respiratory tract, ears and sinuses, malaise and use of antibiotics (see online supplementary figure S1), each parameter gave 1 point/day. The occurrence of x-ray verified pneumonia gave three additional points per day for a period of 7 days. Thus, a pneumonia resulted in 3×7 points=21 extra points. Patients were specifically instructed to record only symptoms related to ongoing respiratory tract infections. Symptoms related to infections at other sites (urinary tract, wounds, etc) as well as non-infectious symptoms were reported as adverse events. Secondary outcomes were serum levels of 25-hydroxyvitamin D_3 (at baseline and after 3, 6, 9 and 12 months), numbers of bacterial cultures, microbiological findings and levels of antimicrobial peptides (LL-37 and HNP1-3) in nasal fluid (at baseline and after 6 and 12 months). In addition, six post hoc genotype analyses were performed in all participants. Analyses of single nucleotide polymorphisms (SNPs) were carried out for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1 and vitamin D binding protein (GC). Safety tests included plasma levels of creatine, calcium, phosphate and albumin, measured at baseline and after 3, 6, 9 and 12 months. At inclusion, urine-HCG (human chorionic gonadotropin) in women was measured and p-parathyroid hormone was measured in both genders. The results of the safety tests were reviewed by an independent and unblinded consultant physician. Two blinded physicians (PB and ACN) were responsible for inclusion and all medical visits to the study site (Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden).

Randomisation and statistical analysis

Participants were randomised to 12 months' treatment with vitamin D_3 (Vigantol, 4000 IU/day) or placebo oil. Block randomisation with a block size of ten was used to ascertain equal group sizes. Staff at Karolinska Trial Alliance was responsible for randomisation procedures. In the statistical analysis, continuous variables were compared using Mann-Whitney U test or linear regression and dichotomous variables by Fisher's exact test or logistic regression. Regressions of log-transformed infectious scores were performed both unadjusted (simple regression) and with adjustment for potential confounders (multiple regression).

Statistical methods: primary analysis

The distribution of the infectious score was found to be skewed, thereby violating the normal assumption of the prespecified t test analysis. Hence, scores were log-transformed prior to analysis. Further, the randomisation had resulted in age distributions that were not entirely balanced between the two groups. Since there might be concerns that such imbalance could influence the results of the study, the original analysis plan was extended with a multivariable analysis adjusting for potential confounders. In this linear regression model based on log-transformed values of the primary outcome (the total infectious score) and its individual components, adjustment was made for age, gender, smoking, type of immune deficiency and significant comorbidities (respiratory or non-respiratory). Because of the transformation procedure, the adjusted effect of vitamin D_3 is expressed as a ratio between the score in the vitamin D_3 and the placebo group. In this multiplicative model, an effect size of 1 indicates identical outcome in the two study groups and statistically non-significant results are recognised by CIs encompassing the value 1.

To explore potential divergent effects on different organ systems, both adjusted and unadjusted analyses were repeated separately for each individual item of the infectious score. In addition, the temporal aspects of the vitamin D_3 effect were investigated by dividing the study period into four 90-day periods (starting on the first day of treatment) and repeating the analyses separately for each time period. 'Ear' and 'sinus' symptoms as well as 'antibiotic use' occurred at low frequencies and for these entities normal distributions could not be achieved despite data transformation. Thus, the adjusted analyses of these individual items were based on multivariable logistic regression, after coding the symptom (or antibiotic therapy) as present or absent during the course of the study. However, this only applies to analysis of the individual items, and not to the primary analysis of the total infectious score, where all item scores were added as originally described.

Most postrandomisation exclusions were due to patients failing to fill out the symptoms diary. Hence, no intention-to-treat (ITT) analysis based on actual outcome data could be performed. However, the potential impact of dropouts was addressed in an ITT analysis based on multiple imputation of missing outcome data. In the imputation process, pooled estimates were derived from 100 datasets created by means of multivariate imputation by chained equations and predictive mean matching for the same covariates as in the adjusted per-protocol analysis.

Detailed descriptions of randomisation and blinding, sampling of nasal fluid, measurement of antimicrobial peptides, measurement of 25-hydroxyvitamin D_3, genotyping and statistical analyses of secondary outcomes are presented in the supplementary methods section.

RESULTS

Baseline data

A total of 286 patients were first assessed for eligibility but 144 were not included because they did not fulfil all inclusion criteria; <42 days with infection/year (n=35), lacked other inclusion criteria (n=42), or declined to participate (n=67). The remaining 142 patients were further screened and 140 patients were included in the study. Of these, 70 were randomised to vitamin D_3 supplementation and 70 to placebo (figure 1). The groups did not differ with regard to gender, IgG replacement therapy, smoking, baseline 25-hydroxyvitamin D_3 levels,

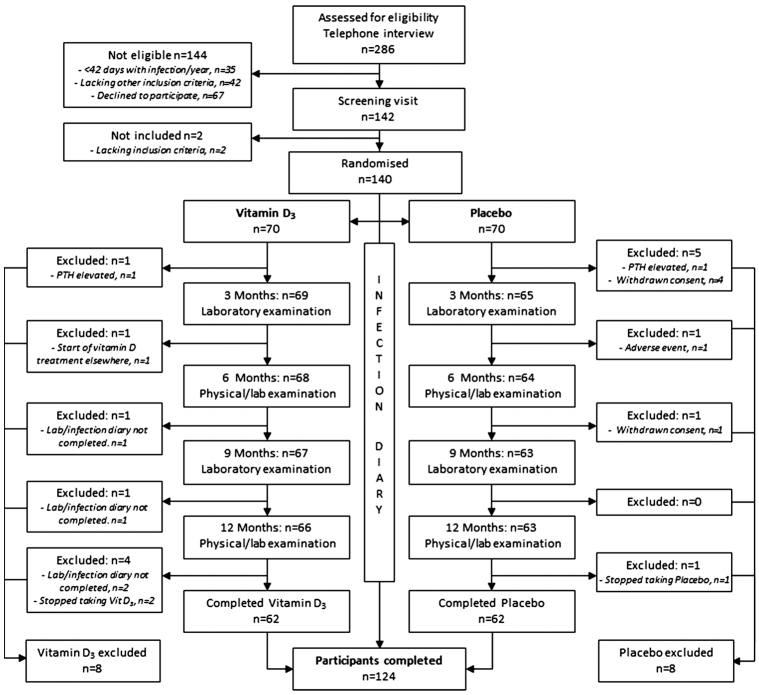

Figure 1 Study outline.

type of immune defect or comorbidities (table 1). Patients with subclass deficiency, selective IgA deficiency (sIgAD), CVID and patients without a defined immunological diagnosis (ND) but with >4 bacterial respiratory tract infections/year were included. IgG replacement therapy was most common in the CVID group (100%) and in the subclass deficiency group (63%), and also frequent in the other groups (ND, 54% and sIgAD, 38%, see online supplementary table S1). Patients allocated to the placebo group were slightly younger than patients in the treatment group (p=0.025, data not shown). During the course of the study, 16 patients left the study prematurely (8 patients from each study group) and consequently 124 patients were included in the main per-protocol analysis. Reasons for dropout included elevated parathyroid hormone (n=2), withdrawn consent (n=5), adverse events (n=1), prescription of vitamin D outside the study (n=1), failure to complete diary (n=4) or non-compliance to study medication (n=3; figure 1).

Table 1 Baseline data

	Vitamin D_3	Placebo
Number	70	70
Age (mean)	55.4	50.8
Female	52/70	50/70
Male	18/70	20/70
IgG-replacement	39/70	42/70
Smoking	4/70	6/70
25-OH levels (mean) (nmol/l)	51.5	46.9
Immunological diagnosis		
sIgA-deficiency	9/70	9/70
IgG subclass	27/70	30/70
CVID	6/70	4/70
ND	28/70	27/70
Concomitant disease		
No other disease	16/70	18/70
Lung: Asthma	27/70	25/70
Lung: BE	5/70	7/70
Lung: COPD	5/70	4/70
Other disease*	17/70	16/70

Mann-Whitney U test was used for comparisons of age and 25-OH vitamin D_3. Fisher's exact test was used for all other comparisons.
*'other disease' includes hypertension, body pain, hypothyroidism and gastritis as most common diagnoses.
BE, bronchiectasis; COPD, chronic obstructive pulmonary disease; CVID, common variable immuno deficiency; ND, increased susceptibility to infections without a defined immunological disorder.

Primary endpoint: infectious score

One year of vitamin D_3 treatment was associated with a significantly reduced total infectious score both in the unadjusted (n=124, p=0.024; table 2) and in the adjusted analyses (n=124, p=0.040; table 2; figure 2A,B and see online supplementary table S2). The

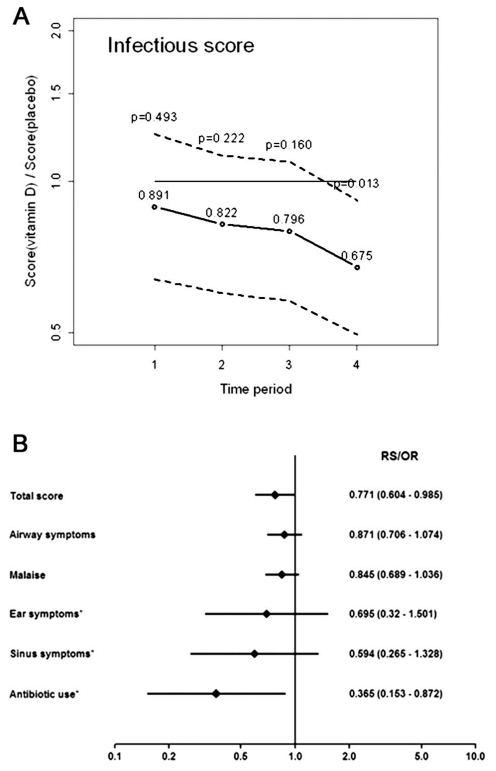

Figure 2 Primary endpoint. The adjusted total relative infectious score (A) is expressed 'per quarter' (3-month periods). The adjusted 1-year scores (total score, airway, malaise, ear, sinus and antibiotics) are depicted in a Forest-plot (B) together with 95% CI. Effects are presented as relative scores (total score, airway and malaise) or OR (ear, sinus, antibiotics and indicated with asterisks).

unadjusted relative score in the intervention group was 0.754 (95% CI 0.591 to 0.963, p=0.024, n=124) corresponding to a 25% reduction and after adjustment for potential confounders, the relative score was 0.771 (95% CI 0.604 to 0.985, p=0.04), corresponding to a 23% reduction (table 2). According to the temporal analysis, the effect of vitamin D_3 supplementation tended to improve with time (figure 2A). The absolute unadjusted score per patient was 202 points for the vitamin D group and 249 points for the placebo group, a significant reduction of 47 points per patient (p=0.023, Mann-Whitney U test, see online supplementary table S3).

When the individual items of the infectious score were analysed separately, all point estimates indicated a reduction in the treatment group (table 2, see online supplementary figure S2), although only antibiotic consumption reached statistical significance (figure 2B and see online supplementary figure S2, panel E). The adjusted OR for antibiotic use was 0.365 (95% CI 0.153 to 0.872, p=0.023, n=124), that is, a 63.5% reduction of the odds of antibiotic use in the intervention group (table 2). The absolute values were 33 days on antibiotics for the placebo group and 16 days for the vitamin D_3 group, that is, a reduction of 17 days in the vitamin D_3 group (see online supplementary table S3). The temporal trends for specific symptoms and antibiotic consumption were similar to the total score and reached statistical significance for 'ear'-symptoms (n=124, p=0.041) and for 'malaise' (n=124, p=0.053) in the final quarter of the study (see online supplementary figure S2, panels B and C).

Analysing the primary outcome according to ITT (n=140) produced results virtually identical to those of the per-protocol analysis. In the unadjusted ITT analysis, vitamin D_3 reduced the total infectious score by 25% (relative score 0.752, 95% CI 0.588 to 0.962, p=0.024) and after adjustment for potential confounders the reduction was 23% (relative score 0.767, 95% CI 0.599 to 0.982, p=0.036).

Serum levels of 25-hydroxyvitamin D_3

Serum 25-hydroxyvitamin D_3 levels did not differ between the groups at baseline (table 1) but already after 3 months

Table 2 Primary endpoint

	Univariable regression model (unadjusted values)			Multiple regression model (adjusted values)		
Endpoint	**Effect**	**95% CI**	**p Value**	**Effect**	**95% CI**	**p Value**
Total score	0.754	0.591 to 0.963	0.024	0.771	0.604 to 0.985	0.040
Airway	0.857	0.697 to 1.053	0.141	0.871	0.706 to 1.074	0.200
Ear*	0.721	0.352 to 1.465	0.367	0.695	0.320 to 1.501	0.357
Sinus*	0.583	0.280 to 1.198	0.144	0.594	0.265 to 1.328	0.204
Malaise	0.845	0.692 to 1.032	0.098	0.845	0.689 to 1.036	0.108
Antibiotics*	0.355	0.154 to 0.784	0.012	0.365	0.153 to 0.872	0.023

Treatment effect calculated as the ratio between infectious scores in the vitamin D_3 and the placebo groups. Due to low frequencies, endpoints marked with asterisks (*) were coded as binary outcomes (ie, present or absent in each patient) and compared by means of logistic regression. In these cases, the effect refers to OR of experiencing the outcome at least once during the course of the study (The data are based on n=124 patients).

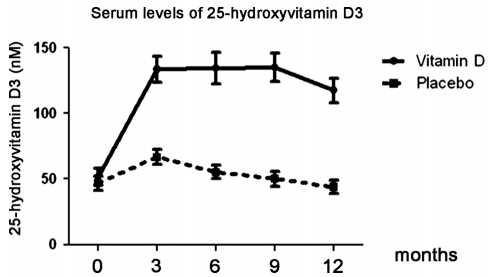

Figure 3 Secondary endpoint. Vitamin D levels. Serum was collected at days 0, 3, 6, 9 and 12 months and levels of 25-hydroxyvitamin D_3 were measured. Values are expressed as mean±95% CI.

the intervention group had a significantly higher level of 25-hydroxyvitamin D_3 (133.4 vs 66.6 nmol/l, p<0.001; figure 3). This increase remained throughout the study (figure 3).

Bacterial cultures and microbiology

During the course of the study, 173 microbiological samples were obtained in the vitamin D_3 group (n=62, 2.79/patient) and 301 in the placebo group (n=62, 4.85/patient; p=0.010; table 3). The number of samples with at least one positive finding was higher in the placebo group, with close to statistical significance (p=0.052), while the fraction of positive samples was similar for both groups (table 3). Significantly more patients had a microbiological sample taken from the respiratory tract (≥1 sample) during the study period in the placebo group; OR 2.63 (95% CI 1.17 to 5.92; table 3).

In total, the vitamin D_3 group generated 76 positive microbiological findings (bacteria or fungi), compared with 159 in the placebo group (p=0.023). There was no difference between the groups for the traditional respiratory pathogens (*Haemophilus influenza*, *Moraxella catharralis* and *Streptococcus pneumonia*), but there were significantly fewer findings of *Streptococcus aureus* (p=0.019) and fungi (p=0.028, *Candida* spp. and *Aspergillus* spp.) in the treatment group (table 4). Likewise, significantly fewer vitamin D_3-treated patients had a bacterial culture positive for *S aureus* (p=0.019) or fungal species (p=0.058), although the latter difference did not reach statistical significance (table 4).

Vitamin D treated patients with subclass deficiency left significantly fewer bacterial or fungal cultures than placebo-treated patients with this diagnosis; seven cultures in the vitamin D group (n=22) versus 47 cultures in the placebo group (n=24) (see online supplementary table S4). Also the number of patients that had ≥1 bacterial culture taken was significantly fewer in the placebo group (12/22 vs 22/24, p=0.0065, see online supplementary table S4). There was no significant effect of other immunological diagnoses on bacterial cultures or microbiology (see online supplementary table S4).

Since concomitant lung disease may be an important factor for vitamin D mediated effects on respiratory immunity, we performed a detailed analysis of bacterial cultures and microbiology of patients with asthma, bronchiectasis (BE) and chronic obstructive pulmonary disease (COPD). The numbers of patients with these diagnoses were quite small, which preclude any firm conclusions regarding any effect. However, there was a trend—however not significant—that vitamin D-treated patients with asthma produced fewer bacterial cultures (average 2.9 cultures/patient vs 7.0 cultures/patients, p=0.080, see online supplementary figure S3) and fewer positive cultures than placebo-treated asthmatics (average 0.6 positive cultures/patients vs 2.7/patient in the placebo group, p=0.052, see online supplementary figure S3). In addition, vitamin D-treated asthma patients showed significantly fewer cultures positive for fungi (*Candida* and *Aspergillus*) compared with placebo-treated asthmatics (p=0.0476, see online supplementary table S5). For BE or COPD patients there was no clear trend or significant effect in bacterial cultures or microbiology.

Table 3 Bacterial cultures

	Vitamin D_3	Placebo	Significance
Number of samples per patient (mean, n=62/62)	2.79	4.85	p=0.010*
Number of positive samples per patient (mean, n=62/62)	1.01	2.02	p=0.052*
Fraction positive cultures (%)	63/173 (36%)	125/301 (41%)	p=0.28**
Patients with ≥1 sample taken	38/62 (61%)	50/62 (81%)	p=0.029**

*Mann-Whitney U test.
**Fisher's exact test.

Levels of antimicrobial peptides in nasal fluid

There was no statistically significant difference between the vitamin D_3 or placebo groups when nasal fluids were analysed for the presence of antimicrobial peptides (AMPs). Initially, the levels of both LL-37 and HNP1-3 tended to be higher in the placebo group (see online supplementary figure S3, panels A and B). However, after 12 months the microbiological pattern was reversed and no primary pathogens could be detected in nasal swabs from vitamin D_3-treated patients (n=25, p=0.039; see online supplementary figure S4, panel C). The placebo-treated patients exhibited the same mix between normal flora and primary pathogens at all three sampling points (0, 6 and 12 months; see online supplementary figure S4, panel C).

SNP variants and treatment effect

Most genetic variants did not affect the primary endpoint. However, patients carrying the 'AA' genotype in

Table 4 Microbiological findings

	Number of findings (total)			Number of patients		
Microorganism	**Vitamin D_3**	**Placebo**	**MW-U**	**Vitamin D_3**	**Placebo**	**Fisher**
Haemophilus influenzae	28	27	p=0.46	10/62	13/62	p=0.64
Moraxella catharralis	8	17	p=0.39	7/62	10/62	p=0.60
Streptococcus pneumoniae	7	6	p=0.74	4/62	5/62	p=1.00
Staphylococcus aureus	6	33	p=0.010	4/62	14/62	p=0.019
Enterobacteriacae	8	8	p=0.39	4/62	7/62	p=0.53
Pseudomonas aeruginosa	8	15	p=0.68	3/62	4/62	p=1.00
Fungal infection	11	53	p=0.028	4/62	12/62	p=0.058
Total	76	159	p=0.023			

Mann-Whitney U test was used to analyse the total number of findings, whereas Fisher's exact test was used for analysis of the number of patients (fraction) with a specific finding.

the CYP2R1-gene, encoding the 25-hydroxylase enzyme, had a larger benefit of vitamin D_3-supplementation (–55%) compared to AG or GG carriers (–6%) (n=124, p=0.046 for interaction, see online supplementary table S6).

Adverse events

In total, the vitamin D_3 group reported 38 adverse events (AEs) versus 56 AEs in the placebo group. The most common symptoms in the treatment group were headache (n=5) and lumbago (n=5), whereas placebo-treated patients reported paresthesias (n=8), diverticulitis (n=4) and urinary tract infection (n=4) as most frequent AEs (table 5, see online supplementary table S7). There was a general trend towards the number of adverse events being higher in the placebo group. Significantly more patients in the placebo group reported cardiovascular problems, such as heart failure, hypertonia and thrombosis (p=0.028). For gastrointestinal and other (non-respiratory) infections there was also a trend favouring the vitamin D_3 group (p=0.058 and 0.09, respectively). No clinically relevant changes in serum levels of calcium, phosphate, creatine or albumin could be observed (see online supplementary figure S5). There was one severe adverse event in each group (rabdomyosarcoma in the vitamin D_3 group and lung bleeding in the placebo group), both judged as being unrelated to the study drug.

Table 5 Adverse events

Organ	**Vitamin D_3 n (%)**	**Placebo n (%)**	**p Value**
CNS	11 (29)	10 (18)	1.00
Gastrointestinal	4 (11)	12 (21)	0.058
Cardiovascular	0 (0)	6 (11)	0.028
Infections (other than RTI)	2 (5)	8 (14)	0.09
Musculoskeletal	10 (26)	10 (18)	1.00
Respiratory (non-infectious)	2 (5)	4 (7)	0.68
Skin	5 (13)	2 (4)	0.44
Other	4 (10)	4 (7)	1.00
Total	38	56	

Number of reports. Fisher's exact test was used for between group comparison (the data are based on AE-reports from n=62 patients/arm).
CNS, central nervous system, RTI, respiratory tract infection.

DISCUSSION

The main conclusion from this long-term RCT is that vitamin D_3 supplementation reduces the total burden of respiratory tract infections. The primary endpoint was composed of five different parameters that patients recorded daily throughout the study year. All point estimates favoured the vitamin D_3 group and a statistically significant effect was seen on both the total score and on the probability of receiving antibiotics ($p<0.05$). The effect on the infectious score was evident both in analysis per-protocol and according to ITT, and withstood adjustment for potential confounders. In addition, the number of bacterial cultures and microbiological findings was significantly reduced in the intervention group. These findings are potentially important and support that Vitamin D_3 supplementation may prevent respiratory tract infections and reduce antibiotic consumption, particularly in patients with hypogammaglobulinaemia or with an increased frequency of respiratory tract infections.

However, our study has several limitations: First, the choice of primary endpoint may be questioned since it relies solely on patient-reported information. To compensate for inherent problems with patient-reported data, the evaluation instrument was designed to cover many aspects of an infectious episode, including various symptoms as well as antibiotic consumption. Together the reported data formed an 'infectious score', which constituted the primary endpoint of the study. Similar composite scores have successfully been applied to different diseases, such as tuberculosis (TB-score[16]), pneumonia (CURB-65[17]) and bacterial meningitis (BMS-score[18]). Notably, vitamin D supplementation had a major effect on the odds of taking antibiotics during the study period (a reduction by 63.5%). In addition, the absolute number of days on antibiotics was reduced by 50% (from 33 days in the placebo group to 16 days in

the intervention group), which was statistically significant both in the adjusted and unadjusted analyses (table 1). However, despite the relatively modest reduction for the other components of the primary endpoint the overall infectious score was significantly reduced—mainly as a result of the large effect on the antibiotic parameter—both in the unadjusted and in the adjusted analyses (table 1 and figure 2). It is important to interpret the statistical significance in light of our power calculation, which was based on a significance level of p=0.02. In the power calculation, the significance level was reduced from 0.05 to 0.02 in order to increase the statistical power at the p=0.05 level. This approach was incorrect, and the targeted power (at the p=0.05 level) should instead have been increased without altering the p-value threshold. However, we have used the widely accepted significance level p=0.05 in the statistical analyses for both the primary and secondary endpoints, respectively. Another potential problem was that the patient population was very heterogeneous with regard to immune deficiency and concomitant diseases. We adjusted for these factors in the multivariable analyses of the primary endpoint, but the sample sizes in each subgroup were too small to draw any conclusions of effects in specific disease groups. However, a detailed post hoc analysis of the relation between immunological diagnosis, concomitant lung disease and the secondary endpoints 'taken bacterial cultures', 'positive bacterial' cultures and 'microbiological findings' was performed. There was a clear trend that vitamin D-treated patients with subclass deficiency and/or asthma produced fewer bacterial cultures, fewer positive cultures and fewer fungal cultures (see online supplementary tables S4 and S5 and figure S3). Although this analysis may lack precision by the small number of patients included, it could have clinical implications regarding target groups for vitamin D_3 supplementation.

Nevertheless, our double-blind RCT has several strengths. For example, we chose a high daily dose of vitamin D_3 based on published calculations on metabolism and effects on immunity.[14 19] Other RCTs using lower doses of vitamin D_3, 400–2000 IU/day, have mainly been negative with regard to the prevention of infections.[20 21] However, one study using 1200 IU/day showed a significant reduction of influenza among school children in Japan.[22] Notably, also studies using higher doses of vitamin D_3 have been negative. Martineau *et al* used 400 000 IU vitamin D_3 during 42 days (9523 IU/day) with the aim of shortening time to sputum conversion in tuberculosis. No significant effect on the primary endpoint could be observed in that study, except in a subgroup with the *tt* genotype in the VDR gene.[23] A recent study investigated whether 100 000 IU vitamin D_3/month (3333 IU/day) could reduce the incidence of COPD exacerbations. There was no significant effect on the primary endpoint, although a post hoc analysis revealed that patients with a low vitamin D_3 level at baseline had a significant effect of vitamin D_3 supplementation.[24]

Importantly, our study is the first to utilise high daily doses for an extended period of one full year. Thus, we covered all four seasons, which was important in Sweden with a known seasonal variation in 25-hydroxyvitamin D_3 levels.[25] Two previous RCTs were performed during the winter season—when vitamin D levels are low—but only during 4[22] and 6 months,[20] respectively. Previous RCTs have been conducted during shorter periods; 42 days,[23] 6 weeks[26] and 12 weeks,[21] respectively. Interestingly, we observed a clear time-dependent effect suggesting that a long-term supplementation approach (>6 months) may be necessary to affect immunity. To expand on the results of a previous study in healthy individuals where no difference between the intervention and placebo groups was observed,[21] we chose a study population with frequent RTIs and at least 42 days with infection during the year prior to inclusion. Notably, patients in the study represent a selected group of individuals with frequent RTI, although the immune disorders that they represent (sIgAD, IgG-subclass deficiency and patients with no defined immune disorder) are generally mild in character and dominated by mucosal RTIs. We also included a small number of CVID-patients, which can be considered to be a more severe immune disorder, but all these patients are treated with IgG replacement therapy and thus well controlled. Hence, the results from this study cannot directly be applied to the general healthy population. Nevertheless, the results provide solid support for additional interventional studies of vitamin D_3, especially in groups consuming large amounts of antibiotics.

The mechanism of the observed effects remains largely unknown. Vitamin D_3 modulates the immune response at many levels, such as induction of AMPs, skewing of T-cells from Th1/Th17 to Tregs as well as general anti-inflammatory effects.[14] Here, we investigated the role of AMPs in nasal fluid. However, we could not detect any significant changes of LL-37 or HNP1-3 during the study period, but noted that placebo-treated patients tended to have higher levels of AMPs after 1 year of treatment. This was paralleled by a shift of the microflora in the nasal compartment that could explain the unexpected finding of higher AMP-levels in the placebo group. Recently, it was shown that 1,25 $(OH)_2$-vitamin D_3 induces both HNP1-3 and LL-37 in nasal fluid of healthy volunteers,[27] supporting that LL-37 may indeed be induced in vivo. However, our study design did not allow such conclusions but rather support that vitamin D_3 affect mucosal immunity, leading to a shift of the microflora. Recently, we showed that the bacterial composition in nasal swabs is an important determinant of AMP-levels in nasal fluid.[28]

Given that vitamin D_3 induces LL-37 in epithelial cells and that LL-37 kills bacteria in vitro, we expected a reduction of the classical bacterial pathogens *H influenza*, *M catharralis* and *S pneumonia* in the intervention group. However, the frequency of these bacteria was not reduced

but a reduction of *S aureus* and fungal species that often colonise the airways was observed. This could be explained by specific effects by vitamin D_3 on immunity against *S aureus*. In fact, vitamin D_3 induces human β-defensin-2 (HBD-2) with bactericidal activity against *S aureus*.[29] A recent study showed that low vitamin D_3 levels were associated with an increased risk of being colonised by this bacterium.[30] Further, vitamin D_3 affects immunity against *C albicans*, which indicates direct effects of vitamin D_3 on human immunity.[31] Alternatively, it is possible that vitamin D_3 may have prevented symptomatic viral infections, which prompted patients to leave a bacterial sample from the airways. Interestingly, there is both mechanistic and clinical evidence that vitamin D_3 can prevent viral infections,[32–34] although we did not address this in the current study.

Notably, we observed a prominent increase in the serum concentration of 25-hydroxyvitamin D_3, which indicated good compliance and tolerability of the study drug. In fact, there was a trend towards adverse events being reported more often in the placebo group, suggesting that vitamin D_3 possibly could be efficient against other diseases, but this observation requires further studies. No clinically relevant changes of blood chemistry (calcium, phosphate, albumin or creatine) were observed. Despite few adverse events and high tolerability, 16 exclusions occurred during the study year. The main reason was problems to adhere to the protocol and 6/16 patients dropped out of the study after a few weeks. The rest failed to send in the diaries, did not leave blood for monitoring of safety parameters or did not take the study drug. One patient was excluded based on symptoms that could be attributed to vitamin D_3 (facial paraesthesia). However, this patient was later confirmed to have been allocated to placebo.

In summary, we found that supplementation with vitamin D_3 reduced the total infectious score with 47 points per patient (23% reduction in the adjusted analysis) during the study year. The observed reduction was lower than the assumed reduction of 70 points per patient (predefined assumption: 210 points=>140 points; a reduction of 30%) that formed the basis for the power calculation. However, despite the predefined level of a reduction of infectious score by 30% as a clinically meaningful effect, we believe that effects lower than this also could be relevant for the individual patient. We base this line of reasoning on the fact that a reduction of 47 points per patient can be translated into 47 days with cough (47 points), 23 days with ear and sinus symptoms (23×2=46 points) or 9 days with cough, sinus and ear symptoms together with malaise and antibiotics (9×5=45 points). In addition, our data indicate that vitamin D_3 supplementation reduces the odds of taking antibiotics by approximately 60% in patients with frequent respiratory tract infections. Thus, supplementation with vitamin D_3 could provide a novel strategy to reduce antibiotic use among high consumers and indirectly prevent the emerging epidemic of bacterial resistance.

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REFERENCES

1. Hess AF, Unger LJ, Pappenheimer AM. Experimental rickets in rats. III. The prevention of rickets in rats by exposure to sunlight. *J Biol Chem* 1922;50:77–81.
2. Holick MF, MacLaughlin JA, Clark MB, *et al*. Photosynthesis of previtamin D3 in human skin and the physiologic consequences. *Science* 1980;210:203–5.
3. Holick MF. Vitamin D deficiency. *N E J M* 2007;357:266–81.
4. Hansdottir S, Monick MM. Vitamin D effects on lung immunity and respiratory diseases. *Vitam Horm* 2011;86:217–37.
5. Hewison M. Antibacterial effects of vitamin D. *Nat Rev Endocrinol* 2011;7:337–45.
6. Gibney KB, MacGregor L, Leder K, *et al*. Vitamin D deficiency is associated with tuberculosis and latent tuberculosis infection in immigrants from sub-Saharan Africa. *Clin Infect Dis* 2008;46:443–6.
7. Martineau AR, Nhamoyebonde S, Oni T, *et al*. Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa. *Proc Natl Acad Sci USA* 2011;108:19013–17.
8. Wilkinson RJ, Llewelyn M, Toossi Z, *et al*. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. *Lancet* 2000;355:618–21.
9. Ginde AA, Mansbach JM, Camargo CA Jr. Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. *Arch Inter Med* 2009;169:384–90.
10. Liu PT, Stenger S, Li H, *et al*. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006;311:1770–3.
11. Daniel C, Sartory NA, Zahn N, *et al*. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. *J Pharmacol Exp Ther* 2008;324:23–33.

12. Lemire JM, Archer DC, Beck L, *et al.* Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. *J Nutr* 1995;125(6 Suppl):1704S–8S.
13. Kreindler JL, Steele C, Nguyen N, *et al.* Vitamin D3 attenuates Th2 responses to Aspergillus fumigatus mounted by CD4+ T cells from cystic fibrosis patients with allergic bronchopulmonary aspergillosis. *J Clin Invest* 2010;120:3242–54.
14. Hewison M. Vitamin D and immune function: an overview. *Proc Nutr Soc* 2012;71:50–61.
15. Yamshchikov AV, Desai NS, Blumberg HM, *et al.* Vitamin D for treatment and prevention of infectious diseases: a systematic review of randomized controlled trials. *Endocr Pract* 2009;15:438–49.
16. Wejse C, Gustafson P, Nielsen J, *et al.* TBscore: signs and symptoms from tuberculosis patients in a low-resource setting have predictive value and may be used to assess clinical course. *Scand J Infect Dis* 2008;40:111–20.
17. Bauer TT, Ewig S, Marre R, *et al.* CRB-65 predicts death from community-acquired pneumonia. *J Intern Med* 2006;260:93–101.
18. Dubos F, De la Rocque F, Levy C, *et al.* Sensitivity of the bacterial meningitis score in 889 children with bacterial meningitis. *J Pediatr* 2008;152:378–82.
19. Heaney RP, Davies KM, Chen TC, *et al.* Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. *Am J Clin Nutr* 2003;77:204–10.
20. Laaksi I, Ruohola JP, Mattila V, *et al.* Vitamin D supplementation for the prevention of acute respiratory tract infection: a randomized, double-blinded trial among young Finnish men. *J Infect Dis* 2010;202:809–14.
21. Li-Ng M, Aloia JF, Pollack S, *et al.* A randomized controlled trial of vitamin D3 supplementation for the prevention of symptomatic upper respiratory tract infections. *Epidemiol Infect* 2009;137:1396–404.
22. Urashima M, Segawa T, Okazaki M, *et al.* Randomized trial of vitamin D supplementation to prevent seasonal influenza A in schoolchildren. *Am J Clin Nutr* 2010;91:1255–60.
23. Martineau AR, Timms PM, Bothamley GH, *et al.* High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *Lancet* 2011;377:242–50.
24. Lehouck A, Mathieu C, Carremans C, *et al.* High doses of vitamin D to reduce exacerbations in chronic obstructive pulmonary disease: a randomized trial. *Ann Intern Med* 2012;156:105–14.
25. Lindh JD, Andersson ML, Eliasson E, *et al.* Seasonal variation in blood drug concentrations and a potential relationship to vitamin D. *Drug Metab Dispos* 2011;39:933–7.
26. Nursyam EW, Amin Z, Rumende CM. The effect of vitamin D as supplementary treatment in patients with moderately advanced pulmonary tuberculous lesion. *Acta medica Indonesiana* 2006;38:3–5.
27. Thijs W, Janssen K, Verhoosel RM, *et al.* Effect of vitamin D treatment on antimicrobial peptides in asthma pathients and healthy controls, Conference Abstract, European Respiratory Society, 2011.
28. Cederlund A, Olliver M, Rekha RS, *et al.* Impaired release of antimicrobial peptides into nasal fluid of hyper-IgE and CVID patients. *PLoS One* 2011;6:e29316.
29. Wang TT, Nestel FP, Bourdeau V, *et al.* Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol* 2004;173:2909–12.
30. Olsen K, Falch BM, Danielsen K, *et al.* Staphylococcus aureus nasal carriage is associated with serum 25-hydroxyvitamin D levels, gender and smoking status. The Tromso Staph and Skin Study. *Eur J Clin Microbiol Infect Dis* 2012;31:465–73.
31. Khoo AL, Chai LY, Koenen HJ, *et al.* 1,25-Dihydroxyvitamin D3 modulates cytokine production induced by Candida albicans: impact of seasonal variation of immune responses. *J Infect Dis* 2011;203:122–30.
32. Beard JA, Bearden A, Striker R. Vitamin D and the anti-viral state. *J Clin Virol* 2011;50:194–200.
33. Hansdottir S, Monick MM, Lovan N, *et al.* Vitamin D decreases respiratory syncytial virus induction of NF-kappaB-linked chemokines and cytokines in airway epithelium while maintaining the antiviral state. *J Immunol* 2010;184:965–74.
34. Sabetta JR, DePetrillo P, Cipriani RJ, *et al.* Serum 25-hydroxyvitamin d and the incidence of acute viral respiratory tract infections in healthy adults. *PLoS One* 2010;5:e11088.

IV

The role of vitamin D in pediatric hematopoietic stem cell transplantation

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Abstract

The importance of vitamin D in immunologic processes has recently emerged, but the role of vitamin D in allogeneic hematopoietic stem cell transplantation (SCT) is not determined. Reports indicate that SCT recipients, particularly children, often suffer from vitamin D deficiency. In addition, others have suggested that adequate levels of vitamin D reduce graft-versus-host disease (GvHD). Here we have investigated the role of vitamin D in children (n=123) undergoing SCT in the years 2004-2011. Vitamin D (i.e., serum calcidiol) was analyzed in prospectively collected cryostored samples. Patients were grouped according to pre-SCT calcidiol level: insufficient (<50 nM, n=38) and sufficient (≥50 nM, n=85). Older children, those transplanted during quarter 1-2 and children with Middle-Eastern or African origin were more commonly found in the insufficient group. Acute GvHD grade II-IV occurred more frequently in the vitamin D sufficient group (47% vs. 30%, p=0.05). No significant difference between the two groups was seen with regard to chronic GvHD. When comparing patients with moderate to severe cGvHD to those without cGvHD, there was a significant difference in calcidiol levels at 6 months post SCT (23nmol/L vs 37 nmol/L, p=0.004). The neutrophil granulocytes rose significantly faster in the vitamin D sufficient group. No differences in lymphocyte counts or infectious disease burden during the first year post-SCT were observed. The overall survival (OS) was comparable between the groups. However, when only comparing patients with malignancies, OS was significantly better in the vitamin D sufficient group (97% vs. 50%, p=0.001). Additionally, rejection (0% vs. 11%, p=0.06) and relapse (4% vs. 33%, p=0.03) rates were more favorable for these patients. To conclude, vitamin D might have a role in pediatric SCT by virtue of its immunomodulatory activity. However, since observational studies may be flawed by confounding factors, randomized and controlled trials on the role of vitamin D in pediatric SCT are highly warranted.

Keywords: bone marrow transplantation, micronutrient, 25-OH-vitamin D

Introduction

Over the years, numerous reports have concluded that vitamin D has biological effects far beyond its hormonal activity in calcium homeostasis, including a role in both innate and adaptive immune responses (1, 2). Observational studies have demonstrated an association between low serum levels of vitamin D and an increased risk of infections, such as tuberculosis and respiratory tract infections (3, 4).

In the context of allogeneic hematopoietic stem cell transplantation (SCT) vitamin D has been suggested to prevent graft-versus-host disease (GvHD). Vitamin D exposure resulted in immature dendritic cell populations with bias towards tolerizing rather than stimulatory T-lymphocyte populations, which could provide a possible mechanism for the beneficial effects against GvHD (5, 6). Humans attain vitamin D mainly from exposure to sunlight and to lesser extent from dietary sources. Following SCT, children have decreased exposure to direct sunlight and they might also suffer from poor nutrition and malabsorption due to SCT-related side-effects. An increased catabolism due to the use of glucocorticoids and other immunosuppressants could also affect the vitamin D levels in serum (7). Low vitamin D levels in children undergoing SCT have recently been reported, but these data were not related to the clinical outcome (8). We believe that the role of vitamin D in the context of pediatric SCT remains elusive. Therefore, we designed a study with the aim of relating the clinical outcome in pediatric SCT with baseline vitamin D levels.

Material and methods

Patients

Between June 2004 and December 2011 163 pediatric patients underwent SCT at our center at the Karolinska University Hospital, Huddinge. Of these 123 were included in this study (exclusion due to lack of follow-up data). The majority of the patients had hematologic malignancies, but patients with

non-malignant conditions (i.e. benign hematologic, metabolic, and primary immunodeficiency disorders) were also included (**Table 1**). The population was 67% male and the ethnic composition was 75% European, 7% African, 15% Middle Eastern and 3% of other ethnic groups. The study was approved by the Regional Ethical Review Board in Stockholm.

SCT procedure

The SCT procedure and conditioning regimens have been published previously (9), and treatment data are presented in **Table 1**. Myeloablative conditioning was frequently applied (n=82, 67%). In most patients cyclosporine A (CsA) and MTX (n=81, 66%) were used as prophylaxis against GvHD. The remaining patients received CsA and prednisolone (n=17, 14%), tacrolimus and sirolimus (n=21, 17%) or other combinations (n=4, 3.2%). Anti-thymocyte globulin (ATG) serotherapy was applied when the stem cell source was an unrelated donor and to all patients with non-malignant disorders (n=96, 78%). Cotrimoxazole was used as *P. jiroveci* prophylaxis, in most cases 3 days per week during the first 6 months post-SCT. In patients who developed hypogammaglobulinemia (i.e., plasma IgG <4 g/L) post-SCT, IgG substitution was applied as previously described (10).

Data collection

Data on each patient were obtained from medical records and patient databases. The collected data includes basic parameters (e.g. sex, age at transplantation, ethnicity etc.), transplant characteristics (e.g. diagnosis, conditioning, GvHD prophylaxis, donor source, and cell dose etc.) and outcome parameters (e.g. survival rate, rejection, relapse, number of infections and GvHD). Data on vitamin D substitution (daily doses and length of treatment), immunoglobulin substitution (number of treatments) and total parenteral nutrition (length of treatment) were extracted from medication administration records. To verify the reproducibility of the abstracted data, 10 cases were selected randomly and reabstracted. Complete agreement between the two abstractions was confirmed.

Outcome data

Vitamin D insufficiency was defined as a serum level of <50 nmol/L of 25-OH-vitamin D (calcidiol) at baseline (11).

The endpoints in this study were: ´presence of acute GvHD´ (aGvHD; graded 1-4 following international criteria (12)), ´presence of chronic GvHD´ (cGvHD; defined by international criteria (13)), ´immune recovery´ (cell counts and measurements of immunoglobulins), ´presence of infections´ (as explained below), ´overall survival´ (OS), ´rejection´ (defined as progressive mixed chimerism reaching >95 % recipient-derived cells in the lineage of interest) and ´relapse´ (defined as increasing minimal residual disease reaching diagnostic criteria).

Infections were recorded if they were registered in the patient's medical record by a physician. Bacterial and fungal infections required positive cultures to be recorded. Viral infections required positive diagnostics with the exception of those with a typical clinical picture (e.g. "winter vomiting disease", a calici virus gastroenteritis). Documented infections that could not be classified as bacterial, viral or fungal were denominated as ´other infections´. Immune recovery was assessed by determining full blood counts and immunoglobulin levels.

Clinical chemistry

Full blood counts and immunoglobulin levels were analyzed routinely for clinical purposes and values were obtained from the patient medical records. Serum samples were collected prospectively pre- and post-SCT (+3, 6 and 12 months) and were cryostored for analysis of calcidiol by a chemiluminescence assay. All laboratory procedures were carried out by accredited methodology at the Department of Clinical Chemistry, Karolinska University Laboratory, Karolinska University Hospital.

Statistics

Continuous variables and proportions were compared using the Mann-Whitney test or the Kruskal-Wallis test and the Fischer's exact test or the χ^2 test, respectively. GvHD, relapse and rejection were estimated using an estimator of cumulative incidence curves, taking competing events into consideration (14, 15). The Cox regression method was used in the predictive analysis for OS and RFS while the method by Fine & Gray was used for predictive analysis for GvHD and relapse. Statistical significance was set at p <0.05. Analysis was performed using the cmprsk software package, S-Plus 6.2 software and Statistica software.

Results

Baseline vitamin D levels and demographics

Patients were divided into two cohorts based on calcidiol level at baseline (i.e. pre-SCT); ´D-low ´ (<50 nmol/L) and ´D-sufficient´ (≥ 50 nmol/L). Clinical outcomes were followed for up to eight years post-SCT. The mean calcidiol level at baseline was 33 nmol/L (range 13-49) in the low level group and 63 nmol/L (range 50-97) in the sufficient level group (P<0.001). Calcidol levels remained significantly higher in the sufficient level group during at least 6 months follow-up post-SCT.

Patients in the ´D-low´ group were older than patients in the ´D-sufficient´ group (mean 10 vs. 5 years with interlacing ranges, P=0.025). There were more patients with myelodysplastic syndrome (MDS) among patients with sufficient calcidiol levels (P<0.001). No differences in other characteristics were seen between the groups at baseline (**Table 1**). The season of transplantation had a significant impact on calcidiol levels, patients transplanted in the first 6 months of the year had significantly lower levels than patients transplanted in the last 6 months (P=0.002, **Figure 1**).

The distribution of ethnic origins differed between groups. Patients with African and Middle Eastern origin were most likely to display low calcidiol levels (100% and 89%, respectively), whereas patients

with European and other origins displayed a more even distribution (63% and 67%, respectively). The differences between groups were significant (European vs. African, P=0.001; European vs. Middle Eastern, P=0.001).

Baseline vitamin D levels, GvHD and immune recovery

Acute GvHD occurred in 62 patients, corresponding to 50% of the patients, out of which 43 patients (35%) suffered from moderate to severe aGvHD (i.e. grade II-IV). In the ´D-sufficient´ group at baseline, moderate to severe aGvHD was more frequently occurring, compared to the ´D-low´ group (47% vs. 30%, p=0.05; **Figure 2A**).

In multivariate analysis of factors affecting aGvHD, disease stage was significant while level of calcidiol at baseline was borderline significant (**Table 2**).

Chronic GvHD occurred in 17 patients (14%), with no significant difference between the ´D-low´ and ´D-sufficient´ group. However, when comparing patients with moderate to severe cGvHD to those without cGVHD, there was a significant difference in calcidiol levels at 6 months post SCT (23 nmol/L, range 18-24 vs. 37 nmol/L, range 10-80, p=0.004).

Neutrophil granulocytes (measured as absolute neutrophil count) rose significantly faster during the first 3 months post-SCT among patients with sufficient calcidiol levels at baseline. During the next 9 months no significant difference was observed (**Figure 2B**).

When comparing patients with the highest baseline levels of calcidiol (> 75 nmol/L) to the others, significantly lower baseline IgG levels at SCT were observed (IgG 5.1 g/L, range 1.6-8.3 vs. IgG 8.3 g/L, range 1.4-19 g/L, p=0.01). This difference remained when the group with calcidiol levels > 75 nmol/L was compared to patients with calcidiol levels 50-74 nmol/L (IgG 5.1 g/L, range 1.6-8.3 vs. IgG 8.6 g/L, range 2.3-15 g/L, p=0.005) and to patients with calcidiol levels < 50 nmol/L (IgG 5.1 g/L, range 1.6-8.3 vs. IgG 7.9 g/L, range 1.4-19 g/L, p=0.03) (**Figure 2C**). The difference in IgG levels did not remain over time post-SCT. IgG substitution was more common in the ´D-sufficient group´ (76% vs. 56%, p=0.04).

Baseline vitamin D and infections

The number of observed clinical infections did not differ significantly between the two groups. In both groups most patients suffered from 1-4 infections during the first year post-SCT. Data on specific viral infections (HSV, CMV and VZV) did not show significant differences other than a lower incidence of HSV and VZV at 3 months post transplantation in the ´D-sufficient´ group (35% vs. 27%, P=0.05 and 37% vs 25%, P=0.04; respectively) compared to the ´D-low´ group.

Baseline vitamin D and survival

Overall survival (OS) in all patients did not differ significantly between groups (69% vs. 87% in the ´D-low´ and ´D-sufficient´ groups, respectively). Nor did OS differ between ´D-low´ and ´D-sufficient´ patients with non-malignant diseases (87% in both groups). In patients with malignant diseases, sufficient calcidiol levels were associated with better OS compared to patients with low calcidiol levels (87% vs. 50%, p=0.01; **Figure 3A**).

There was no difference in transplant related mortality (TRM) between the groups. In a multivariate analysis factors affecting OS were baseline calcidiol levels, unrelated donor, and patients with MDS (**Table 2**). Graft rejection did not occur among patients in the sufficient level group but among 11% of patients in the low level group, though this difference was only borderline significant (p=0.06). Relapse was more common among patients in the ´D-low´ group (33% vs. 4%, p=0.03; **Figure 3B**).

Analysis by a multivariate regression model found that baseline calcidiol levels were significantly associated with relapse (**Table 2**). Relapse-free survival (RFS) was significantly higher in patients with sufficient levels of calcidiol (87% vs. 41%, p=0.002). Factors affecting RFS in the multivariate analysis were calcidiol levels at baseline and the use of an unrelated donor (**Table 2**).

Discussion

It has been known for decades that the steroid hormone vitamin D affects immunologic processes but over the years more precise mechanisms of action have been uncovered (16). Gathering evidence has shown that sufficient vitamin D levels may be beneficial for patients undergoing SCT (5, 6, 8). In the present study we have followed 123 pediatric patients for up to eight years post-SCT and recorded clinical outcomes. We have related theses outcomes to baseline vitamin D levels that have been obtained by analyzing cryopreserved serum samples. This is one of the first reports describing a correlation between vitamin D and clinical outcome parameters in pediatric SCT.

We chose to define vitamin D deficiency as baseline calcidiol (25(OH)-vitamin D) levels in serum below 50 nmol/L, based on previous reports and guidelines [11-14]. To date there is no consensus regarding sufficient levels, but several authors claim 75 nmol/L or higher to be sufficient in both children and adults (17). Most recommendations are based on studies of vitamin D and bone health; however levels for optimal influence on immunologic processes might be even higher (18). It has been observed that intestinal calcium absorption is maximized above 80 nmol/L, in postmenopausal women and that parathyroid hormone (PTH) concentrations in adults continue to decline and reach their nadir at 75-100 nmol/L (19-21). This shows an inverse relationship between vitamin D and PTH.

Young children, institutionalized patients and non-western immigrants are usually regarded as risk groups for vitamin D deficiency (22), but also up to a third of American children and adolescents are vitamin D deficient with seasonal variation (18, 23). In adult SCT patients numbers are even higher, 70-89% of adult SCT patients are vitamin D deficient prior to transplantation (24, 25). Duncan et al followed 67 pediatric SCT patients and found that 37% were deficient at baseline, but in the subgroup of patients ≥11 years 67% were deficient (8). This is in accordance with our findings (69%). SCT patients are more prone to develop vitamin D deficiency due to sun avoidance, hospitalization, corticosteroid treatment, diminished uptake from intestines affected by GvHD and bacterial overgrowth (8, 26-28). The lowest baseline levels in our study were found in patients transplanted

during winter until early summer, which is before sun exposure at the latitude in which the study took place is enough to raise serum levels. In brief, our patients with insufficient baseline levels were older, came from certain ethnic groups (African and Middle Eastern) and were transplanted during time of year when vitamin D levels tend to be lower. The groups did not differ significantly regarding SCT-parameters (i.e., conditioning, donor, stem cell source etc.). Comparable and diverging results were reported by others (8, 25, 26, 29), and might be explained by intrinsic and extrinsic study population differences. However, it is clear that certain patients are at risk for developing vitamin D deficiency, which easily can be treated.

Since GvHD is a major obstacle to successful SCT great efforts are made to understand and prevent this condition. T-lymphocyte responses, which can be modified by vitamin D, are central in the GvHD pathogenesis. Pakkala et al showed that a vitamin D analogue decreased signs of aGvHD in mice, probably by down-regulation of both T-lymphocyte activation and inflammatory effector mechanisms (30). *In vitro* studies have shown that active vitamin D dose-dependently inhibits mixed lymphocyte cultures (31) and affects dendritic cell (DC) maturation resulting in a Th2 polarized T-lymphocyte population (5). Active vitamin D also alters DC surface phenotype and morphology, which may compromise contacts between DC and T-lymphocytes and thereby diminish interaction and T-lymphocyte cytokine secretion (32). Together this would lead to tolerating rather than stimulated T-lymphocyte populations. These findings serve as a rationale for treating or preventing GvHD with vitamin D. Rosenblatt et al reported two cases of adult patients suffering from steroid refractory GvHD, where improvement occurred when vitamin D levels were corrected (5). Contradictory to this, the frequency of aGvHD was higher and moderate to severe cGvHD lower in patients with sufficient calcidiol levels in our material. These findings emphasize that the two forms of GvHD have different pathogenesis, but may also underline the fact that vitamin D has dual roles – both immune stimulatory and inhibitory. The linkage between vitamin D levels and cGvHD are supported by observations in adult SCT (6, 33). We found that calcidiol levels were significantly lower in patients with moderate to severe cGvHD at 6 months post-SCT compared to patients without

cGvHD (p=0.004). The difference can partially be explained by inferior nutritional status and lower sun exposure among the severely ill patients. Since low levels of calcidiol in this context are unfavorable, substitution with cholecaliferol may be considered.

Systemic or locally produced calcitriol (1,25(OH)2-vitamin D) affects several types of immune cells. In addition to showing inhibitory actions on parts of the adaptive immune system, active vitamin D can stimulate monocyte proliferation and enhance synthesis of the antibacterial peptide cathelicidin (1, 2, 34, 35). The effect of vitamin D on immune reconstitution after SCT is not well established. In our material neutrophil and lymphocyte counts were significantly higher first 3 months post-SCT in the ´D-sufficient´ group. This might be interpreted as a vitamin D-mediated stimulatory effect on immunity in the early phases of immune recovery. Notably, this period is when aGvHD is observed and our data suggests that higher calcidiol levels are associated with an increased frequency of aGvHD. The difference in neutrophil and lymphocyte recovery did not remain beyond the 3-month period. Calcitriol has been shown to suppress B-lymphocyte proliferation, plasma cell differentiation and IgG-secretion (1, 2), which was in accordance with our findings. Patients with the highest levels of calcidiol at baseline had the lowest IgG levels during follow-up and more commonly needed IgG replacement. A faster normalization of immune cell counts might have beneficial effect on infection susceptibility but no significant impact of this parameter was discerned in our material. One reason for this could be that our study population had relatively low baseline calcidiol levels. Effects on infection susceptibility might only occur in individuals with vitamin D levels > 75 nmol/L (6, 34, 36, 37). In addition, studies have suggested that very high vitamin D serum concentrations activate feedback systems effectively blocking the effect of vitamin D (38, 39). A cross-sectional trial from Greenland , recently published, demonstrates that both low (< 75 nmol/L) and high (> 140 nmol/L) serum concentrations were associated with an increased risk of tuberculosis (40).

Overall survival did not differ between the two groups, probably because of the good survival rate in patients with non-malignant disease. However, in patients transplanted due to malignancies OS was

significantly higher in the ´D-sufficient´ group. Notably, another study on adult patients could not detect significant difference with regard to OS and baseline vitamin D levels (33). Also relapse was significantly more common in the ´D-low´ group suggesting that low levels of vitamin D is a risk factor, which should be taken into account in the transplant setting. Since sufficient levels of vitamin D appears to be associated with the triad of better OS, less risk of relapse and more events of aGvHD it can be postulated that vitamin D has immune stimulatory effects mediating expansion of alloreactive cells including those with a capacity of killing malignant cells (i.e., graft-vs.-leukemia reaction). An alternative explanation to the association between vitamin D levels and OS might be that calcidiol levels constitute a surrogate marker for patient wellbeing and performance status at start of SCT (41). However, in multivariate analysis calcidiol levels remained significantly associated to OS, which supports a true association. Rejection did only occur among patients in the ´D-low´ group in our material. This suggests a beneficial role of vitamin D in allograft survival. In analogy with the hypothesis of OS and relapse, higher vitamin D levels might stimulate expansion of alloreactive cells killing recipient cells giving rise to full donor chimerism development. Clinical relevant immunomodulatory actions of vitamin D seems to request higher levels of active vitamin D (31). These findings merit further experimental studies as well as clinical trials in larger patient cohorts.

This study is based on a large pediatric cohort with a high degree of available data. We conclude that baseline vitamin D levels seem to have a role in the clinical course of children undergoing SCT. Vitamin D deficiency was associated with an increased risk of death, relapse and cGvHD. Higher calcidiol levels were not always beneficial, since aGvHD occurred more frequently among patients with sufficient baseline levels of vitamin D. There were no clear effects on immune reconstitution and risk of infection. Since this study is a retrospective and observational study, no causality can be inferred by these associations. Thus, further studies are needed to confirm our findings and to establish whether vitamin D could impact immunologic processes in SCT and the subsequent clinical outcome.

References

1. Di Rosa M, Malaguarnera M, Nicoletti F, Malaguarnera L. Vitamin D3: a helpful immuno-modulator. Immunology. 2011 Oct;134(2):123-39. PubMed PMID: 21896008. Pubmed Central PMCID: 3194221.
2. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nature reviews Immunology. 2008 Sep;8(9):685-98. PubMed PMID: 19172691. Pubmed Central PMCID: 2906676.
3. Khoo AL, Chai L, Koenen H, Joosten I, Netea M, van der Ven A. Translating the role of vitamin D3 in infectious diseases. Critical reviews in microbiology. 2012 May;38(2):122-35. PubMed PMID: 22304022.
4. Ginde AA, Mansbach JM, Camargo CA, Jr. Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. Archives of internal medicine. 2009 Feb 23;169(4):384-90. PubMed PMID: 19237723. Pubmed Central PMCID: 3447082.
5. Rosenblatt J, Bissonnette A, Ahmad R, Wu Z, Vasir B, Stevenson K, et al. Immunomodulatory effects of vitamin D: implications for GVHD. Bone marrow transplantation. 2010 Sep;45(9):1463-8. PubMed PMID: 20081878.
6. Silva F, Perez-Simon JA, Caballero-Velazquez T, Sanchez-Guijo FM, Villanueva-Gomez F, Vazquez L, et al. Effect of vitamin D treatment in chronic GVHD. Bone marrow transplantation. 2011 Oct;46(10):1395-7. PubMed PMID: 21243033.
7. Sato T, Fukagawa M, Uchida K, Katayama A, Nagasaka T, Matsuoka S, et al. 1,25-dihydroxyvitamin D synthesis after renal transplantation: the role of fibroblast growth factor 23 and cyclosporine. Clinical transplantation. 2009 Jun-Jul;23(3):368-74. PubMed PMID: 19519602.
8. Duncan CN, Vrooman L, Apfelbaum EM, Whitley K, Bechard L, Lehmann LE. 25-hydroxy vitamin D deficiency following pediatric hematopoietic stem cell transplant. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2011 May;17(5):749-53. PubMed PMID: 20951818.
9. Remberger M, Ackefors M, Berglund S, Blennow O, Dahllof G, Dlugosz A, et al. Improved survival after allogeneic hematopoietic stem cell transplantation in recent years. A single-center study. Biol Blood Marrow Transplant. 2011 Nov;17(11):1688-97. PubMed PMID: 21620989. Epub 2011/05/31. eng.
10. Sundin M, Nordin K, Jostemyr Y, Winiarski J. Subcutaneous IgG replacement after pediatric SCT. Pediatric transplantation. 2012 Dec;16(8):866-71. PubMed PMID: 22931490.
11. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism. 2011 Jul;96(7):1911-30. PubMed PMID: 21646368.
12. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone marrow transplantation. 1995 Jun;15(6):825-8. PubMed PMID: 7581076.
13. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. Biol Blood Marrow Transplant. 2005 Dec;11(12):945-56. PubMed PMID: 16338616. Epub 2005/12/13. eng.
14. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med. 1999 Mar 30;18(6):695-706. PubMed PMID: 10204198. Epub 1999/04/16. eng.
15. Kalbfleisch JD, Prentice RL. The statistical analysis of failure time data. Second edition ed. Hoboken, NJ: John Wiley & Sons, Inc.; 2002.

16. Hodler B, Evequoz V, Trechsel U, Fleisch H, Stadler B. Influence of vitamin D3 metabolites on the production of interleukins 1,2 and 3. Immunobiology. 1985 Nov;170(4):256-69. PubMed PMID: 3002965.
17. Holick MF. Vitamin D deficiency. The New England journal of medicine. 2007 Jul 19;357(3):266-81. PubMed PMID: 17634462.
18. Holick MF. The D-lightful vitamin D for child health. JPEN J Parenter Enteral Nutr. 2012 Jan;36(1 Suppl):9S-19S. PubMed PMID: 22179524. Epub 2011/12/20. eng.
19. Heaney RP, Dowell MS, Hale CA, Bendich A. Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. Journal of the American College of Nutrition. 2003 Apr;22(2):142-6. PubMed PMID: 12672710.
20. Chapuy MC, Preziosi P, Maamer M, Arnaud S, Galan P, Hercberg S, et al. Prevalence of vitamin D insufficiency in an adult normal population. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 1997;7(5):439-43. PubMed PMID: 9425501.
21. Holick MF, Siris ES, Binkley N, Beard MK, Khan A, Katzer JT, et al. Prevalence of Vitamin D inadequacy among postmenopausal North American women receiving osteoporosis therapy. The Journal of clinical endocrinology and metabolism. 2005 Jun;90(6):3215-24. PubMed PMID: 15797954.
22. van Schoor NM, Lips P. Worldwide vitamin D status. Best Pract Res Clin Endocrinol Metab. 2011 Aug;25(4):671-80. PubMed PMID: 21872807. Epub 2011/08/30. eng.
23. Rajakumar K, Holick MF, Jeong K, Moore CG, Chen TC, Olabopo F, et al. Impact of season and diet on vitamin D status of African American and Caucasian children. Clin Pediatr (Phila). 2011 Jun;50(6):493-502. PubMed PMID: 21565884. Pubmed Central PMCID: 3296802. Epub 2011/05/14. eng.
24. Joseph RW, Alousi A, Konda B, Komanduri K, Neumann J, Trevino C, et al. High incidence of vitamin D deficiency in patients undergoing allogeneic stem cell transplantation. American journal of hematology. 2011 Nov;86(11):954-6. PubMed PMID: 21948087.
25. Urbain P, Ihorst G, Biesalski HK, Bertz H. Course of serum 25-hydroxyvitamin D(3) status and its influencing factors in adults undergoing allogeneic hematopoietic cell transplantation. Annals of hematology. 2012 May;91(5):759-66. PubMed PMID: 22080146.
26. Robien K, Strayer LG, Majhail N, Lazovich D, Baker KS, Smith AR, et al. Vitamin D status among long-term survivors of hematopoietic cell transplantation. Bone marrow transplantation. 2011 Nov;46(11):1472-9. PubMed PMID: 21243026. Pubmed Central PMCID: 3131431.
27. El-Hajj Fuleihan G, Muwakkit S, Arabi A, Daouk LE, Ghalayini T, Chaiban J, et al. Predictors of bone loss in childhood hematologic malignancies: a prospective study. Osteoporos Int. 2012 Feb;23(2):665-74. PubMed PMID: 21476039. Epub 2011/04/09. eng.
28. Sproat L, Bolwell B, Rybicki L, Dean R, Sobecks R, Pohlman B, et al. Vitamin D level after allogeneic hematopoietic stem cell transplant. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2011 Jul;17(7):1079-83. PubMed PMID: 21193053.
29. Simmons JH, Chow EJ, Koehler E, Esbenshade A, Smith LA, Sanders J, et al. Significant 25-hydroxyvitamin D deficiency in child and adolescent survivors of acute lymphoblastic leukemia: treatment with chemotherapy compared with allogeneic stem cell transplant. Pediatric blood & cancer. 2011 Jul 1;56(7):1114-9. PubMed PMID: 21488156. Pubmed Central PMCID: 3135735.
30. Pakkala I, Taskinen E, Pakkala S, Raisanen-Sokolowski A. MC1288, a vitamin D analog, prevents acute graft-versus-host disease in rat bone marrow transplantation. Bone marrow transplantation. 2001 Apr;27(8):863-7. PubMed PMID: 11477445.
31. Vanham G, Van Baelen H, Tan BK, Bouillon R. The effect of vitamin D analogs and of vitamin D-binding protein on lymphocyte proliferation. Journal of steroid biochemistry. 1988 Apr;29(4):381-6. PubMed PMID: 3374126.
32. Ferreira GB, Overbergh L, Verstuyf A, Mathieu C. 1alpha,25-Dihydroxyvitamin D(3) and its analogs as modulators of human dendritic cells: A comparison dose-titration study. The Journal of steroid biochemistry and molecular biology. 2012 Oct 23. PubMed PMID: 23098690.

33. Glotzbecker B, Ho VT, Aldridge J, Kim HT, Horowitz G, Ritz J, et al. Low levels of 25-hydroxyvitamin D before allogeneic hematopoietic SCT correlate with the development of chronic GVHD. Bone marrow transplantation. 2013 Apr;48(4):593-7. PubMed PMID: 23000654.
34. Schwalfenberg GK. A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. Mol Nutr Food Res. 2011 Jan;55(1):96-108. PubMed PMID: 20824663. Epub 2010/09/09. eng.
35. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. Journal of immunology. 2004 Sep 1;173(5):2909-12. PubMed PMID: 15322146.
36. Lehouck A, Mathieu C, Carremans C, Baeke F, Verhaegen J, Van Eldere J, et al. High doses of vitamin D to reduce exacerbations in chronic obstructive pulmonary disease: a randomized trial. Annals of internal medicine. 2012 Jan 17;156(2):105-14. PubMed PMID: 22250141.
37. Sabetta JR, DePetrillo P, Cipriani RJ, Smardin J, Burns LA, Landry ML. Serum 25-hydroxyvitamin d and the incidence of acute viral respiratory tract infections in healthy adults. PloS one. 2010;5(6):e11088. PubMed PMID: 20559424. Pubmed Central PMCID: 2885414.
38. Henry HL. Regulation of vitamin D metabolism. Best practice & research Clinical endocrinology & metabolism. 2011 Aug;25(4):531-41. PubMed PMID: 21872796.
39. Hollis BW, Conrad HR, Hibbs JW. Changes in plasma 25-hydroxycholecalciferol and selected blood parameters after injection of massive doses of cholecalciferol or 25-hydroxycholecalciferol in non-lactating dairy cows. The Journal of nutrition. 1977 Apr;107(4):606-13. PubMed PMID: 191582.
40. Nielsen NO, Skifte T, Andersson M, Wohlfahrt J, Soborg B, Koch A, et al. Both high and low serum vitamin D concentrations are associated with tuberculosis: a case-control study in Greenland. The British journal of nutrition. 2010 Nov;104(10):1487-91. PubMed PMID: 20553638.
41. Shrank WH, Patrick AR, Brookhart MA. Healthy user and related biases in observational studies of preventive interventions: a primer for physicians. Journal of general internal medicine. 2011 May;26(5):546-50. PubMed PMID: 21203857. Pubmed Central PMCID: 3077477.

Table 1 Patient characteristics (n=123)

Characteristic	Calcidiol at baseline <50 nM (n=85)	≥50 nM (n=38)	p
Age (years)	10 (0-19)	5 (0-15)	0.03
Sex (male/female)	57 (67%)/28 (33%)	26 (68%)/12 (32%)	
BMI	18.5 (12.0-45.1)	17.0 (13.1-26.7)	
Ethnic origin			
European	57 (67%)	34 (90%)	
African	9 (11%)	0	
Middle Eastern	16 (19%)	3 (8%)	
Other	3 (4%)	1 (3%)	
Diagnosis			
Non malignant	42 (49%)	15 (39%)	
AML/ALL	8/22 (9%/26%)	2/6 (5%/16%)	
CML	3 (4%)	0	
Lymphoma	3 (4%)	0	
MDS	7 (8%)	13 (34%)	<0.001
Other	0	2 (5%)	
Stage (early/late)	58/27	20/18	
Conditioning			
fTBI + Cy	26 (31%)	7 (18%)	
Bu + Cy	30 (35%)	19 (50%)	
Flu + Bu	4 (5%)	1 (3%)	
Flu + Cy	7 (8%)	7 (18%)	
Flu + fTBI + Cy	4 (5%)	1 (3%)	
Flu + Treo	16 (19%)	3 (8%)	
ATG	67 (79%)	29 (76%)	
Donor			
Age (years)	22 (0-55)	22 (0-46)	
Female to Male	20 (24%)	8 (21%)	
MRD	29 (34%)	14 (37%)	
MUD	38 (45%)	19 (50%)	
MM	18 (21%)	5 (13%)	
Stem Cell Source			
Bone marrow	55 (65%)	29 (76%)	
PBSC	16 (19%)	5 (13%)	
Cord blood	14 (16%)	4 (11%)	
Cell dose			
Nucleated cells, $\times 10^8$/kg	4.1 (0.3-37)	5.2 (0.4-34)	
CD34 cells, $\times 10^6$/kg	4.8 (0.1-44)	5.5 (0.1-15)	

GvHD prophylaxis		
CsA + MTX	52 (61%)	29 (76%)
CsA + prednisolone	12 (14%)	5 (13%)
Tacrolimus + sirolimus	18 (21%)	3 (8%)
Other	3 (4%)	1 (3%)
Acute GvHD		
0	46 (54%)	15 (39%)
I	14 (16%)	5 (13%)
II-IV	25 (29%)	18 (47%)
Chronic GvHD (Y/N)	11/73	6/32
IgG replacement	48 (56%)	29 (76%)

Table 1. Abbreviations: BMI denotes body mass index; AML, acute myeloid leukemia; ALL, acute lymphatic leukemia; CLL, chronic lymphatic leukemia; MDS, myelodysplastic syndrome; fTBI, fractionated total body irradiation; Cy, cyclophosphamide; Bu, busulphan; Flu, fludarabine; Treo, treosulphan; ATG, anti-thymocyte globuline serotherapy; MRD, human leukocyte antigen (HLA) matched related donor; MUD, HLA matched unrelated donor; MM, HLA mismatched donor; PBSC, peripheral blood stem cells; GvHD, graft-versus-host disease; CsA, cyclosporine A; MTX, methotrexate; IgG, immunoglobulin G.

Figure 1

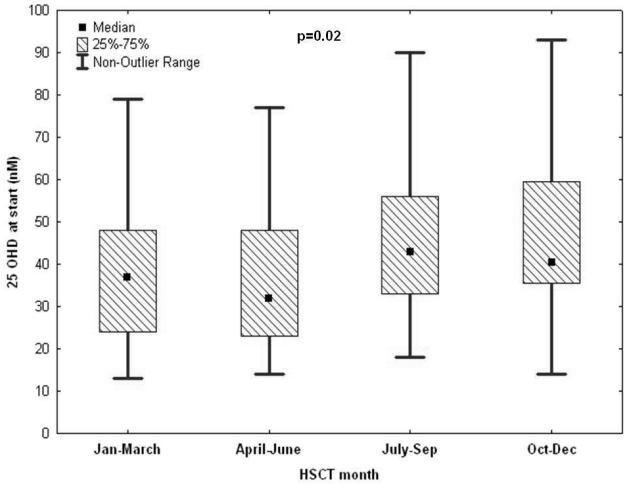

***Figure 1*:** Time of transplantation had significant impact on levels of calcidiol at baseline. The p–value denotes a comparision of all four groups with Kruskal-Wallis test.

Table 2 **Multivariate analysis* of factors associated to acute GvHD, mortality, relapse and relapse-free survival (RFS).**

aGvHD grade II-IV	**HR**	**95% CI**	**P**
calcidiol >50 nM	1.72	0.96-3.13	0.065
High risk disease stage	2.59	1.42-4.71	0.002
Mortality	**HR**	**95% CI**	**P**
calcidiol >50 nM	0.15	0.04-0.57	0.005
URD	3.77	1.11-12.8	0.03
MDS	2.93	1.09-7.88	0.03
Relapse	**HR**	**95% CI**	**P**
calcidiol >50 nM	0.08	0.01-0.63	0.02
Relapse-Free Survival	**HR**	**95% CI**	**P**
calcidiol >50 nM	0.14	0.04-0.50	0.002
URD	3.58	1.23-10.4	0.02

Table 2. Abbreviations: *, corrected for differences in characteristics (age, stage and MDS); aGvHD; acute graft-versus-host disease, RFS; relapse-free survival, HR; hazards ratio, 95% CI; 95% confidence interval, URD; unrelated donor, High risk; beyond CR (complete remisson) 1/CP (chronic phase) 1, MDS; myelodysplastic syndrome.

Figure 2A

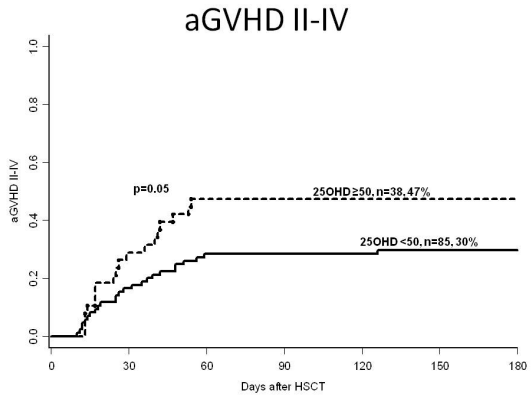

Figure 2B

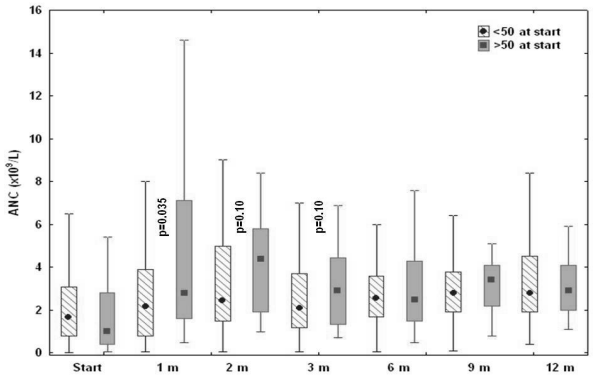

Figure 2C

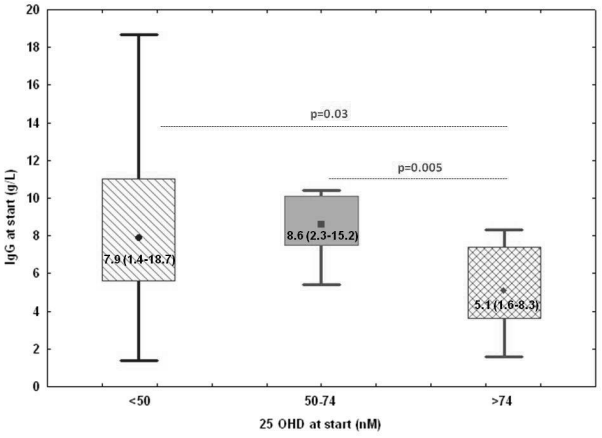

Figure 2: (A) Moderate to severe aGvHD occurred more commonly among patients with sufficient levels of calcidiol at baseline. (B) Absolute neutrophil count rose faster during the first 3 months post-SCT among patients with sufficient levels of calcidiol at baseline. (C) Levels of IgG were significantly lower in a group with calcidiol above 74 nM at baseline compared to other groups.

Figure 3A

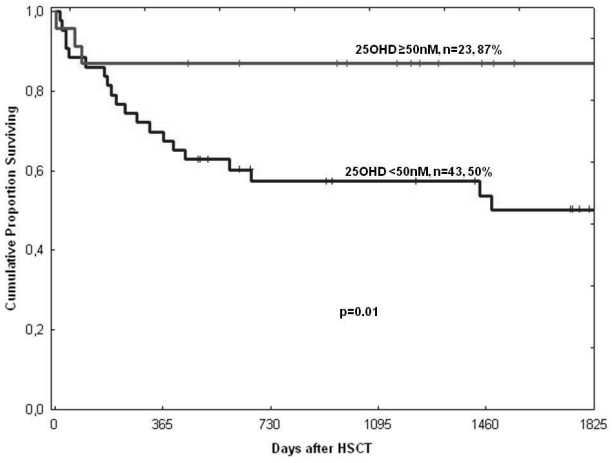

Figure 3B

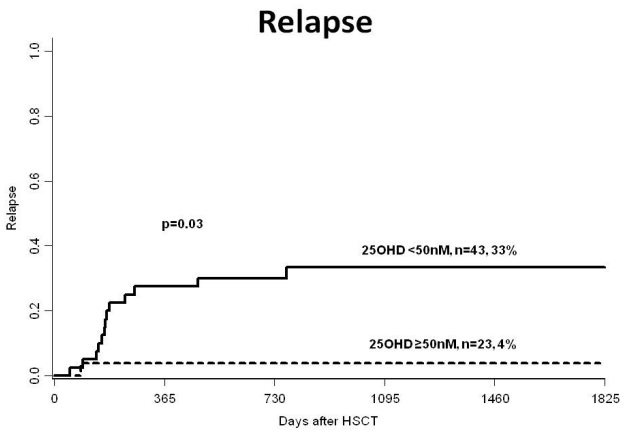

Figure 3: (A) In patients with malignant diseases sufficient calcidiol levels at baseline were associated with greater overall survival. (B) Relapse was less common among patients with sufficient levels of calcidiol at baseline.