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**CONDITIONING ASSOCIATED
EFFECTS ON ORAL MUCOSA AND
SALIVARY FUNCTION IN
ALLOGENEIC HEMATOPOIETIC
STEM CELL RECIPIENTS**

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To all the allogeneic HSCT patients who have been part of this study—people struggling to fight their disease; and while standing on the edge of medical science, they were able to smile and show unbelievable courage.

Courage that could not help but inspire us all.

ABSTRACT

Allogeneic hematopoietic stem cell transplantation (HSCT) is an effective treatment for patients with a range of disorders of the immunohematopoietic system. In HSCT recipients, acute complications in the oral cavity are common. They are related to the disease itself, its treatment, the pre-transplant status of the oral cavity, and nutritional problems during the neutropenic phase. As the results of treatment improve and survival rates increase, not only cure of the disease but also a variety of delayed side effects become apparent that make the compromised patient require special oral care. In order to improve the patient's quality of life, this problem must be solved. The aim of this thesis was to investigate conditioning related effects on salivary function and on the oral mucosa, and also the impact of other risk factors for salivary dysfunction and oral mucositis in allogeneic HSCT recipients.

In patients conditioned with fractionated total body irradiation (fTBI) or single-dose total body irradiation (sTBI), we found that fTBI resulted in less reduction of salivary secretion rate one year after HSCT than sTBI, despite the higher total dose of radiation. In addition, we found that risk factors for a low stimulated salivary secretion rate (SSSR) one year after HSCT were sTBI and seropositivity of recipients for 3–4 herpes viruses. A cumulative increase in risk factors resulted in less salivary output.

We found no difference in long-term salivary function after HSCT in 15-year-olds who received conditioning with sTBI, fTBI, or busulfan (Bu). There was a negative correlation between age at conditioning with sTBI and salivary function. This correlation was not seen using fTBI or Bu. We also found a negative correlation between total systemic exposure to Bu and the SSSR. Female sex was a risk factor for salivary dysfunction at 15 years of age after HSCT.

Comparing myeloablative conditioning (MAC) to reduced intensity conditioning (RIC), we found that MAC was associated with a higher prevalence of oral mucositis (OM). Severe OM prolonged hospitalization. The introduction of a new intensive oral hygiene protocol was associated with lower OM score and the two scoring systems used for grading of OM showed good correlation. We also identified several risk factors for OM. In multivariate analysis, all donor-recipient gender combinations except the female-donor-male-recipient situation and year of transplantation—especially before the year 2011 when oral care was intensified—was significantly associated with a higher OM score.

In HSCT recipients, we found no difference in volume of gingival crevicular fluid (GCF) before, during, or after HSCT. When monitoring pro-inflammatory cytokines, we observed that cytokines are activated in the GCF. Patients conditioned with MAC or RIC had different patterns of pro-inflammatory cytokines, both in GCF and serum. There was a correlation between oral mucositis and an increase in IL-6 in the serum. Finally, we found no correlations between GCF and serum levels of cytokines at any time point.

In conclusion, both acute and long-term oral side effects are common after allogeneic HSCT. There is a lack of comprehensive and effective oral management regimens. By developing evidence-based recommendations that might improve the appropriateness of clinical practice, the acute oral complications might be reduced,

patient outcomes would be better, and cost-effectiveness and quality of life would improve.

Our findings also suggest that it is necessary to have long-term follow-up after allogeneic stem cell transplantation because some children will have permanently reduced salivary function. These people may require additional preventive measures throughout their lives in order to maintain proper oral health.

LIST OF PUBLICATIONS

- I. **Garming Legert K**, Remberger M, Ringdén O, Heimdahl A, Dahllöf G. Salivary secretion in children after fractionated or single dose TBI. *Bone Marrow Transplant* 2011 May 9 [Epub ahead of print]
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LIST OF ABBREVIATIONS

aGVHD	Acute graft-versus-host disease
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
ALL	Acute lymphoblastic leukemia
ATG	Anti-thymocytglobuline
AUC	Area under the plasma concentration time curve
BM	Bone marrow
Bu	Busulfan
cGVHD	Chronic graft-versus-host disease
CB	Cord blood
CML	Chronic myeloid leukaemia
CLL	Chronic lymphatic leukaemia
CMV	Cytomegalovirus
CR	Complete remission
CsA	Cyclosporine
Cy	Cyclophosphamide
DLI	Donor lymphocyte infusion
EBV	Epstein-Barr virus
fTBI	Fractionated total body irradiation
Flu	Fludarabin
GCF	Gingival crevicular fluid
G-CSF	Granulocyte colony stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia effect
Gy	Gray
HSV	Herpes simplex virus
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
HSCs	Hematopoietic stem cells
IL-1 β	Interleukin 1-Beta
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-10	Interleukin 10
LFS	Leukemia free survival
MAC	Myeloablative conditioning
MDS	Myelodysplastic syndrome
MTX	Methotrexate
MUD	Matched unrelated donor
NF- κ B	Nucleor factor kappa-light-chain-enhancer of activated B cells
NCI-CTCAE	NCI - Common Terminology Criteria For Adverse Events
OM	Oral mucositis
OMAS	Oral mucositis assessment scale
PBSC	Peripheral blood stem cells
RIC	Reduced intensity conditioning

ROS	Reactive oxygen species
SAA	Severe aplastic anemia
SSSR	Stimulated salivary secretion rate
sTBI	Single dose total body irradiation
TBI	Total body irradiation
TNF- α	Tumor necrosis factor alfa
TRM	Transplantation related mortality
USSR	Unstimulated salivary secretion rate
VZV	Varicella zoster virus
VAS	Visible analogue scale
WHO	World health organization

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is an effective and well-established treatment for patients with a range of disorders of the immunohematopoietic system. During recent decades, the survival rate has steadily improved and what was earlier an experimental therapy in patients with end-stage leukemia is currently the treatment of choice for many patients with severe hematological diseases and immunological deficiencies.

When treatment results improve and survival rates increase, not only cure of the disease but also a variety of delayed side effects become apparent. Acute complications due to the treatment and long-term side effects because of impaired immune defense are still a major problem to be solved in order to improve the patient's quality of life.¹⁻²

The studies presented here focus on both acute and long-term oral complications in recipients with malignant disorders treated with various conditioning regimens in preparation for HSCT, and also children who have survived for a long time after undergoing different conditioning regimens.

Allogeneic hematopoietic stem cell transplantation

HSCT is the transplantation of multipotent hematopoietic stem cells (HSCs) derived from bone marrow, peripheral blood, or umbilical cord blood from a donor. Allogeneic HSC donors must have a tissue human leukocyte antigen (HLA) type that is compatible with the patient. Even if there is a good match, the patient will require immunosuppressive medications to prevent graft-versus-host disease (GVHD). Immediately before transplantation, the patient is conditioned with chemotherapy, irradiation, or both, and also with immunosuppressive therapy. Several weeks after engraftment in the bone marrow, expansion of HSCs and their progeny is sufficient to normalize the blood cell counts and reinitiate the immune system.³

One of the first attempts to transplant bone marrow was described as early as 1891 by Brown-Sequard and d'Arsonval, who treated a leukemic patient by oral administration of bone marrow.⁴ Over the years, other forms of administration were tried.

The first intravenous infusion of bone marrow was performed by Thomas et al. in 1957, in a patient with end-stage leukemia, where there was evidence of engraftment of the transplanted bone marrow.⁵ The real breakthrough came, though, with the discovery of the human leukocyte antigen (HLA) that enabled matching between donors and recipients⁶. The first successful HSCTs were performed 1968 in patients with severe combined immunodeficiency disorders, using HLA-identical donors.⁷⁻⁸ The success of HSCT was limited in the 1960s because of GVHD, and attempts to prevent GVHD were made by Thomas et al. in the late 1960s. This resulted in evidence that

administration of methotrexate (MTX) could markedly reduce the severity of this complication in dogs.⁹

The first successful HSCTs in the treatment of severe aplastic anemia using genotypically HLA-identical sibling donors was reported by a group in Seattle in 1972.¹⁰ In 1966, Santos and Owens reported that cyclophosphamide (Cy) was effective against GVHD.¹¹ Conditioning with Cy and total body irradiation (TBI) were adopted in the conditioning regimens during the 1970s. In 1977, Thomas et al. showed that a few patients with otherwise lethal leukemia could be cured using conditioning with Cy and TBI before HSCT from HLA-identical siblings.¹²

In 1990, Professor E. D. Thomas received the Nobel Prize in Physiology or Medicine for his pioneering work in HSCT.

As necessary for the improved results as the discovery of HLA and the possibility of enabling matching between donors and recipients was the development of means to support the patient during the aplastic phase. This became possible with the introduction of new antimicrobial agents during the 1970s and 1980s.

The human leukocyte antigen system

The human leukocyte antigen system is the name of the major histocompatibility complex (MHC) in humans. The super locus contains a large number of genes related to immune system function in humans. This group of genes resides on chromosome 6, and encodes cell-surface antigen-presenting proteins and many other proteins. The proteins encoded by certain genes are also known as antigens, as a result of their historic discovery as factors in organ transplants.

HLAs are not typical antigens, like those found on surfaces of infectious agents. HLA antigens are alloantigens, which means that they can be thought of as an antigen that is present in some members of the same species, but is not common to all members of that species. If an alloantigen is presented to a member of the same species that does not have the alloantigen, it will be recognized as foreign. The different classes of major HLAs have different functions: HLAs corresponding to MHC class I (A, B, and C) present peptides from inside of the cell (including viral peptides, if present) on its surface. If the antigens are foreign, they attract CD8- positive T-cells that destroy the antigen-carrying cell. HLAs corresponding to MHC class II (DR, DM, DP, DQA, and DQB) present antigens from outside of the cell to T-lymphocytes. These particular antigens stimulate the multiplication of T-helper cells, which in turn stimulate a specific antibody-producing B-cell. That particular clone becomes expanded and produces antibodies to the specific antigen. Self-antigens are suppressed by regulatory T-cells. Any cell displaying some other HLA type is “non-self” and an invader, resulting in rejection of the cells bearing that HLA and of tissues containing these cells.

Apart from the genes encoding antigens, a large number of other genes are located on the HLA complex, many of which are involved in immune function.¹³

Diversity of HLAs in the human population is one aspect of defense against disease, and, as a result, the chance of two unrelated individuals having identical HLA molecules at all loci is very low. Because of the importance of HLA in transplantation, the HLA loci are typed by serology or by polymerase chain reaction (PCR) before HSCT, to find as perfect an HLA-matched unrelated donor as possible.

Cancer and metabolic disorders treated with hsct

Indications for HSCT include (1) first complete remission (1CR) in patients with acute myeloid leukemia (AML) and (2) patients with acute lymphoblastic leukemia (ALL) with features associated with poor response to conventional chemotherapy. More advanced stages of leukemia are also indications for HSCT. Other indications for patients with hematological malignancies include myelodysplastic syndrome (MDS), high-risk lymphomas, and chronic lymphocytic leukemia (CML).¹⁴⁻¹⁶ Severe aplastic anemia, other hemoglobinopathies, and some rare inborn errors of metabolism, where HSCT can provide the missing enzyme, can also be cured, or the disease progression may be stopped, by HSCT.¹⁷⁻¹⁹ HSCT has also been used in patients with metastatic solid tumors because of the well-known anticancer effect of this therapy, but the place of HSCT in this treatment needs to be established further.²⁰

Conditioning regimens

To eradicate malignant cells and to suppress the host immune system, conditioning therapy is given before transplantation. The conditioning regimens differ depending on the patient's diagnosis, age, and general medical status (Table 1).

Myeloablative conditioning

In patients with hematological malignancies, the aims of myeloablative conditioning (MAC) are (1) to eradicate the malignant cells, (2) to provide space for the transplant, and (3) to obtain immunosuppression in order to rescue the patient from pancytopenia and toxic side effects of the graft.

The two most commonly used MAC regimens are cyclophosphamide (Cy) (60 mg/kg) for 2 consecutive days followed by total body irradiation (TBI) given as single or fractionated doses.^{12,21-22} As an alternative, busulfan (Bu) (4 mg/kg/day) is given for four consecutive days followed by Cy (120–200 mg/kg).²³⁻²⁵ To prevent relapse, more intensive conditioning has been used. With this regimen, a lower relapse rate was achieved—but also more toxicity—and the leukemia-free survival was found to be the same in patients conditioned with standard MAC regimens.²⁶⁻²⁷

For patients with severe aplastic anemia, Cy (50 mg/kg) on 4 consecutive days with or without anti-thymocyte globulin (ATG) is commonly used for conditioning.²⁸ Patients with inborn errors of metabolism are generally conditioned with Cy combined with Bu.²⁹⁻³⁰

Reduced intensity conditioning

The standard MAC regimens have been challenged by reduced intensity conditioning (RIC) in recent years. RIC is now used more often as a means of achieving some level of initial donor chimerism without the organ toxicity typically associated with conventional conditioning regimens as MAC.^{20,31-33} The idea behind RIC is that the conditioning serves to induce immunosuppression and pave the way for donor stem cell engraftment. If patients with hematological malignancies have relapse, donor lymphocyte infusions (DLIs) are given to induce a graft-versus-leukemia effect (GVL) of the immunocompetent cells in the graft.^{20, 34}

Studies comparing the intensity of the conditioning regimens have shown that RIC is associated with lower transplant-related mortality but higher risk of relapse. The balance of these two factors has resulted in similar overall survival with RIC and MAC.^{35,36} Not only the higher risk of relapse, but also the morbidity and mortality from GVHD remains with RIC. Another disadvantage of using RIC is the increased risk of graft failure. This less toxic approach enables transplantation in elderly patients and patients with organ impairment who can not tolerate full MAC but also in younger patients where strategies to decrease relapse are not needed.

A large variety of RIC regimens are used; most of them include fludarabine (Flu) combined with other cytotoxic drugs such as Bu, melphalan or Cy. One of the most commonly used RIC protocols was suggested by Slavin and co-workers, and consists of Flu (30 mg/m²) for 6 consecutive days followed by 2 days of oral Bu (4 mg/kg/day) and anti-T-cell immunoglobulin.³³

Table 1. Differences between the two different conditioning regimens: myeloablative conditioning (MAC) and reduced intensity conditioning (RIC).

	MAC	RIC
Aim	To eradicate malignant cells and provide space for the transplant. To obtain immunosuppression.	To induce immunosuppression. To pave the way for donor stem cell engraftment.
Indications	Young patients with no other organ impairment. Patients where relapse is not wanted.	Elderly patients. Young patients where strategies to reduce relapse are not needed.
+	Lower risk of relapse. Lower risk of graft failure.	Less toxic Lower TRM. Less GVHD?
-	Increased toxicity. Higher TRM. More GVHD? Similar LFS as RIC.	Higher risk of relapse. Higher risk of graft failure. Similar LFS as MAC.

GVHD, chronic graft-versus-host disease; TRM, transplantation-related mortality; LFS, leukemia-free survival.

Total body irradiation

Total body irradiation as conditioning for HSCT was introduced in the 1950s.³⁷ Regarding myeloablative conditioning regimens, there are three objectives of TBI in HSCT of patients with leukemia. The first objective is to provide adequate immune suppression to prevent rejection of the donor transplant. The second is to provide physical space for donor stem cells to engraft, although it is now doubted if space is needed (personal communication Olle Ringdén, Karolinska Institutet), and the third objective is to eradicate malignant cells.³⁸ Total body irradiation is administered in one

single session, or fractionated over several days (fTBI) together with one or several myelosuppressive or immunosuppressive drugs.³⁸⁻⁴¹ When the fractionated technique is used, the total dose can be increased relative to the single-dose technique. The higher total dose that can be achieved with fTBI allows greater mortality of leukemic cells and lower doses per fraction reduce the morbidity in normal tissue, resulting in reduction of negative side effects.^{29,42-43} Before 1998, fTBI was seldom used at our centre.

The dose rate is important for the outcome of the treatment. A higher dose rate will give rise to more pronounced and more frequent undesirable side effects. If the dose rate during single-session TBI with 10 Gy is increased from 0.04 to 0.07 Gy/min, increased mortality due mainly to septicemia and pulmonary complications was observed.⁴⁴

Some structures in the body, such as the lungs, are more sensitive to radiation, making it necessary to protect them during the irradiation procedure.⁴⁵ This is achieved by shielding of the lungs during the irradiation procedure. The mean dose rate during the entire treatment is needs to be tolerable.^{24,44}

There are also differences in the doses absorbed by different organs, depending on inhomogeneities in body structures, differences in the density of different structures, and the direction of the radiation beam.^{46,47} The position of the patient and the direction of the beam thus influence the doses absorbed by particular organs.⁴⁰

The effect of sTBI compared to fTBI on salivary glands and salivary secretion has not previously been studied.

Busulfan

Busulfan (Bu) together with Cy is an alternative conditioning regimen used to avoid the detrimental effects of radiation on growth and central nervous system development.²² In a randomized study it was observed that Bu increased the risk of veno-occlusive disease of the liver, hemorrhagic cystitis, chronic GVHD, obstructive bronchiolitis, and permanent alopecia compared to TBI.²⁴

A significant problem with oral Bu is the wide inter-patient variability in pharmacokinetics depending on unpredictable intestinal absorption, age, and metabolism.⁴⁸ By analyzing Bu plasma concentrations, it has been observed that high area under the plasma concentration time curve (AUC) correlates with increased toxicity, mainly hepatic veno-occlusive disease and seizures, and a low AUC results in a higher risk of graft rejection and relapse.⁴⁹ Young children have a lower systemic exposure than adults if given identical doses of Bu based on their body weight.⁵⁰

Bu is distributed equally to saliva and to plasma, and therefore salivary glands are exposed to high concentrations of an alkylating agent for four days.⁵¹ Monitoring of blood levels of Bu followed by dose adjustment to achieve a targeted steady-state concentration is important to reduce toxic side effects.

The effect of Bu on oral side effects such as mucositis and salivary secretion has not previously been studied.

Donors

In most cases, the best donor for HSCT is an HLA-identical sibling, which is available in approximately one third of all patients. During the last decades, HLA-matched unrelated donors (MUD) have been used more frequently.⁵²⁻⁵³ About 15 million registered volunteer donors are available worldwide today. With genomic

typing for HLA class I and II, it is possible to find a well-matched, unrelated donor for most patients.³⁹⁻⁵³ Improved tissue typing and better matching have resulted in improved outcome using unrelated donors.⁵⁴

Stem cell sources

For several decades, bone marrow (BM) aspirated from the iliac crest of the donor was the main source of hematopoietic stem cells for transplantation.⁵⁵ During the last decade, granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cells (PBSCs) are increasingly being used and have now replaced BM as a source of stem cells for many patients with hematological malignancies.⁵⁶⁻⁵⁸ Advantages of using PBSCs instead of BM include (1) the fact that no anesthesia is needed for the donor, (2) faster engraftment in the recipient, and possibly also (3) a reduced risk of leukemic relapse. The data on the probability of relapse are conflicting. One disadvantage of using PBSCs instead of BM is an increased risk of cGVHD, which may be due to that grafts of PBSCs rather than bone marrow contain a several-fold higher content of nucleated cells, CD34 cells, natural killer cells, and especially T-cells.^{56,58-59} Because of the increased risk of cGVHD, PBSCs are not recommended for patients with non-malignant disorders because these patients do not benefit from cGVHD and the associated graft-versus-leukemia effect. Furthermore, children and young adults appear to do better if they receive BM instead of PBSCs as the source of stem cells from HLA identical sibling donors.⁶⁰

Banks of cryopreserved cord blood (CB) have been established as an alternative to BM or PBSCs.⁶¹⁻⁶² A potential advantage is the rapid availability. CB is relatively deficient in mature T-cells; there is therefore a lower risk of GVHD, and HLA matching does not need to be as stringent as with BM and PBSCs. The use of CB is, however, associated with slower engraftment, an increased risk of graft failure, and more infections compared to other sources of stem cells (Table 2).

Table 2. Comparison of different sources of stem cells. CB requires less stringent HLA matching (+) than BM (+++). Engraftment is faster with PBSCs (+++). The risk of relapse is approximately the same in all sources. The risk of cGVHD is higher with PBSCs (+++) than with BM (+) and CB (+), and the risk of graft failure is higher with CB (+++) than BM.

	HLA match	Engraftment	Relapse	cGVHD	Rejection
BM	+++	++	++	+	(+)
PBSCs	+++	+++	+(+)	+++	(+)
CB	+	+	++	+	+++

HLA, human leukocytes antigen; cGVHD, chronic graft-versus-host disease; BM, bone marrow; PMSCs, peripheral stem cells; CB, cord blood

Supportive care

As a result of myeloablative conditioning, all patients become pancytopenic and consequently susceptible to infections and other toxic side effects. Also, after RIC patients become cytopenic. Transfusions of erythrocytes and platelets are given prophylactically.⁶³ During this period, patients are kept in reversed isolation or in home care.⁶⁴⁻⁶⁵ Despite this, infectious complications caused by bacteria, viruses, and fungi are common shortly after HSCT and are treated accordingly. Hematopoietic growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) are used to accelerate neutrophil recovery and hopefully reduce the risk of infection.⁶⁶ A major concern with the use of G-CSF is the increased risk of GVHD.⁶⁷ Impaired defense mechanisms and oral mucosal lesions predispose the patient to local mucosal infections that can be hazardous for the outcome of HSCT. Support in oral care, careful monitoring of the status of the oral cavity, and early detection of oral infections are therefore important.

Infections

During the aplastic period after HSCT, bacteremia with α -streptococci and coagulase-negative staphylococci is common.⁶⁸ Invasive fungal infections occur in 10% of patients, and they are especially common in patients with severe GVHD. Viral infections, particularly reactivation of herpesviruses, commonly occur after HSCT.⁶⁹ Herpes simplex viruses often reactivate during the pancytopenic phase, but are manageable using prophylaxis or treatment with acyclovir. A major cause of morbidity and mortality is reactivation of cytomegalovirus (CMV), which may cause pancytopenia, hepatitis, gastroenteritis, or life-threatening pneumonitis.⁷⁰ CMV infection also causes profound immunosuppression.⁷¹ This may pave the way for other bacterial and fungal infections.⁷² CMV infection is most commonly caused by reactivation of latent CMV in a CMV-seropositive patient, or after transmission of CMV from a CMV-seropositive donor.⁷³ Prior to transplantation, it is important to determine the serology of patients and donors regarding the four most common herpes virus [herpes simplex virus (HSV), Epstein-Barr virus (EBV), CMV and varicella zoster virus (VZV)].

Immunosuppression

Immunosuppressive treatment/prophylaxis is necessary to prevent GVHD. The purpose of the GVHD prophylaxis is to reduce the reactivity of transplanted immunocompetent T-cells without destroying the stem cells necessary for the engraftment. Methotrexate and Cyclosporin (CsA) are often used in combination or alone.⁷⁴⁻⁷⁵ Side effects of MTX include neutropenia, mucositis, and liver toxicity. Side effects of CsA are nephrotoxicity, tremor, gingival hyperplasia, and hirsutism.⁷⁶ Other regimens include the addition of prednisolone to CsA and MTX or replacement of MTX with prednisolone.

An effective way to prevent GVHD is T-cell depletion, but that may increase the risk of graft failure and leukemic relapse.⁷⁷⁻⁷⁸

Tacrolimus, which is a calcineurin inhibitor like CsA, has been used in combination with CsA. In recent years, tacrolimus has been combined with sirolimus, a macrolid immunosuppressant, which has resulted in low incidence of acute GVHD.⁷⁹⁻⁸⁰ Common side effects of sirolimus are hypertriglyceridemia, mild reversible cytopenia, and edema.

ATG is also used as an immunosuppressant in HSCT, and reduces the risk of acute GVHD and transplantation related mortality in unrelated donor transplants.⁸¹

Graft-versus-host disease

Acute graft-versus-host disease

Acute graft-versus-host disease (aGVHD) is one of the major hazards in HSCT.⁸²⁻⁸⁵ Donor T-cells trigger aGVHD after activation by recipient HLA antigens. Antigen-presenting cells present the alloantigens to helper T-cells, which release IL-2 and activate cytotoxic T-cells, inducing killing of HLA class I-positive target cells. Natural killer cells and macrophages participate in the reaction. HLA disparity between recipient and donor is a major risk factor for aGVHD. Other risk factors are transplantation from a female donor to a male recipient, seropositivity for several herpesviruses in the recipient and donor, certain HLA alleles, and the host environment.⁸⁵⁻⁸⁶ The main target organs for aGVHD in humans are the skin, the gut, and the liver.⁸⁴⁻⁸⁷ It generally appears during the first 3 months after HSCT and is graded on a five-point scale from 0 to IV. The first-line treatment for aGVHD is high doses of steroids.⁸² When this treatment fails, the outcome is poor.

Chronic graft-versus-host disease

GVHD may also appear in a chronic form (cGVHD), which generally appears from 3 months to 1 year after HSCT.⁸⁸ Risk factors associated with cGVHD include previous aGVHD, high donor or recipient age, PBSC graft, transplantation from a female donor to a male recipient, CML, and seropositivity for several herpesviruses in the recipient or donor.^{89,90-91} The manifestations of cGVHD include skin disease, keratoconjunctivitis, generalized sicca syndrome, oral lesions, esophageal and/or vaginal stricture, malabsorption, pulmonary insufficiency, and immunodeficiency.⁸⁵⁻⁸⁸ Infections with gram-positive bacteria are common and may cause septicemia. The classification of cGVHD can be mild, moderate, or severe according to the judgement of the treating physician.^{89,92} This classification correlates with the clinical outcome in the patient.

Graft versus leukemia effect

The ability of the immune system to control cancer is most evident in the graft-versus-leukemia effect seen after HSCT.^{85, 93-94} Patients with GVHD, and especially cGVHD, have a lower risk of relapse than patients without GVHD. There is also a graft-versus-leukemia effect after HSCT in patients without GVHD.⁹³⁻⁹⁵ A graft-versus-leukemia effect may be induced by decreasing the immunosuppression and discontinuing immunosuppression within 3 months after HSCT.

Immune reconstitution

After HSCT, patients suffer from a deficient immune system for 12 months or longer. They have an increased risk of infectious complications and may develop secondary malignancies. Immune recovery is linked to histocompatibility and GVHD. Immunosuppression is necessary to reduce recipient T-cell reactivity against minor and major histocompatibility antigens of the donor. Immunity against viral, bacterial, and fungal antigens is also impaired. The cellular immune system is deficient and immunoglobulin levels are reduced.⁹⁶⁻⁹⁷

General complications following hsct

Acute general complications after HSCT are rejection,⁹⁸⁻⁹⁹ infections,¹⁰⁰⁻¹⁰¹ acute GVHD,¹⁰² relapse,¹⁰³ feeding problems, malnutrition,¹⁰⁴ nausea, vomiting, and psychological problems.¹⁰⁵ Following TBI, several patients experience reversible hair loss, parotitis, pancreatitis, diarrhea, erythema, hyperpigmentation or mucositis.

Among the long-term complications of treatment with HSCT are cataract formation,¹⁰⁶⁻¹⁰⁷ pulmonary insufficiency,¹⁰⁸ renal dysfunction,¹⁰⁹ affected cardiovascular system,¹¹⁰ chronic GVHD,^{89,111} dysfunction of the immunological system, and increased risk of secondary malignant disease,¹¹²⁻¹¹³ Amongst the neurophysiological changes in children such as retarded motor development,¹¹⁴ there can be a reduction in IQ score.¹¹⁵ Endocrinological sequelae of childhood HSCT have also been observed,¹¹⁶ sometimes resulting in height reduction,¹¹⁷ disturbed timing of onset of puberty,^{116,118-119} and changed craniofacial development.¹²⁰

Current results

Today, more than 200,000 HSCTs have been performed worldwide, with an annual rate of around 20,000 transplants, and the results have been, and still are, improving continuously.^{20,121-122} More than 60% of patients become long-term survivors.¹²³

Relapse of malignant disease is the most common cause of treatment failure after HSCT. The risk is related to the diagnosis, disease stage at the time of transplantation, and the type of conditioning. Furthermore, with more effective immunosuppression the risk of relapse increases. Combined efforts to improve outcome after HSCT have been very effective. Although older patients with more advanced disease are being treated and more alternative HLA non-identical donors are used, the overall survival and transplantation-related mortality (TRM) have improved. The problem of relapse still has to be remedied, even though significantly lower TRM and improved survival after HSCT has been observed in recent years.¹²³

ORAL COMPLICATIONS

In HSCT recipients, acute complications in the oral cavity are common and include soreness of the mucosal membranes, infections, pain, changes in taste sensation, salivary gland dysfunction, and xerostomia. The acute complications are related to the pretransplant status of the oral cavity and nutritional problems during the neutropenic phase, and may interfere with the treatment and the engraftment.¹²⁴⁻¹²⁶

Approximately 80% of children treated for cancer become long-term survivors.¹²⁷ Long-term side effects include (amongst others) salivary gland dysfunction, growth anomalies, and graft-versus-host disease, and make the compromised pediatric patient require special oral care.¹²⁸⁻¹²⁹

Acute oral complications

Except for systemic side effects such as vomiting, fever, infections, and acute GVHD, many patients report the oral and pharyngeal tissues as sites for major problems during the neutropenic phase after HSCT. The oral cavity is reported to be one of the most common sites of complications after HSCT.^{124,130} Chemotherapy and TBI may induce a complex sequence of biological events and also toxic reactions in the oral mucosa, with subsequent development of mucosal lesions. The conditioning procedures also result in a low immune defense and altered microbial colonization of the oral cavity.

Treatment with immunosuppressive drugs to prevent GVHD may further aggravate the lesions in the oral mucosa, and increase the risk of microbial dissemination into the circulation.¹³¹

Alterations in the quality of saliva, reduced salivary flow, and an impaired swallowing mechanism due to pain also promote colonization of potentially pathogenic gram-negative bacteria. Changes in secretory immunoglobulin A because of reduced total saliva volume may reduce the antimicrobial activity of saliva. Antimicrobial therapy with subsequent alterations in the indigenous flora also facilitate colonization by exogenous microorganisms.¹³²⁻¹³⁴

The alteration in the oral environment and the immunosuppressive status of the patient, with low numbers of neutrophils, can lead to microorganisms entering the circulation.¹³⁵ The risk of local and sometimes life-threatening systemic infections derived from the oral cavity is therefore high in HSCT recipients immediately after HSCT.¹³⁶⁻¹³⁷

The side effects of HSCT in the oral cavity are experienced by the patient as being very uncomfortable and painful and they often necessitate total parenteral nutrition.^{126,138}

Many conditioning regimens have been used, but no single regimens have been shown to be superior. It is necessary to evaluate the impact of different conditioning regimens

and other risk factors on the oral side effects. The effect of conditioning with high dose compared to reduced dose chemotherapy on oral mucositis in allogenic stem cell recipients has not previously been investigated.

Long-term oral complications

The prevalence of long-term oral complications has been reported to vary between 60% and 100% in patients treated with HSCT.¹³⁹ The long-term oral complications are related either to the disease process, the transplant immunobiology, or the transplant-preparative regimens. The conditioning regimen used also influences the number of secondary complications. The long-term oral complications can seriously affect the patient's quality of life. Frequently reported long-term oral complications are: salivary gland dysfunction, gingivitis, periodontal involvement, taste acuity, gingival hyperplasia, and oral ulcers.¹⁴⁰ There is also an increased risk of secondary oral malignancies both in patients with and without cGVHD.¹⁴¹

Salivary gland dysfunction has been reported several years after pediatric HSCT.¹⁴² If salivary dysfunction is a permanent condition following pediatric HSCT, this is an important information for dentists responsible for the oral care of these patients. To optimize the preventive treatment procedures, and to enable further improvements in oral health, quality of life, and well being, it is important to register changes in the secretion rate of saliva during and after the HSCT procedure.

Impaired root development has been reported in 95–100% of the pediatric patients.^{128,142} Enamel hypoplasia has been found in 25–42% of pediatric HSCT recipients, microdontia in 68%, and dental aplasia in 58%.^{128,142} Craniomandibular dysfunction, including reduced mouth opening and translation movement of the condyles, with subsequent muscle pain and headaches, have been reported in 84% of pediatric HSCT patients as compared to 54% of healthy controls.¹²⁹ Oral ulcers have been reported in 37% of pediatric HSCT recipients.¹²⁵

Oral mucosa

The skin and the mucosal membranes are the first line of defense. The epithelium of the mouth can be divided into three categories: (1) the masticatory mucosa, with a para-keratinized stratified squamous epithelium, found on the dorsum of the tongue, the hard palate, and attached gingiva, (2) the lining mucosa, a non-keratinized stratified squamous epithelium found almost everywhere else in the oral cavity, and (3) the specialized mucosa, found specifically in the regions of the taste buds on the dorsum of the tongue.

The oral epithelium is composed mainly of keratinocytes, but melanocytes and immunocompetent cells also reside within the oral epithelium. The keratinocytes are renewed by proliferation of immortal stem cells in the basal layer and the cells differentiate into mature keratinocytes higher up in the epithelium. The renewal turnover rate has been estimated to be 10–14 days in humans.¹⁴³ The cells are active in the recognition of antigens and signaling to underlying tissue, and also in receiving signals from these tissues. Several cytokines are involved in this process.¹⁴⁴⁻¹⁴⁶

The basal lamina underlies all epithelium, separating epithelial cells from underlying connective tissue. The basal lamina influences many functions, e.g. cell metabolism, differentiation, and migration.

The submucosa is the layer of dense irregular connective tissue or loose connective tissue that supports the mucosa, and that joins the mucosa to the bulk of underlying smooth muscle. In the submucosa blood vessels, lymphatic vessels, and nerves can be seen—all of which supply the mucosa.

The innate immunity often provides the first reaction after an antigen challenge.¹⁴⁷ Macrophages are normally found in the lamina propria of the mucosa. Dendritic cells of the adaptive immune system are found mostly in the stratum basale and in the lamina propria of the connective tissues. In healthy individuals, the mucosal membranes are also protected against infections by motility, e.g. the swallow reflex and secretion.

The mucosal membranes are easily damaged by chemotherapy and radiation.¹⁴⁸⁻¹⁴⁹ The epithelium and the immune system constitute a selective barrier system in the oropharyngeal and gastrointestinal tract, as does the bacterial equilibrium of these sites. If one or more of the barrier functions are affected, it may give rise to severe local or systemic adverse effects. A lesion in the oral mucosa consists of a break in the first line of defense, which will make the patient predisposed to local and systemic infections derived from the oral cavity.⁶⁹ Cytotoxic drugs affect malignant cells as well as normal cells with a high turnover rate, causing an impaired capacity to induce immune defense reactions.¹⁵⁰ Tissues that are constantly renewed are thus likely to be affected by cytotoxic drugs. In the oral cavity, cells of the epithelium and many cells of the immune system—as well as bacteria—have a high turnover rate. Sensitivity to the anti-neoplastic agent 5-fluorouracil, for example, varies depending on the tissue the macrophages, dendritic cells, and T-cells reside in. This may be due to differences in cell origin or in antigen load.¹⁵⁰

The main goal of cytotoxic drugs is to cause cell injury, leading to death of tumor cells. Apoptosis is a normal physiological process in proliferating tissues, balancing mitosis in the maintenance of tissue homeostasis.¹⁵¹ However, apoptosis can also be triggered in a cell by a distorted gene, as an intrinsic suicide process to dispose of cells with potentially dangerous gene mutations. A distorted gene in a potentially malignant cell or a virus-infected cell is discovered at cell-cycle checkpoints during proliferation. If the DNA in the cell cannot be repaired, the intrinsic cell death program is induced via the protein p53 and caspase activation.¹⁵⁰ Cytotoxic insults such as radiation and chemotherapy may also kill cells through apoptosis. During treatment with cytotoxic drugs, the drug is concentrated in proliferating cells. New gene damage is created within the proliferating cell and it may go into apoptosis. In this way, the drug can assist the body in disposing of malignant cells.¹⁵⁰

Oral microflora

The oral cavity normally harbors a complex microflora consisting of more than 400 different bacterial species. The microflora is composed of both aerobic and anaerobic microorganisms, where the anaerobes outnumber the aerobes by a factor of 10.¹⁵² The normal microflora is important for the local oral immune defense through its ability to compete against other potential pathogens.¹⁵² In immunocompromised patients, infections frequently occur as a consequence of impaired defense mechanisms. Colonization resistance is dependent on the competition between the normal anaerobic microflora and potential pathogens for space and nutrition on the mucosal membranes.^{134,152} The microorganisms isolated from infections in HSCT recipients

either belong to the patient's indigenous oropharyngeal or gastrointestinal microflora or they are acquired from the hospital environment. Gram-negative aerobic rods and fungi are the microorganisms most frequently isolated.¹⁵³⁻¹⁵⁴

Oral mucositis

In describing the changes in the oral mucosa in relation to cancer treatment, a widely used expression is oral mucositis. The criteria used with this expression are not always defined, however, and the term oral mucositis (OM) may cover different signs and symptoms in different studies.¹⁵⁵⁻¹⁵⁷ It would be more adequate to define the oral mucosal lesions according to their etiology.¹²⁵ Mucosal lesions in the neutropenic HSCT patient result from epithelial damage due to the cytotoxic effects of chemotherapy and radiation as well as from superficial oropharyngeal infection, mechanical trauma, GVHD, and hemorrhages.¹²⁴⁻¹²⁵ To reduce the severity of oral mucosal lesions, several studies have been performed but no method or drug has been shown to be superior. Palifermin, a recombinant human keratinocyte growth factor (KGF), is currently the most effective drug, but it is expensive. KGF reduces the incidence and duration of severe OM by protecting cells that line the surface of the mouth and intestinal tract, and stimulates the growth of new epithelial cells to build up the mucosal barrier. The problem of oral mucosal lesions during the aplastic period, especially after myeloablative conditioning, still remains.

For patients treated with chemotherapy or TBI, OM begins around 3–5 days after drug infusion and has been shown to worsen until a peak is reached. It then declines gradually until it is completely resolved after approximately 2 weeks. The onset and duration of mucositis reflects the course of neutropenia (Figure 1).¹⁵⁸ About 60–100% of HSCT recipients are reported to be affected.¹⁵⁹⁻¹⁶¹

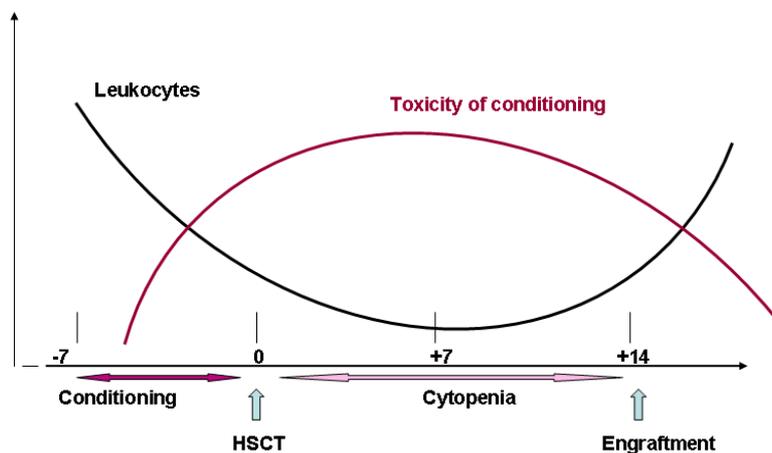


Figure 1. Toxicity expressed as oral mucositis in relation to neutrophil number.

Oral mucositis is characterized by direct cell injury mediated by chemotherapy or radiation. More specifically, it is a consequence of a complex cascade of biological events starting with clonogenic cell death and the release of reactive oxygen species, progressing through a series of steps in which biological pathways are activated and amplified, and culminating in ulcer development.¹⁶²

Pathobiology of oral mucositis

In an attempt to describe the pathobiological process, Sonis¹⁶² has proposed five phases. In the first phase—the *initiation phase* and during the primary damage response—radiation and chemotherapy directly injure DNA and cause strand breaks, resulting in clonogenic death of basal epithelial cells. Reactive oxygen species (ROS) generate and initiate a series of interacting biological events. A number of transcription factors such as NF- κ B, Wnt, and p53 are activated. Chemotherapy and radiation can directly activate NF- κ B and it can be activated indirectly by ROS. Among the 200 genes whose expression is governed by NF- κ B are those associated with the production of molecules that are known to be active in the pathogenesis of OM, including upregulation of key cytokines such as tumor necrosis factor alfa (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6). Apoptosis is an important consequence of the effect of NF- κ B in normal cells. Radiation and chemotherapy also affect other pathways that lead to indirect cell death. All of these processes begin within seconds after administration of radiation or chemotherapy, but there is a lag between the damage at the cellular and molecular level and the clinical manifestations (Figure 2).

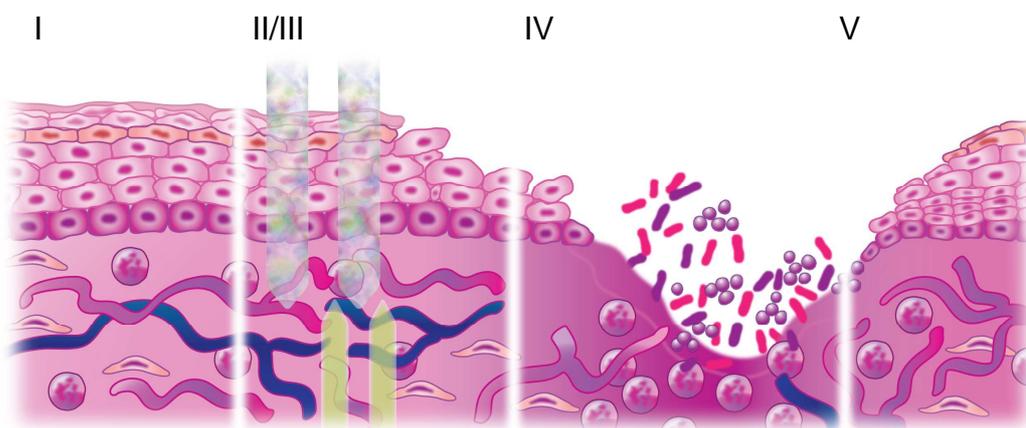


Figure 2. The pathobiology of mucositis described as a five-stage process. The key biological processes associated with the pathogenesis of oral mucositis can be arbitrarily divided into five stages: initiation (I), the primary damage response (messages and signalling) (II), amplification (III), ulceration (IV), and healing (V).

In the second and third phases, the *signalling phase* and the *amplification phase*, many of the molecules induced by the primary response positively or negatively feed back and alter the local tissue response. TNF may feed back positively on NF- κ B to amplify its response, and initiate mitogen-activated protein kinase signalling. As a consequence, this will inhibit intermittent resolution and lead to ulceration.

Ulceration is the major event associated with OM and is described by Sonis as the fourth phase. Prevention of ulceration can minimize pain, risk of infection, use of feeding tubes, and the length of hospital stays. Ulceration develops as a consequence of the direct and indirect mechanisms noted above, causing damage and apoptotic changes to mucosal epithelium. Mucositis ulcers are deep, and oral bacteria quickly colonize them. The bacteria on the surface of the ulcer are active contributors to the mucositis process. Microbial cell wall products penetrate into the submucosa, now rich

in macrophages, to stimulate those cells to further secrete pro-inflammatory cytokines. In granulocytopenic patients, there is a risk that live bacteria will invade submucosal vessels and induce bacteremia or septicemia.

The fifth phase is the *healing* phase. The majority of cases of mucositis heal spontaneously. Ulcer resolution is the result of an active biological process in which signaling from the submucosal extracellular matrix guides the proliferation, migration, and differentiation (Figure 3).

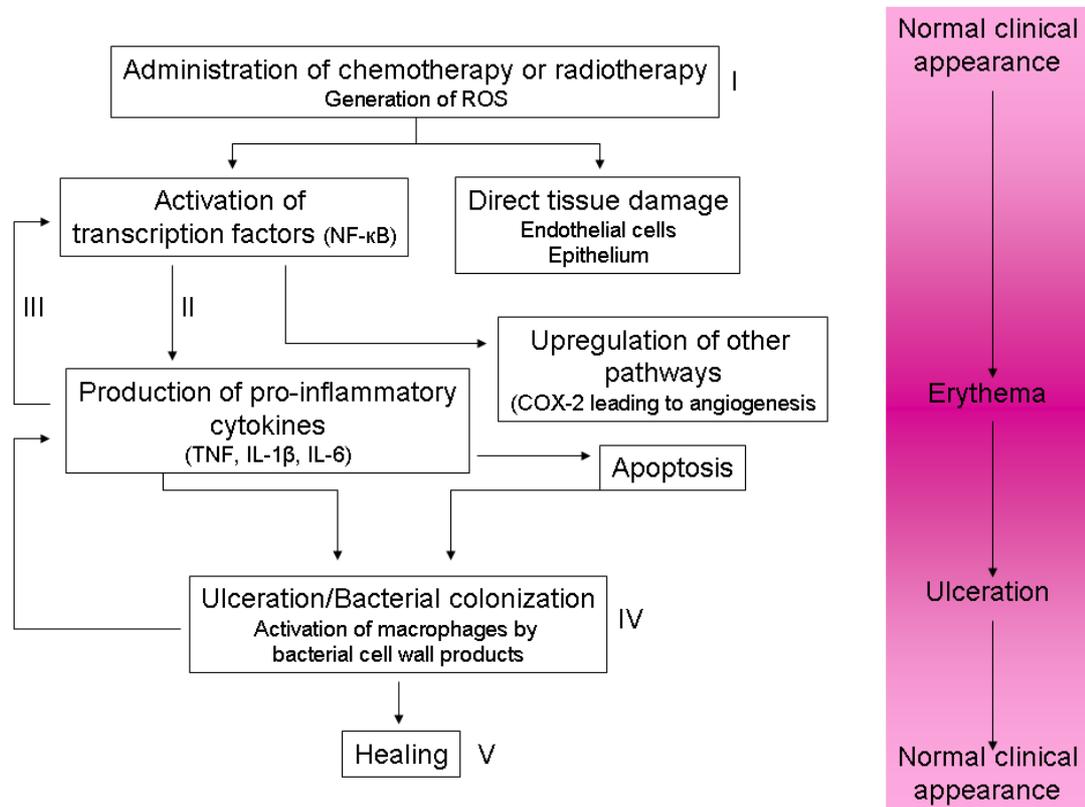


Figure 3. Diagram illustrating the mucosal and clinical changes that lead to mucositis according to the current hypothesis. The five overlapping phases are demonstrated as; initiation (I), upregulation and message generation (II), signaling and amplification (III), ulceration (IV), and healing (V). Adapted from Logan et al.¹⁶³

The pro-inflammatory cytokines involved are thought to play an important role in the overlapping phases of mucositis development, in particular upregulation and message generation, signal amplification, and ulceration. Different drugs act through different pathways, though, and by themselves they may promote or inhibit different pro-inflammatory cytokines.¹⁶⁴

Suggested risk factors for OM during cancer treatment vary and often reflect the modality of treatment, the type or dose of chemotherapy, or combinations of drugs. The use of MTX for GVHD prophylaxis has been shown to be associated with a high severity of OM.¹⁶⁵ Patients with grafts from unrelated donors are more likely to experience severe OM than those who receive grafts from related donors.¹⁶⁵ Other predisposing factors are acute myelogenous leukemia, acute lymphocytic leukemia, myelodysplastic syndrome, and prolonged neutrophil recovery. Patient-related factors such as age, systemic disease, and local mucosal factors may also affect risk.¹⁶⁶

The clinical and economic impact of oral and gastrointestinal mucositis has been shown to be high.¹⁶⁷ Severe OM may prolong hospitalization, require parenteral opioids for pain control, interfere with oral nutrition, and limit oral hygiene and/or speech. In patients who experience more severe OM (i.e. of WHO grade 3–4), approximately 35% will have a delay in chemotherapy, 60% will have a reduced dose of chemotherapy, and 30% will have the regimen discontinued.¹⁶⁸

Gingival crevicular fluid

The gingival crevicular fluid (GCF) is a serum-derived transudate or inflammatory exudate that can be collected from the gingival crevice surrounding the teeth (Figure 4). GCF contains substances from the host and from microorganisms in the subgingival and supragingival plaque. Constituents from the host include molecules from blood and contributions from cells and tissues of the periodontium. The latter includes the vasculature, epithelium, connective tissues, and inflammatory and immune cells that have infiltrated the periodontal tissues. Among the important host-derived constituents are markers of inflammation, including cytokines and enzymes. Products of tissue breakdown can also be detected in the GCF.¹⁶⁹

The volume of GCF present at a given site may be directly related to tissue inflammation, permeability, and the nature and ulceration of the crevicular epithelium. Sites characterized as being moderately or severely inflamed have a greater volume of GCF than less inflamed sites.¹⁷⁰

The analysis of specific constituents in the GCF provides a quantitative biochemical indicator for the evaluation of the local cellular metabolism. GCF can also be analyzed to determine whether specific markers of systemic disease can be identified in the oral cavity.¹⁶⁹ Increased levels of pro-inflammatory cytokines in GCF have been reported in obese individuals reflecting the elevated levels in serum.¹⁷¹

Furthermore increased cytokine levels are considered to be potential tools for predicting mucosal damage because of the time constraints between detectable serum changes and mucosal damage.¹⁷² Certain key cytokines are, as described above, important in the development of OM as radiation and chemotherapy induce activation of those cytokines within the mucosa. If levels of these cytokines are measurable in the GCF has not been investigated before.

The most common clinically applicable method used is the use of precut methylcellulose filter paper strips. The fluid is absorbed by the strip, which is first placed in the sulcus and then eluted and analyzed. This method offers a non-invasive means of assessing the host response in GCF. The filter strip method can be time-consuming, though, and the technique is sensitive. The sample must be relatively free from plaque and not contaminated with saliva or blood. The strip must also remain in the sulcus for a long enough time to absorb an adequate sample of fluid. Sampling of GCF often involves collection of the entire volume of fluid at the sampling site. This volume varies from one tooth site to another tooth site, Lamster et al. therefore developed an approach to GCF sampling that standardizes the time of collection and reports the data as the total amount in the timed sample.¹⁶⁹

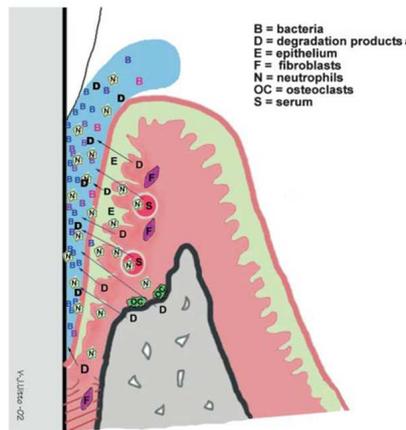


Figure 4. Gingival crevicular fluid is composed of substances derived from serum, leukocytes, bacteria, activated epithelial cells, connective tissue cells, and bone cells. Adapted from Uitto.¹⁷³

The oral mucosa in cGVHD

Chronic GVHD presents with a spectrum of disorders that may change with the length of time after transplantation. There is oral involvement in 80–90% of patients with extensive chronic GVHD.⁸⁸ In the oral cavity, typical lichen planus-like eruptions are generally found and can range from fine white reticular striae on the buccal mucosa to large plaques on the buccal surface or lateral aspect of the tongue.¹⁷⁴⁻¹⁷⁵ In other forms, such as lupus erythematosus-like lesions, ulcerations may be observed with erythematous borders and generalized mucosal atrophy. Xerostomia is part of the clinical spectrum of GVHD and a Sjögren-like syndrome is often present. Xerostomia increases the risk of development of extensive dental caries. The filiform papillae of the tongue are often affected, which can lead to abnormal changes in taste sensation.¹⁷⁵ Histologically, the oral mucosa shows atrophic necrosis of squamous cells and infiltration of mononuclear cells, resembling what is seen in patients with oral lichenoid reaction.¹⁷⁵

Saliva

Saliva is composed of the secretions from the major and minor salivary glands and the gingival crevicular fluid. Saliva also contains desquamated epithelial cells, leukocytes, food residues, blood, viruses and bacteria, and their products.¹⁷⁶ Together with the three pairs of major salivary glands—the parotid, submandibular, and sublingual glands—multiple minor salivary glands in the mucosa all over the oral cavity contribute to the secretion of saliva. The size of the parotid gland is approximately twice that of the submandibular gland, which in turn is five times the size of the sublingual gland.

The secretion of saliva is controlled by the autonomic nervous system. The average unstimulated salivary flow rate is 0.3 ml/min during daytime and less than 0.1 ml/min during sleep. The stimulated salivary secretion rate during food intake is about 4.0 ml/min.¹⁷⁷ The major salivary glands produce about 90% of the total salivary volume.¹⁷⁸ The remaining saliva is produced by the numerous minor salivary glands.

The type of secretion varies according to the different glands. The parotid secretion is serous, while the sublingual and minor salivary glands produce viscous, glycoprotein-containing saliva. The saliva produced by the submandibular glands is mixed.

From the acinar lumen, the saliva passes through the narrow intercalated ducts and then through the wider striated ducts, which are lined by cuboidal cells rich in mitochondria, where a modification of the saliva occurs. Ion exchange makes the secretion change from an isotonic solution to a hypotonic one. Sodium is actively absorbed; chloride is passively absorbed, while potassium and bicarbonate are secreted. Further modification of the saliva occurs in the excretory ducts, where sodium is absorbed and potassium secreted, making the saliva still more hypotonic.¹⁷⁹ The various cell functions give rise to saliva of different compositions coming from the different glands. The parotid gland produces a watery fluid, rich in electrolytes, containing amylase and proline-rich polypeptides. The minor salivary glands produce a fluid rich in mucopolysaccharides (glycoproteins) and they produce up to 70% of the mucin found in the oral cavity.¹⁷⁸

The saliva has several different functions in the oral cavity. Speech and swallowing are facilitated through saliva acting as a lubricant. The mucosa and teeth are coated with saliva as a protection against trauma. Salivary proteins form a pellicle on the surface of the tooth, acting as a protective diffusion barrier. The time during which demineralization can occur is reduced through neutralization of pH after intake of food, and remineralization of teeth is achieved through saliva. The saliva also helps the mouth to maintain an appropriate ecological balance.¹⁸⁰ The mucosal surface is protected by specific immunoglobulins (sIgA)¹³³ and unspecific antimicrobial systems (e.g. lysozyme, lactoferrin, and sialoperoxidase)¹⁸¹ in the saliva, and indirectly through swallowing. Mucins and sIgA have antimicrobial properties. Through salivary aggregating factors, bacterial cells are clumped together and are thereby more easily removed from the oral cavity. Parotid saliva has an anti-fungal capacity, reflecting the properties of basic and neutral peptides.¹⁸² The enzymes amylase and lingual lipase in the saliva have digestive activity.¹⁸³ A specific zinc-binding protein, gustin, and the low ionic strength of saliva are important for the taste function.¹⁸⁴ Taste is further facilitated by the dissolution and transport of tastants.¹⁸⁵ The saliva protects the oral mucosa from dehydration, but under conditions of systemic dehydration salivary flow is reduced. Dryness of the mouth and information to the CNS from osmoreceptors in the intraoral mucosa result in reduced urine production and increased thirst.¹⁸⁶

Clinical complications of salivary dysfunction

Several complications secondary to salivary dysfunction can occur, such as experienced difficulty in eating and swallowing, digestive disorders, speech problems, alterations in taste, problems of oral hygiene, trauma and ulceration of the oral mucosa, a burning sensation in the mucosa, bacterial and fungal infections, dental caries, and gingivitis.¹⁸⁷ These problems may seriously affect the patient's quality of life, which makes salivary gland dysfunction an important issue in transplant patients.

Diagnosis of salivary dysfunction

There are many different methods to estimate salivary function (sialometry). For estimation of stimulated secretion of whole saliva (SSSR), chewing of a standardized paraffin bolus is used, which is a simple and rapid method. Unstimulated secretion of whole saliva (USSR) is estimated with the patient sitting in a passive position drooling

or spitting into a collection vessel. Parotid gland secretions can be obtained using a specially designed cup positioned over the parotid papilla.¹⁸⁸ No ideal device for collection of the secretion from the submandibular/sublingual glands has been presented, although different attempts have been made to use plastic devices for suction of saliva.¹⁸⁹⁻¹⁹¹

Measurements of both USSR and SSSR are considered to be stable on an individual basis when standardized procedures are used. Heintze et al. reported a highly significant correlation between duplicate tests of both USSR and SSSR.¹⁹² Pedersen et al. showed high reliability for both USSR and SSSR.¹⁹³

Some reports have, however, shown a small increase in SSSR when the sampling procedure was repeated. The lower secretion value found at the first examination has often been reported not to be significantly different from that obtained after repeated measurements.^{192,194} Le Bell et al. found a significant intra-individual difference between the two first measurements of SSSR in 41 nine-year-old children.¹⁹⁵ In very young children, some saliva may initially be swallowed before they learn to spit,¹⁹⁶ but in older individuals the small increase in secretion rate during the second measurement is most probably due to psychological factors.¹⁹⁷

Sialometry is the most frequently used method to evaluate salivary function clinically. For functional study of the salivary glands, both magnetic resonance tomography (MR) and sialography are often used.

Xerostomia

Today, the term xerostomia is limited to defining the patient's subjective experience of dry mouth.^{187,193-198} The patient's subjective complaints of dry mouth should be followed by objective measurement of salivary secretion rate, since symptoms of xerostomia do not always correlate with salivary dysfunction.¹⁹⁸

Factors affecting salivary flow rate

Several factors influence the flow of saliva. USSR is influenced by e.g. water balance, body position, light, previous stimulation, heart rhythm, medication, and changes in circadian and circannual rhythms.¹⁷⁷ SSSR is influenced by thought, sight, smell, taste,¹⁹⁹ mechanical intra- and extraoral stimulation, gland size, gag reflex, vomiting, and smoking.²⁰⁰ Damage to the salivary glands through e.g. irradiation,²⁰¹ autoimmune disease,²⁰² and HIV infection²⁰³ are other causative factors that can reduce flow rate. Medication,²⁰⁴ trauma, and decrease in chewing²⁰⁵ can result in an interference with neural transmission, which may in turn inhibit the secretory function of the salivary glands.¹⁸⁷ Other causes of salivary dysfunction are protein caloric malnutrition²⁰⁶ and dehydration.²⁰⁷

Gender

Girls have a lower SSSR than boys in all age groups of children.¹⁹⁶ Andersson et al. found the USSR to be slightly lower in girls than in boys at 10 years of age. The difference was significant at 13 years of age.¹⁹⁶ In adults, conflicting results have been presented regarding the correlation between gender and salivary flow rate. Ship and Baum found that there was no significant difference between major salivary secretion in healthy males and females while Heintze et al. observed that females have lower

salivary secretion rates.^{192, 208}

Age

The influence of age on salivary function in healthy children was studied by Crossner.¹⁹⁶ A significant increase in salivary secretion rate up to the age of 15 years was found. From this age, Dawes found no correlation with salivary flow rate, indicating that the salivary glands are fully developed at the age of 15 years.¹⁷⁷

Acinar atrophy and ductal irregularities occur with increasing frequency in older people.²⁰⁹ The decrease in salivary flow rate, which has sometimes been reported in the elderly, has been correlated with general health status and medication rather than with the normal ageing process.^{187,204} The salivary glands have residual capacity and can maintain the salivary secretion rate despite reduction in acinar cell numbers due to ageing.²⁰⁹

Diseases

Both local and systemic diseases are associated with salivary gland dysfunction.^{176,210} Autoimmune disorders such as rheumatoid arthritis, Sjögren's syndrome, systemic lupus erythematosus, and scleroderma will cause lymphoepithelial lesions that interfere with salivary gland function.²⁰² Other systemic diseases that may reduce salivary flow rate are sarcoidosis, hypertension, hyperlipidemia, and anxiety disorders.²¹¹

Viruses

Elevated levels of EBV have been found in saliva and salivary glands from patients with Sjögren's syndrome,²¹² and this has been suggested to be involved in the destruction of the salivary glands.²¹³ It has been proposed that EBV is reactivated by some kind of trigger, resulting in a chronic autoimmune attack on the salivary glands.²¹⁴

In a study by Shillitoe et al., CMV was suggested to be responsible for glandular dysfunction in patients with Sjögren's syndrome.²¹⁵

The salivary glands are also thought to be the site of replication of human herpesvirus 6 (HHV-6), but the role of HHV-6 in the pathology of the salivary glands is not clear.²¹⁶

Drugs

Today, more than 400 different medical drugs are known to induce reduced salivary flow. In general, drug-induced salivary dysfunction is reversible, and normal flow rates are regained after elimination of the medication.¹⁸⁷ Examples of drugs known to reduce the salivary secretion rate are: neuroleptics,²¹⁷ antidepressants,²¹⁷⁻²¹⁸ antihistamines/anticholinergics,²¹⁹ diuretics,²¹⁰ benzodiazepines,²¹⁹ antihypertensives,²²⁰ and gastric antisecretory drugs.²²¹

Chemotherapy

Treatment of malignant diseases induces reduced salivary secretion and changed composition of the saliva.²²² Kosuda et al. showed that chemotherapy used alone only had a minor influence on salivary function, as determined by salivary gland scintigraphy. When chemotherapy was combined with radiation, there was a more pronounced disturbance of salivary function than with radiation alone, indicating that chemotherapy makes the gland tissue more susceptible to radiation injury.²²³

Radiation

Radiosensitivity of an organ is usually related to a fast rate of turnover of the cells. The cells of the salivary glands have a relatively slow turnover rate²²⁴ but in spite of this, the salivary gland is ranked as one of the most radiosensitive organs in the body. The mechanism of this increased radiosensitivity is not understood²²⁵. The submandibular/sublingual and parotid glands differ in radiosensitivity, the serous parotid glands being more sensitive to irradiation.²²⁵ Salivary dysfunction may result from radiation effects on connective tissues—causing fibrosis²²⁶, which affects the vascular supply, neurologic innervation, and the secretory acinar cell itself.²²⁷ Acinar cell death caused by apoptosis has been suggested to be one factor in the functional disturbances after radiation.²²⁸ The expression of neuropeptides is known to be changed after radiation therapy, and after high doses of radiation noradrenaline-stimulated secretion of electrolytes is reduced.²²⁹ In addition to DNA injury, the radiation-induced lethal damage to the acinar cells may be caused by disturbances in the cell membrane.²³⁰ A reduction in total protein content and qualitative changes in the protein composition of saliva has also been noted.²³¹

AIMS OF THE THESIS

General aim

The aim of this thesis was to investigate conditioning-associated effects on salivary function and the oral mucosa of hematopoietic allogeneic stem cell recipients.

Specific aims

Study I

The aim of study I was to investigate whether children conditioned with fTBI have a significantly better salivary secretion rate one year after allogeneic HSCT than those conditioned with sTBI. A secondary aim was to investigate the contribution of other known risk factors for low salivary secretion rate one year after allogeneic HSCT.

Study II

The aim of study II was to investigate whether conditioning with fTBI or Bu would result in less long-term salivary dysfunction compared to sTBI in pediatric allogeneic HSCT recipients. Furthermore, we wanted to investigate whether other known risk factors for low salivary secretion rate after allogeneic HSCT also contributed.

Study III

The aim of study III was to investigate whether RIC would induce less severe OM than MAC. Secondary aims were to determine the effect of a new oral hygiene protocol and the impact of other risk factors for OM in allogeneic HSCT recipients. Furthermore, we wanted to examine how closely the WHO mucositis score and the OMAS score were correlated.

Study IV

The aim of study IV was to investigate the relationship between OM and production of pro-inflammatory cytokines, both in GCF and in serum, in relation to different conditioning regimens and other risk factors for OM in adult allogeneic hematopoietic stem cell recipients.

MATERIAL AND METHODS

This section gives a brief overview of the methods used to obtain the results presented in this thesis. The first two studies evaluated salivary dysfunction as long-term oral complications in children treated with different kinds of conditioning before HSCT. The two subsequent studies concerned oral mucositis as an acute complication in patients with different kinds of conditioning in preparation for allogeneic HSCT.

Patients

Study I

Pediatric allogeneic HSCT recipients between the age of 4 and 13 years, grafted at Karolinska University Hospital, Huddinge during the period January 1994 to December 2005, were included in the study. During this period, a total of 165 children in this age group received HSCT. As 54 children did not cooperate at the salivary secretion test at baseline, 57 children died during the one-year follow-up period, and 10 more received other types of conditioning, the final study group consisted of 44 children. The conditioning procedure included single-dose total body irradiation (sTBI) and cyclophosphamide (Cy) in 27 patients. Chemotherapy protocols with fractionated total body irradiation (fTBI) were used in 17 patients. Of the donors, 12 were matched siblings, 22 were matched unrelated donors (MUDs), and 10 were mismatched unrelated donors. Diagnosis and other baseline characteristics of the patients are shown in table 3.

Most of the patients in both groups received MTX, CsA, or both as prophylaxis for GVHD. The sTBI/Cy-treated children had a mean age of 8.9 ± 2.4 years, and the fTBI group had a mean age of 9.0 ± 2.3 years at HSCT.

Study II

A total of 309 children under the age of 15 underwent allogeneic HSCT between January 1980 and December 2006 at Karolinska University Hospital, Huddinge. One hundred twenty-nine of them died before the age of 15, and 23 children were not available for evaluation since they were only in Sweden for the transplantation. Thirty-three children received other types of conditioning that did not include radiotherapy or Bu and were therefore excluded from the study. Fifty other children were not available for follow-up for other reasons. The mean age of the 74 participants in the study was 7.2 ± 3.3 years in the sTBI group, 8.8 ± 4.0 years in the fTBI group, and 7.7 ± 3.7 years in the Bu group. Thirty-five of the patients had been conditioned with sTBI/Cy, 14 with fTBI, and 25 with Bu, in combination with other chemotherapeutic agents depending on their diagnosis. The diagnosis and other baseline characteristics are shown in table 3.

The majority of patients in the three groups were treated with MTX, CsA, or both for GVHD. Forty-five of the donors were HLA-identical sibling/related donors and 22 were MUDs. Four donors were an allele- or antigen-matched unrelated donor, and in 3 cases the donor was a mismatched related donor. Most of the patients received bone marrow as stem cell source.

The children were divided into three different groups: patients treated with sTBI, those treated with fTBI, and those who received Bu, in combination with various cytotoxic drugs, mainly Cy.

Study III

One hundred eighty-three HSCTs in 166 patients aged ≥ 12 years were performed at Karolinska University Hospital, Huddinge between October 2007 and May 2011. The study involved 171 patients. Twenty-one of these patients had previously undergone HSCT. Two patients died before the study period ended, and 12 were not available for inclusion. Fifty-five patients were partly treated in home care and could thus not be followed under the whole study period. MAC was given to 72 of the patients and 99 received RIC, depending on their diagnosis and other contributing factors. The median age of the patients receiving MAC was 40 years (15–58) and it was 55 years (12–71) for patients treated with RIC. Most of the patients had malignant diseases. Diagnoses and other patient characteristics are given in table 3. Of the donors, there were 57 HLA-identical sibling/related donors and 101 MUDs. Thirteen patients had an allele- or antigen-mismatched unrelated donor. The majority of patients received peripheral stem cells. MTX and CsA were used in 116 patients as prophylaxis for GVHD and sirolimus and tacrolimus were used in 42 patients. Patients with a cord blood graft ($n = 9$) received CsA and steroids. CsA and MTX in combination with Cy were given to 4 patients. Patients treated with MTX as prophylaxis for GVHD also received folinic acid to prevent mucositis.

The patients were divided in two groups, those receiving MAC and those receiving RIC.

Study IV

Adult patients undergoing HSCT were included in the study. A total of 110 patients received HSCT at Karolinska University Hospital, Huddinge between October 2007 and May 2009, 77 of whom were between 20 and 67 years of age. Of these, 19 were not available for inclusion and 1 patient died before the study period ended. Fourteen patients were partly treated in home care, and could not be followed. The mean age of the 43 participating patients was 49 ± 11 . MAC was given to 19 patients and 24 received RIC, depending on the diagnosis and other contributing factors. The diagnosis and other baseline characteristics are given in table 3. There were 14 HLA-identical sibling/related donors and 24 MUDs. In 5 cases, a mismatched unrelated donor was used. Thirty-four patients were given prophylaxis for GVHD with MTX and CsA, and 4 with CsA; 13 received sirolimus and tacrolimus. Patients treated with MTX were also given calcium folinate as prevention against mucositis.

Table 3. Baseline characteristics of the HSCT recipients examined.

	I	II	III	IV
Variables	(n = 44)	(n = 74)	(n = 171)	(n = 43)
Mean age ± SD	9±2	8±4	46±14	49±11
Age range (years)	(5–13)	(1–13)	(12–71)	(20–67)
Male / Female	30/14	39/35	91/80	18/25
Underlying disease				
AML	5	17		22
ALL	26	25		1
CML	1	4		4
Other hematological malignancies	5	4		12
Severe aplastic anemia	7	5		2
Immunodeficiencies, hematological defects, or metabolic disorders	0	19		2
Non-malignant disease	7	24	10	4
Malignant disease	37	50	161	39
Conditioning regimen				
sTBI + chemo	27	35	37	6
fTBI + chemo	17	14	13	2
Bu-based		25	38	16
Other chemo			19	83
RIC	0	3	99	19
MAC	44	71	72	24

AML; acute myeloid leukaemia, ALL; acute lymphoblastic leukaemia, CML; chronic myeloid leukaemia, sTBI; single dose total body irradiation, fTBI; fractionated total body irradiation, chemo; chemotherapy, Bu; busulfan, RIC; reduced intensity conditioning, MAC; myeloablative conditioning

In all four studies, serology for the most common viruses in the herpes virus family was examined before HSCT in recipients and donors. The viral serology is shown in table 4.

Table 4. Total herpes group serology in all patients and donors prior to HSCT (studies I–IV). Subjects seropositive to 0–2 or 3–4 herpesvirus family members (percent) are shown.

Herpes virus family load	Recipient 0–2	Recipient 3–4	Donor 0–2	Donor 3–4
Study I (n = 44)*				
sTBI	30	70	56	48
fTBI	35	65	13	80
Study II (n = 74)**				
sTBI	41	59		
fTBI	29	71		
Bu	42	58		
Study III (n = 171)	8	92	74	26
Study IV (n = 43)	2	98	21	79

*In Study I, serology was not evaluated in 4 of the donors

** In Study II, serology was not evaluated in 1 patient in the sTBI and Bu groups each

Methods

Unstimulated and stimulated salivary secretion rate (I, II)

A clinical examination was performed at the Division of Pediatric Dentistry at baseline, usually about 2 weeks prior to HSCT, and 3, 6, 12 months after HSCT; and thereafter at yearly intervals. The patients were instructed not to eat, drink, put anything in the mouth, or brush their teeth at least one hour before the examination. The saliva was collected in a quiet examination room. An attempt was made to hold the examination in the morning, preferably before lunch.

Unstimulated whole saliva was collected over 10 min. The patients were asked to sit still, bow their head, and try not to move during this time. Immediately before the test, they were instructed to swallow any remaining saliva in the mouth. The saliva was allowed to accumulate in the mouth and was collected in the vessel approximately once a minute. The volume was recorded, expressed as ml/min. A USSR of less than or equal to 0.1 ml/min was considered low.¹⁷⁷ After a 5-min break, paraffin chewing-stimulated whole saliva SSSR was collected over 5 min. The patient was asked to chew a standard piece of paraffin without swallowing, and to put the stimulated saliva in a collecting vessel. (Before the collecting procedure started, the paraffin wax was chewed for one minute, and then the patient was instructed to swallow all the saliva in the mouth). The volume of saliva then produced was recorded and SSSR was expressed in ml/min. An SSSR below or equal to 0.5 ml/min was considered low.^{177,196}

Busulfan concentration determination (II)

The concentration of Bu in plasma was determined for each patient. The AUC for the first and the last dose of Bu was calculated according to a one-compartment open model using Win Non Lin software.²³²

Oral examination (III, IV)

Prior to HSCT and before the start of conditioning, a clinical examination of the oral mucosa and a radiographic examination of the jaws and teeth were performed. The clinical examination was performed at the Department of Dental Medicine, Karolinska Institutet, Huddinge. The mucosa, teeth, and saliva were examined for any pathological changes. The gingiva was only inspected visually as probing of gingival pockets could not be performed because of cytopenia. The presence of gingival inflammation was noted as 0 (normal), 1 (mild), or 2 (severe). Supragingival calculus was recorded for all teeth as being present or absent, and was removed before conditioning.

Intraoral radiographs and a panoramic radiograph (OPG) were taken. Dental infectious foci were treated conservatively and marginal bone loss was recorded from intraoral radiographs using standardized techniques.²³³ Pathological periodontal bone loss was considered when the patient had a distance from the cemento-enamel junction to alveolar bone crest of > 2 mm.²³³ The presence of subgingival calculus was recorded for proximal surfaces of premolars and molars (Figure 4).

Sampling of gingival crevicular fluid (IV)

On three occasions—before conditioning, during, and after HSCT—gingival crevicular fluid (GCF) was collected at two sites, 16 and 36, from each patient using a paper strip (Periopaper). The sampling was performed before the clinical oral examination. Where there was a missing tooth or periodontal disease, the adjacent premolar was used. Before the sampling, the surface of the tooth was gently dried with cotton pellets or air to remove supragingival plaque. The strip was then inserted in the gingival crevice and left there for 30 seconds. The strip was analyzed using a Periotron 8000 (Pro Flow) and the volume of fluid was calculated by interpolation from a standard curve and expressed as μl GCF. The Periopaper was placed in Eppendorf tubes containing 120 μl buffered saline, and kept frozen at -70°C (Figure 4).

Serum sampling (IV)

On the same day as the gingival crevicular fluid was collected, 10 ml serum was also taken. The serum sample was immediately centrifuged and then kept frozen at -70°C .

Analysis of cytokines (IV)

All samples from GCF and serum were analyzed for levels of IL-1 β , TNF- α , IL-6, IL-7, and IL-10 (pg/ml) using commercially available kits (Bio-Plex Cytokine) in accordance with the manufacturer's instructions. For higher validity regarding cytokine levels, the measurements from the two sites were pooled when being analyzed statistically.

Bedside clinical examinations (III, IV)

Oral mucositis

The oral cavity was clinically examined daily from 3 days prior to HSCT until 25 days after HSCT or discharge (Figure 5). Clinical features of OM were recorded daily using toxicity grading of oral mucositis according to WHO criteria and also the Oral Mucositis Assessment Scale (OMAS) three times a week (Figures 6 and 7).¹⁶⁷ In Study IV, the NCI Common Terminology Criteria of Adverse Events (NCI-CTCAE) version 3 validated scale was also used (Figure 8).¹⁶⁸

The nursing staff were trained, by the investigating dentist, in daily diagnosis of oral mucositis (OM) according to WHO criteria.²³⁴ The investigating dentist or a dental hygienist collaborating with the dentist examined the oral cavity three times a week (Figure 5).

Pain in the oral cavity and in the throat was recorded using the validated visual analog scale (VAS).²³⁵ By asking the patient about his or her ability to swallow (yes/no), the oral function was evaluated. Patients were also asked about their subjective opinion about the consistency and amount of saliva (0 = normal, 1 = thickened, 2 = reduced).

Sampling of GCF

Follow-up bedside examinations for sampling of gingival crevicular fluid and serum were performed 7–10 days and 20–30 days after HSCT at the Center for Allogeneic Stem Cell Transplantation, Karolinska University Hospital, Huddinge (Figure 5).

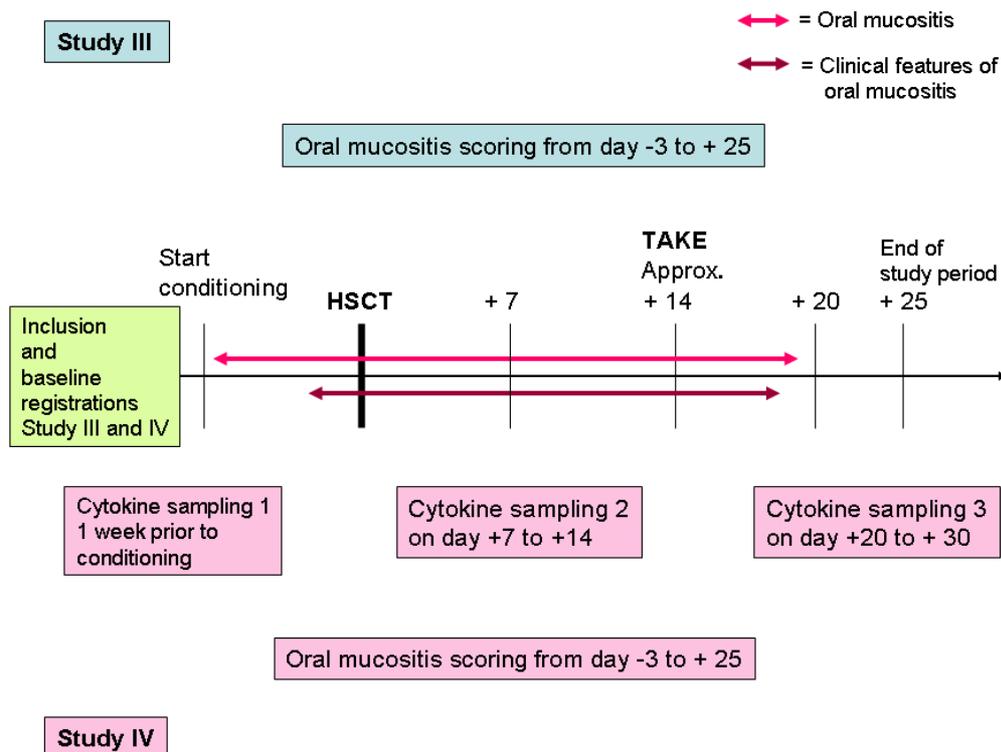


Figure 5. Time scale of oral examinations and cytokine sampling performed in studies III and IV.

Oral complication scoring methods (III, IV)

To measure and describe the severity of OM and other oral complications, objective findings, subjective findings, and a combination of both are used. The WHO score and the OMAS score are both validated scores that are commonly used.

Toxicity grading according to World Health Organization (III, IV)

The World Health Organization (WHO) grading scale was developed to describe toxicities associated with particular chemotherapy regimens or radiation therapy. It uses both objective assessment of mucosal changes (redness and ulceration) and functional outcome (inability to eat because of pain in the oral cavity or throat) to arrive at a score²³⁴ (Figure 6).

Toxicity grading according to World Health Organization				
Pain:				
VAS (0-10) Oral Cavity:	_____			
VAS (0-10) Throat:	_____			
Ability to swallow:				
Question: Can you eat?	_____			
Question: Can you drink?	_____			
Analgetics for oral pain?	Yes	No		
Circle:				
Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
None	Soreness/ erythema	Erythema/ ulcers can eat solids	Ulcers, requires liquid diet only	Alimentation not possible

Figure 6. Toxicity grading according to World Health Organization, which was used every day from day -3 to day +25 in studies III and IV.

The Oral Mucositis Assessment Scale

The Oral Mucositis Assessment Scale (OMAS) score was developed for investigative applications. This scale separates objective findings from subjective ones. Primary indicators of OM are the degree of ulceration and redness, measured at specific sites in the mouth. A single score is not produced from this scale; instead, there are scores for ulceration and redness based on different locations in the mouth (Figure 7).¹⁶⁷

Oral Mucositis Assessment Scale
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Location	Ulceration /pseudomembrane				Erythema		
Upper lip	0	1	2	3	0	1	2
Lower lip	0	1	2	3	0	1	2
Right cheek	0	1	2	3	0	1	2
Left cheek	0	1	2	3	0	1	2
Right ventral and lateral tongue	0	1	2	3	0	1	2
Left ventral and lateral tongue	0	1	2	3	0	1	2
Floor of the mouth	0	1	2	3	0	1	2
Soft palate/fauces	0	1	2	3	0	1	2

Ulceration/pseudomembrane: 0 = no lesion
 1 = < 1 cm²
 2 = 1 – 3 cm²
 3 = > 3 cm²

Erythema: 0 = None
 1 = Not severe
 2 = Severe

Figure 7. Oral Mucositis Assessment Scale (OMAS) used three times a week from day -3 to day +25 in studies III and IV.

NCI-CTCAE v. 3

NCI Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3 was used for scoring pain in the mouth, together with the VAS scale described above. The NCI-CTCAE v. 3 displays grades 1 through 5 with clinical descriptions of severity for each adverse event²³⁶⁻²³⁷ (Table 5).

Table 5. The NCI-CTCAE v. 3 grading scale, based on the general guideline, which was used for scoring of pain in the oral cavity every day from day -3 to day +25 in study III.

GRADE	SEVERITY
0	None
1	Mild
2	Moderate
3	Severe
4	Life-threatening or disabling
5	Death related to adverse event(s)

Oral hygiene protocol (III)

From the start of the study in October 2007 to October 2010, patients (n = 142) followed a dental hygiene protocol including tooth brushing twice a day with a super-soft toothbrush only. The patients were further instructed not to brush on their gingival mucosa and not to use interdental brushes and flossing. Mouth rinses were not allowed. From the end of October 2010 to May 2011, the remaining 29 patients in the study received a more intensive oral hygiene protocol including the use of interdental brushes or flossing in addition to brushing with a soft toothbrush twice a day. Furthermore, the patients were instructed to suck on ice chips every second or third hour of being awake and also to rinse the mouth with isotonic saline solution every second hour of being awake, from transplantation until a neutrophil blood cell count of $> 0.5 \times 10^9/l$ was reached. The patients were not allowed to suck on ice chips during administration of chemotherapeutic agents. The new oral hygiene protocol was a modification of the recommendation of the Multiprofessional Association of Supportive Care in Cancer.²³⁸

Collaboration in scoring of oral mucositis (III, IV)

The investigating dentist was educated in OM scoring using the both scales (WHO and OMAS) both from an experienced colleague and by reading the literature.²³⁹ After this education, the dentist was trained against other colleagues skilled in OM scoring—using patients and cases on paper or in photographs.

A dental hygienist was educated and trained by the investigating dentist, using both scoring methods in the same way as described above. The agreement in scoring was controlled by several blind tests in patients with different scores of OM.

The nurses were trained repeatedly in using the WHO OM score (by the investigating dentist)—both by lectures and by training on real cases at the ward. The agreement in scoring was tested against the dentist and the dental hygienist and also against each other, using several blind tests in patients with different scores of OM.

Risk factors (I-IV)

Several risk factors for complications in the setting of HSCT are known. Of those, some are associated with the prognosis of the transplantation but most are related to the risk of developing different kinds of complications including acute and chronic oral complications. In studies I and II, possible risk factors for low salivary secretion rate after HSCT such as gender, age, conditioning regimen, seropositivity for herpesviruses, and GVHD were tested in a multivariate analysis against unstimulated and stimulated whole saliva secretion.²⁴⁰ Known possible risk factors for oral mucositis such as age, gender, conditioning regimen, MTX, donor source, and aGVHD were tested in studies III and IV, in multivariate analysis against OM.^{165,166}

Statistical analyses (I-IV)

Study I

Power analysis

With a power of 90%, an α -error of 0.05, and a calculated mean saliva production of 1.0 and 0.4 ml/min (SD 0.5) in the two groups, we calculated that we would require 16 patients in each group.

Studies I and II

As cut-off points for salivary dysfunction, a stimulated whole-salivary secretion rate of below or equal to 0.5 ml/min was chosen. Comparisons between groups of patients were performed using Fisher's exact test and the Mann-Whitney U-test. The logistic regression model was used in univariate analyses of possible risk factors that might contribute to a low salivary secretion rate. Furthermore, we used multivariate logistic regression analysis, where significant variables at the 5% level from the univariate analyses were included.

Study III

The patient baseline characteristics (RIC vs. MAC) were compared using Fisher's exact test and the Mann-Whitney U-test. For possible risk factors associated with a higher summarized OM score on days 9–12 after HSCT, a multiple regression model was used in uni- and multivariate analyses. Maximum possible summary score was 16. Significant variables at the 5% level from the univariate analyses were included in the multivariate regression analysis.

Study IV

For comparison of concentrations of GCF between the different time points, the Wilcoxon matched-pair test was used. Fisher's exact test and the Mann-Whitney U-test were used for comparisons between groups of patients. For possible risk factors for a higher total OM score during days 9–12, univariate and multivariate analyses were performed using the multiple regression method. Significant variables at the 5% level from the univariate analyses were included in a multiple regression analysis.

All statistical analyses in studies I–IV were performed using Statistica software.

Ethical considerations

The local ethical committee at the Karolinska University Hospital, Huddinge, approved the protocol for this study.

RESULTS

The four studies included in this thesis evaluated conditioning related effects on salivary function and the oral mucosa as well as other risk factors for salivary dysfunction and OM related to allogeneic HSCT. In studies I and II, we focused on salivary dysfunction in children treated with different conditioning regimens before allogeneic HSCT. In study III, we investigated severity of OM in patients treated with MAC or RIC in preparation for allogeneic HSCT. Furthermore, we determined other risk factors for OM in HSCT. Finally, in study IV we investigated the relationship between OM and production of pro-inflammatory cytokines both in serum and in GCF before, during, and after HSCT and in relation to different conditioning regimens and other risk factors for OM. This section gives a brief overview of the results of these studies followed by a discussion of the findings in relation to the current literature.

Unstimulated and stimulated salivary secretion rate after HSCT (I, II)

In study I, we found no significant differences between USSR and SSSR in the sTBI and fTBI groups at baseline. In the sTBI-treated group, the median USSR was reduced by 74% (0–94) one year after HSCT as compared to a median reduction of 33% (0–80) in the fTBI group ($p = 0.003$). The median reduction in SSSR in the sTBI group one year after HSCT was 56% (0–93) as compared to 12% (0–70) in the fTBI group ($p = 0.003$). Sixty-three percent (17/27) of the children in the sTBI group and 24% (4/17) in the fTBI group had an SSSR of < 0.5 ml/min one year after HSCT ($p = 0.015$).

Fifteen children in the sTBI group and 6 in the fTBI group were diagnosed with chronic GVHD, but they showed no statistically significant reduction in salivary flow rate compared to children with no chronic GVHD.

In study II, we found that irrespective of the type of conditioning—sTBI, fTBI or Bu—there were no significant differences in USSR or SSSR at 15 years of age. Forty-seven percent of the children (16/35) in the sTBI group, 47% (7/15) in the fTBI group, and 42% (10/24) in the Bu group had an SSSR less than or equal to 0.5 ml/min.

In the entire cohort, the SSSR in girls ($n = 35$) was significantly lower at 15 years of age (0.7 ± 0.3 ml/min) than in boys ($n = 39$; 1.1 ± 0.4 ml/min) ($p < 0.001$). Furthermore, the USSR was significantly lower in girls (0.3 ± 0.2 ml/min) than in boys (0.5 ± 0.4 ml/min) ($p < 0.05$). Sixty-six percent of the girls (23/35) had an SSSR of ≤ 0.5 ml/min at 15 years of age, as compared to 25% (10/39) in boys ($p < 0.001$).

In children conditioned with sTBI/Cy, there was also a significant correlation between the age at stem cell transplantation and the SSSR at 15 years of age ($p = 0.02$). The younger the patient was at conditioning, the lower the salivary secretion

rate at 15 years of age. Children conditioned with fTBI/Cy or Bu/Cy did not show this correlation (both $p = 0.35$).

Twenty-one children in the cohort were diagnosed as having GVHD. There was no difference in salivary secretion between patients with or without chronic GVHD (cGVHD). In patients with cGVHD, the mean USSR was 0.36 ± 0.21 ml/min and the corresponding value in patients without cGVHD was 0.36 ± 0.22 ml/min ($p = 0.93$). The mean SSSR in patients with cGVHD was 0.84 ± 0.40 ml/min and in patients without cGVHD it was 0.91 ± 0.52 ml/min ($p = 0.90$).

Stimulated salivary secretion and the distribution of plasma AUC of busulfan (II)

The distribution of the plasma AUC of Bu expresses the total exposure to Bu. In the patient group, AUC varied between 3,301 and 8,986 ng/ml/h. There was a significant inverse correlation between the plasma AUC of Bu and the stimulated salivary secretion rate measured at 15 years of age.

Risk factors for salivary dysfunction (I, II)

In study I, significant risk factors for a low stimulated salivary secretion rate in the univariate logistic regression analysis one year after HSCT were conditioning regimens including sTBI, and recipient seropositivity for 3–4 herpesviruses (as compared to 0–2) prior to HSCT. None of the viruses examined (HSV, EBV, CMV, or VZV) were individually correlated with salivary dysfunction.

Both the identified risk factors remained significant in the multivariate analysis; sTBI (OR = 6.49, 95% CI = 1.40–30, $p = 0.014$) and seropositivity of recipients for 3–4 herpes viruses (OR = 6.57, 95% CI = 1.26–34, $p = 0.021$).

There was an inverse relationship between the number of risk factors present and the mean degree of stimulated salivary secretion. With no risk factor present ($n = 6$), the median SSSR was 1.2 ml/min (0.80–1.75); for one risk factor present ($n = 19$), median SSSR was 0.67 ml/min (0.08–1.70), and if both risk factors were present ($n = 19$), it was 0.3 ml/min (0.07–0.80).

The univariate logistic regression analysis in study II showed that female gender was associated with a low salivary secretion rate (≤ 0.5 ml/min) at 15 years of age (OR = 4.89, 95% CI = 1.76–13.61, $p = 0.002$). The female-donor-male-recipient situation was associated with higher salivary secretion (OR = 0.23, 95% CI = 0.06–0.95, $p = 0.039$).

Both of the significant risk factors were included in the multivariate model and female gender remained significantly correlated with low stimulated salivary secretion rate at 15 years of age (OR = 3.93, 95% CI = 1.21–12.79, $p = 0.021$).

Oral mucositis (III, IV)

Of all the 171 patients included in study III, 24 (14%) did not develop any subjective or clinical signs of OM during the study period. The peak mean WHO OM score was 1.7 and occurred on days 10–11. On day 11, the observed distribution of WHO OM score was as follows: score 0, $n = 37$ (24%); score 1, $n = 28$ (18%); score 2, $n = 53$ (34%); score 3, $n = 23$ (15%); and score 4, $n = 11$ (9%) (Figure 8). On days 9–12, when the data were combined, 21 patients had a total OM score of 0, 82 patients had

a total OM score of 1–8, 28 patients had a total OM score of 9–12, and 14 patients had a total score of 13–16; 6 patients had the maximum total score of 16. Data are missing in 26 patients.

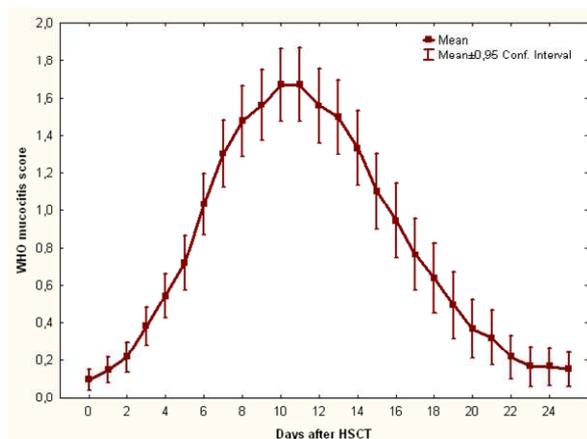


Figure 8. Oral mucositis score according to WHO, in 171 allogeneic HSCT recipients in Study III.

All 43 patients in study IV experienced OM of some score during the transplantation. The peak in mean WHO score was 2.0 (95% CI: 1.6-2.4) and that peak occurred on day 11. Most of the patients ($n = 22$) (51%) had a WHO score of 2, and 6 patients (14%) had the highest WHO OM score of 4.

Conditioning regimens and oral mucositis (III, IV)

In the multivariate analysis, MAC remained significantly correlated with higher total OM score (> 2) on days 9–12 after HSCT (RH = 1.57, 95% CI = 1.37–1.80, $p < 0.001$).

Furthermore, in patients conditioned with Bu, a significantly higher total WHO OM score at days 9–12 was seen in the univariate analysis (1.19, $p = 0.043$) comparing RIC with MAC. There were no differences between the groups, RIC and MAC, whether the conditioning protocol included TBI ($n = 51$) or whether the patients were treated with cytotoxic drugs only ($p = 0.50$).

Risk factors for oral mucositis (III, IV)

In study III, we identified several risk factors associated with higher total WHO OM score on days 9–12 after HSCT in the univariate analysis. Excluding MAC (described earlier), these were; all donor-recipient gender combinations—except for the female-donor-male-recipient situation (hazard ratio (HR) = 1.27, $p = 0.003$), female patient (HR = 1.19, $p = 0.04$), and recipient seropositive for 3–4 herpesviruses (HR = 1.21, $p = 0.02$). Also, patients receiving their second HSCT had a lower OM score than during the first HSCT (HR = 0.82, $p = 0.017$). In patients with different diseases, malignant or non-malignant, there was no difference in the severity of OM.

Thirty-five patients were diagnosed as having aGVHD of grade I, 58 patients as having aGVHD of grade II, and 11 patients as having aGVHD of grades III–IV. Sixty-seven patients were diagnosed as not having aGVHD. There was no difference in severity of OM between patients with and without aGVHD. Patients who were treated with drugs including MTX as GVHD prophylaxis ($n = 120$), did not develop

more severe OM than those treated with other immunosuppressive agents (n = 51). Other suggested risk factors for OM such as stem cell source and nucleated cell dose did not significantly affect the severity of OM; nor did prolonged time to engraftment compared to those with shorter time to engraftment. The median number of days to reach a neutrophil count of $> 0.5 \times 10^9$ was 17 (0–47).

In the multivariate analysis, MAC, as described above, all donor-recipient gender combinations except the female-donor-male-recipient situation (RH = 1.26, 95% CI = 1.10–1.44, p = 0.001), and year of HSCT (RH = 0.84, 95% CI = 0.73–0.96, p = 0.013) remained significantly correlated with total WHO OM score for days 9–12. There was also a significant relationship between the number of risk factors present and the OM score after HSCT (p < 0.001).

In study IV, MAC was found to be significantly associated with higher WHO OM score from day 8 to day 11 after HSCT (p < 0.05). Patients undergoing an HSCT protocol including TBI (n = 8) did not develop higher OM scores than patients who were conditioned with cytotoxic drugs only. Treatment with ATG (n = 31) was associated with less OM on days 6–14 compared to patients not treated with ATG (n = 12) (p < 0.05).

A total of 25 patients were diagnosed as having aGVHD. Of those, 6 patients had aGVHD of grade I and 19 had grades II–IV. There was no difference in severity of OM between patients with different grades of aGVHD; nor was there a difference in patients treated prophylactically with MTX against GVHD (n = 26) or with other agents (n = 17).

In the multivariate analysis, MAC remained significantly correlated with the total OM score during days 9–12 after HSCT (OR = 1.37, 95% CI = 1.03–1.82, p = 0.035). Patients with a donor positive for 3–4 herpesviruses also had a higher OM score for days 9–12 in the multivariate model (OR = 1.42, 95% CI = 1.08–1.87, p = 0.02). The number of risk factors and higher WHO OM score (> 2) showed a significant relationship (p = 0.01). With more risk factors present, the risk of having a higher OM score increased.

Oral care and hospitalization (III)

The year when patients were treated with HSCT was significantly associated with the total OM score (HR = 0.82, p = 0.015). Patients treated during the last year, 2011 (n = 29), with the new oral care protocol had a significantly lower OM score than those treated in earlier years. There was also a significant correlation between OM for days 13–24 and hospitalization (day 15: r = 0.31, p < 0.001; day 23: r = 0.45, p < 0.001). However, when comparing the number of days of hospitalization between patients transplanted in 2011 (mean 25, 95% CI 21–29 days) and patients transplanted in 2007–2010 (mean 24, 95% CI 22–26 days), no significant difference was found (p=0.25).

Other acute oral complications (III, IV)

In both studies, pain in the oral cavity and throat and the patients' experience of the quality of their saliva, was associated with severity of OM.

The reported pain in the oral cavity and throat on day 11 in study III, using VAS and NCI-CTCAE v.3, are presented in table 6. All patients who had a WHO OM score of 0 also scored 0 using VAS or NCI-CTCAE v.3 scale.

In study IV, the mean reported pain in the oral cavity on day 11 was ≥ 5 in 21 patients (49%) using VAS. The corresponding mean value for pain in the throat was ≥ 5 in 23 patients (53%).

Also at day 11, twenty-five patients (58%) experienced that their saliva was reduced, 12 (28%) described it as thickened, and 6 (14%) described it as normal.

Table 6. Pain in the oral cavity and throat measured with VAS and NCI-CTCAE v.3 (only oral pain) on day 11 in Study III. Data are missing for 18 patients.

	Pain, oral cavity n =	Pain, oral cavity %	Pain, throat n =	Pain, throat %
VAS				
0–2	86	56	78	51
3–4	29	19	29	19
5–6	21	14	27	18
7–8	12	8	14	9
9–10	5	3	5	3
NCI-CTCAE				
0	37	22		
1	66	39		
2	33	19		
3	12	7		
4	5	3		
5	0	0		

Correlation between WHO oral mucositis scoring system and OMAS (III)

Comparing the two oral mucositis grading scales used, i.e. grading of toxicity according to WHO and the OMAS, there was a good correlation (Figure 9) (day 10: $r = 0.74$, $p < 0.001$).

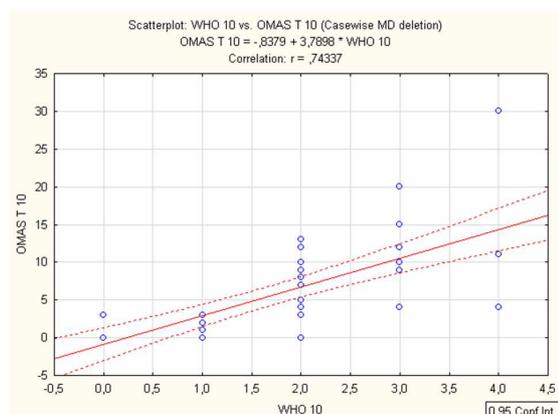


Figure 9. Correlation between toxicity grading according to WHO and OMAS on day 10.

Cytokines in gingival crevicular fluid and serum (IV)

There was no difference in the volume (μl) of GCF observed before, during, and one month after HSCT or in patients with different conditioning, MAC or RIC. The mean GCF volume was 0.40 ± 0.20 at T0, 0.45 ± 0.21 at T1, and 0.40 ± 0.17 at T2. In GCF, levels of IL-1 β tended to increase during transplantation but compared to the baseline examination, the value did not reach statistical significance ($p = 0.06$). After HSCT, the GCF levels decreased significantly below baseline values ($p = 0.004$). IL-6 was significantly increased during the transplantation relative to the baseline examination ($p < 0.001$) and then returned to baseline levels after transplantation ($p < 0.001$). IL-10 levels were reduced during transplantation ($p < 0.000$), and then remained unchanged at the examination one month after HSCT. The GCF levels of TNF- α and IL-7 did not change during the study period.

In serum samples, the levels of IL-6 were significantly increased during transplantation ($p = 0.001$), and then returned to baseline values after HSCT ($p = 0.04$). IL-10 levels remained unchanged during HSCT and then increased after HSCT ($p = 0.02$). Levels of IL-1 β , IL-7, and TNF- α only showed small variations that were not statistically significant.

There was an increase in serum levels of IL-6 that correlated with severity of oral mucositis on days 8 ($p < 0.05$). There were no significant correlations between cytokines in GCF and severity of OM.

Cytokines in gingival crevicular fluid and serum and risk factors for oral mucositis (IV)

Conditioning

In patients conditioned with MAC, there was a significant increase in IL-10 during transplantation compared to conditioning with RIC ($p = 0.024$). No other statistically significant variations were observed.

In serum, the level of IL-6 was increased during HSCT in patients conditioned with MAC as opposed to RIC ($p = 0.016$). TNF- α levels were reduced during HSCT with MAC ($p = 0.004$), as were those of IL-7 at all three time points examined: T0 ($p = 0.015$), T1 ($p = 0.004$), and T2 ($p = 0.003$) relative to RIC.

Septicemia

Ten patients were diagnosed as having septicemia during HSCT. Five of these patients had α -streptococci in the blood. There were no statistically significant changes in cytokine levels in GCF in patients with or without septicemia.

In serum, IL-6 was significantly increased during HSCT in patients with septicemia in comparison to those without septicemia ($p < 0.01$).

There were no significant correlations between cytokine levels in GCF and in serum.

GENERAL DISCUSSION

HSCT and salivary secretion rate

The influence of the conditioning on salivary secretion rate during the first year in study I was significant. The sTBI children had a significantly lower secretion rate than those in the fTBI group one year after HSCT. Chaushu et al. reported a similar immediate effect on the parotid secretion rate whether the patients had received TBI or not during the conditioning procedure, but in contrast to the TBI-treated patients, there was recovery in the patients not treated with TBI already after two to five months.²⁴¹ Previous studies from our group have shown that 79% of the children treated with sTBI have at least one or several symptoms of xerostomia five years after HSCT.²⁴² Adkins et al. have shown that there is an inverse relationship between the intensity of TBI as conditioning regimen on the one hand and the toxicity, treatment-related mortality, and the risk of relapse on the other.³⁰ The optimal regimen for TBI in allogeneic HSCT is still to be determined, because it depends on many different variables such as patient age, conditions regarding co-morbidity, disease characteristics, dose rate and fractionation, source and dose of stem cells, and also GVHD prophylaxis. In our study 24% of the children in the fTBI group also showed evidence of salivary dysfunction one year after HSCT. It is evident that fTBI also can cause damage to progenitor secretory cells with subsequent loss of their capacity to multiply. The present investigation describes the effect of dose fractionation of TBI on salivary secretion rate for the first time.

There is an increase in salivary secretion rate until the age of 15 years.¹⁹⁶ Girls have significantly lower salivary secretion rates than boys of all age groups; because of their size.¹⁹⁶ The risk of reduced salivary secretion rate after HSCT is therefore higher in girls. Female sex was also the second most important risk factor for low salivary secretion rate one year after HSCT. In this group of long-term survivors treated with HSCT, the level of salivary output was lower than levels reported in healthy teenagers. The stimulated salivary secretion rate at 15 years of age varied between 0.2 ml/min and 2.2 ml/min. About 45% had a stimulated secretion rate equal to or below 0.5 ml/min. In the present study, the mean follow-up time was 8 years. Oeffinger et al. observed that the incidence of health conditions reported in this group of patients increases with time.¹²⁷ Furthermore, in a study by Dahllöf et al., 70% of the children conditioned with TBI/CY had a salivary secretion rate that never exceeded the baseline value for the irradiated children during the four-year follow-up period, indicating that the reduced salivary secretion rate might be permanent in this group of children.²⁴³ Surprisingly, in our study there was no difference in salivary output between the three groups investigated, sTBI, fTBI, and Bu, at 15 years of age. This indicates that the sTBI-induced decrease in salivary secretion rate may be transient and that same capacity of the progenitor secretory cells to multiply is maintained. Furthermore, this finding shows that all regimens investigated cause damage to the salivary glands in the long term.

A negative correlation between age at conditioning and the salivary secretion rate was found in the sTBI group when measuring saliva at 15 years of age in the patients. This correlation was not seen in the fTBI and Bu groups. Hematopoietic cells are less capable of DNA repair than other tissue cells. In order to reduce the damage and risk of late organ toxicity, fTBI is used.²⁴⁴ In this study, the results indicated that the damage to the salivary glands was more extensive and more permanent the younger the child was when treated.

In children conditioned with Bu, salivary dysfunction was significantly correlated with the total systemic exposure of Bu, probably due to the fact that Bu is equally distributed to saliva and plasma. Over a period of four days, the salivary glands are exposed to high concentrations of Bu.⁵¹ This is in agreement with previous studies on other side effects of Bu.⁴⁹ To reduce all these side effects, is it important to monitor blood levels of Bu with dose adjustment to achieve a targeted steady-state concentration.

Reduced salivary secretion rates have been found following cGVHD,²⁴⁵ but it has not been possible to differentiate the reduced salivary secretion rates caused by cGVHD from those caused by irradiation.²⁴⁶ In the present studies, and also in a study by Jones et al., no reduction in salivary secretion rate was found in patients with cGVHD when they were off immunosuppressive treatment for cGVHD.²⁴⁷

Measurements of unstimulated and paraffin-stimulated whole saliva were used in the first two studies. The methods are simple, and the variation during the day is slight.²⁴⁸ Greater volumes of chewing-stimulated saliva than of unstimulated saliva can be collected in a reasonable amount of time. That is one of the reasons why stimulated secretion is usually used in examinations of salivary function. Unstimulated saliva has been suggested as the sialometric method of choice for the evaluation of Sjögren's syndrome.²⁴⁹ A good correlation has previously been reported between resting and paraffin-stimulated salivary flow.¹⁹²⁻¹⁹³ This was also found in our studies.

Risk factors for salivary dysfunction

In study I, in the univariate analysis we identified sTBI and recipient seropositivity for 3–4 herpesviruses as being risk factors for low salivary secretion. When using a multivariate logistic regression model, both risk factors remained significantly correlated with a stimulated salivary secretion rate below or equal to 0.5 ml/min one year after HSCT. The second most important risk factor, after TBI, was seropositivity of the recipient for 3–4 herpesviruses. In Sjögren's syndrome and non-specified sialadenitis, CMV and EBV have been suggested to be correlated with hypofunction of the salivary gland.²⁵⁰ Another herpesvirus found in patients with Sjögren's syndrome is HHV-6, which has been suggested to have salivary glands as its site of replication.²¹⁶ There may be a possibility of reactivation of latent herpesviruses in the salivary glands, during conditioning with TBI or chemotherapy and during the period of immunosuppression following HSCT, which might contribute to salivary dysfunction. Herpesviruses, especially HHV-6, are also a causative factor in the development of GVHD.²⁵¹⁻²⁵² GVHD is often accompanied by salivary gland dysfunction during the active phase of disease, within five months of HSCT.²⁵³ In this study, GVHD was not a significant risk factor for salivary dysfunction one year after HSCT. With more risk

factors present, the risk of having a reduced salivary secretion rate one year after HSCT was higher.

Female gender was found to be associated with a salivary secretion rate at 15 years of age of less than or equal to 0.5 ml/min, while a female-donor-male-recipient transplantation was associated with higher salivary secretion. Female donor has been reported earlier to be a risk factor for HSCT with increased transplantation-related mortality, relapse, and reduced leukemia-free survival.²⁵⁴

In the multivariate model, only the variable female gender remained significantly correlated with low stimulated salivary secretion rate. This is not surprising, and was probably due to the fact that girls are smaller and consequently have a lower salivary gland secretion than boys.

Oral mucositis

The majority of patients included had OM. In study III, 86% were affected with OM and in study IV, 100%. The mean severity in both studies was approximately WHO score 2 with a small decrease during recent years. The findings in our studies are in agreement with those in earlier studies.

Ulcers in the oral mucosa in patients conditioned with MAC have been found to affect between 29% and 100%, and range from aphthous-like lesions to generalized desquamation.¹⁶⁰ In study III, 34% of patients had a mean WHO score of 2 and in study IV, 51% had a mean WHO score of 2 at the mean peak (day 11). When summing the WHO OM scores for days 9 to 12, 71% of the patients had a total score of between 1 and 8. The result shows a decrease in severity of OM thus the patients in study IV were included first and subsequently scored in earlier year than patients included in study III. We found that there was a significant correlation between year of transplantation and severity of OM. This was probably due to RIC being used more in recent years, but also to the intensified standard of oral care that was introduced. From 2007, the standard of oral care was intensified at our center. The patients were visited three times a week by a dentist or dental hygienist and supported in their oral care. We believe that this support in oral care, careful monitoring of the status of the oral cavity, and early detection of oral infections were important factors for the decrease in OM in later years.²³⁸⁻²³⁹ During the last seven months of our study, the oral care protocols were intensified further. Also, a modified oral cryotherapy was added that included sucking on ice chips every second or third hour of being awake.²⁵⁵ Furthermore, the patients were instructed to rinse their mouth with isotonic saline solution every second hour of being awake until a neutrophil blood cell count of $> 0.5 \times 10^9/l$ was reached. The incidence and severity of OM clearly decreased after the introduction of this protocol. Despite the short period of observation, the results were statistically significant. This demonstrates the importance of an interdisciplinary approach to systematic oral care protocols that takes the individual needs of the patient into account. Optimal oral hygiene may help to avoid secondary infection from ulcers in the oral mucosa and systemic infections, and also improve the patient's quality of life. The results show the importance of regular education in OM and oral care of health care professionals and also the need for dental professionals. The peak in mean WHO OM score was on days 10–11 in study III and day 11 in study IV, which is to be expected in patients conditioned in preparation for allogeneic HSCT.

In both oral mucositis studies, pain in the throat and the oral cavity was correlated with the severity of the OM score. Using the NCI-CTCAE v.3 to measure pain in the oral cavity, the results showed that at day 11, 103 patients (67%) had NCI-CTCAE of grade 1. This was probably due to effective analgetics or mild pain in the patients with mild OM or no OM at all.

The majority of patients in both studies experienced that their saliva was reduced (study III: 46%; study IV: 58%). This is to be expected because of the pharmacokinetic effect on the salivary glands of the drugs administered, and radiation given.

Risk factors for oral mucositis

Several risk factors were identified in both studies III and IV. There was a significant relationship between the number of risk factors present and WHO OM score of > 2 after HSCT. With more risk factors present, the risk of having a higher OM score increased. This is in agreement with the assumption that toxicity is multifactorial and also genetically determined.²⁵⁶⁻²⁵⁷

In both studies, MAC was significantly associated with a higher WHO OM score in the multivariate analysis compared to RIC. Historically, risk of mucositis has been associated with the treatment and with host factors.²⁵⁸ Treatment-related variables include those associated with the type of therapy, dose, and route of transmission. To a large extent, treatment type and dose can be overwhelming risk factors. Patients receiving conditioning regimens in preparation for HSCT have been considered to be at high risk of mucositis, especially with treatment including TBI or high doses of stomatotoxic drugs. In an attempt to ameliorate risk, several centers have adopted less toxic protocols.

In both study III and IV, patients undergoing an HSCT protocol that included TBI did not develop a higher OM score than patients conditioned with cytotoxic drugs alone. When radiation is part of RIC, the total dose of TBI delivered is much lower. In study III, 40% of patients received TBI as part of MAC. Despite this, there were no differences in OM in patients treated with or without TBI.

In the univariate analysis in study III, there was a significant correlation between Bu and a higher OM score compared to other conditioning regimens. Busulfan is distributed equally to saliva and plasma, the oral mucosa is exposed to high concentrations of this alkylating agent for four days.⁵¹ Tissues with a high turnover rate, such as the cells of the oral mucosa, are more likely to be affected by cytotoxic drugs.¹⁵⁰ It is possible that this high concentration of Bu both in serum and in the saliva causes an impaired ability to induce immune defense reactions, and therefore more severe OM.

It is clear that factors other than therapy are critical and determine risk, and it is still unclear why patients of the same age—with the same malignancy and chemotherapy regimens—develop mucositis of different severity and with different frequency.²⁵⁶

In both studies, seropositivity to herpesviruses was significantly associated with OM. Seropositivity to 3–4 herpesviruses in the recipient prior to HSCT was significantly associated with a higher WHO OM score on days 9–12 in the univariate analysis in Study III. The role of herpesviruses in the etiology of mucositis has been the subject of speculation for some time, and remains controversial. Woo et al. showed that development of OM was unrelated to HSV antibody status or positive viral culture, and that acyclovir prophylaxis was ineffective in preventing OM.²⁵⁹ While radiation

and chemotherapy are successful activators of latent viruses in seropositive patients, Djuric et al. found that the rate of HSV-1 reactivation was not different before or after chemotherapy. They also found that there was no relationship between the rate of viral reactivation and the presence or absence of OM.²⁶⁰

In study IV, seropositivity to 3–4 herpesviruses in the donor was significantly associated with a higher WHO score in the multivariate analysis for days 9–12. It can be speculated that cells infected with virus that reactivates may induce a tissue attack in a similar way as herpes viruses can induce an allogeneic effect in donor lymphocytes and stimulate aGVHD.²⁵²

In the multivariate analysis, we found that female-donor-male-recipient situation compared to other all donor-recipient gender combinations, was significantly associated with a lower WHO OM score on days 9–12, which we cannot explain. The statistical significance of this finding was too great for it to have occurred by chance ($p=0.001$). Female-donor-male recipient has been found to be a risk factor for GVHD possible due to the Y-chromosome being a target for GVHD.^{67,261}

Gender has been identified as a possible patient-associated risk factor for OM.²⁵⁸ In agreement with this assumption; we found that being female was associated with a higher OM score in the univariate analysis in study III. Toxicity risk is to a large extent genetically controlled, and it seems likely that differences in the expression of genes associated with OM pathogenesis affect risk.¹⁶²

Patients receiving their second HSCT developed less severe OM than after the first. This is probably due to the fact that most patients received RIC in their second HSCT, and that the second HSCT was performed in recent years with more intensive oral care protocols. In contrast to this, it is known that the risk of OM increases with subsequent cycles of treatment.¹⁶²

Acute GVHD of grades I–IV was diagnosed in 60% of patients in study III and 58% in study IV. There were no signs of oral manifestation of aGVHD. Acute GVHD can manifest as oral erythema, atrophy and sometimes ulcerations—resembling those seen in some autoimmune connective tissue diseases such as lupus erythematosus.¹⁷⁵ The prevalence of acute oral GVHD is reported to range from 20% to 33%.¹⁶⁰ There was no difference in severity of OM between patients with and without aGVHD. This may be surprising, because damage to the tissue, as OM, can pave the way for aGVHD.^{87,165} Some studies have also shown that patients conditioned with MAC have more acute GVHD than those receiving RIC. Patients who received GVHD prophylaxis including MTX did not develop more severe OM than those treated with other immunosuppressive agents. MTX inhibits DNA synthesis, and thereby causes reduced cell renewal and cell replacement. This may lead to ulceration and an impaired barrier function.²⁶²⁻²⁶³ As a consequence, the mucosal immune system is exposed to an increased amount of microbial stimuli. Since the 1980s, calcium folinate has been given to patients at our center as prophylaxis against toxicity of MTX. It is possible that our findings are due to this prophylaxis.

The median number of days to reach a neutrophil count of $> 0.5 \times 10^9$ was 17 (0–47). We found no difference in OM between patients with malignant or non-malignant disease, and between patients with short or prolonged time to neutrophil engraftment. This contrasts with studies that have shown that prolonged neutrophil recovery is a risk factor for OM.¹⁶⁵⁻¹⁶⁶ Stem cell source and nucleated cell dose did not have any significant effect on severity of OM.

Oral mucositis and hospitalization

The clinical and economic impact of oral and gastrointestinal mucositis are high. In our study, a higher OM score for days 13–23 tended to result in prolonged hospitalization. This is not unexpected, since of the patients who experience more severe oral mucositis (i.e. of WHO grade 3–4), approximately 35% will have a delay in chemotherapy, 60% will have a reduced dose of chemotherapy, and 30% will have the regimen discontinued.¹⁶⁸ The development of severe mucositis will necessitate use of a feeding tube to maintain nutrition in 70% of patients, result in fever in 60% of patients, and necessitate hospitalization in 62% of patients. Additionally, with mucositis of WHO grade 3 or 4, 70% of patients receiving standard-dose chemotherapy and 87% receiving high-dose chemotherapy with a stem cell transplant (HSCT), will require feeding tubes to maintain adequate nutrition.¹⁶⁸ Mucositis associated with autologous bone marrow transplants can extend the hospital stay by 6 days.²⁶⁴

Correlation between WHO oral mucositis scoring system and OMAS

There are multiple scoring methods to grade OM and other oral complications. A major problem has been the lack of a validated and objective scoring system for OM. The WHO scoring system and the OMAS are both validated scales.^{167,234} The WHO grading scale was developed to describe toxicities associated with a particular chemotherapy regimen or radiation therapy. The OMAS score was developed for the purpose of investigative applications. In present study, both of these scales were used in parallel and they showed good correlation. Both scales can thus be used to improve management strategies, since it is important to educate relevant target audiences so that barriers such as knowledge deficits can be avoided. The correlation between the scales also indicates that the education of the healthcare professionals has been successful. The WHO grading scale is easier to use for non-dental professionals and is not especially time-consuming. Thus, it should be easy to incorporate oral mucositis scoring as a standard routine at the hospital clinic.

Cytokines and oral mucositis

There was no difference in GCF volume between the different time points in each patient, which may indicate the low level of inflammation in the gingival or periodontal mucosa. This is also in agreement with earlier clinical observations; thus, OM is known to be most severe on the buccal and labial mucosa and the lateral and ventral surfaces of the tongue rather than on the fixed mucosa of the gingiva.²⁶⁵ The development of mucositis is thought to be driven through the activation of NF- κ B, which promotes the upregulation of certain key cytokines such as IL-1 β , TNF- α , and IL-6. Increased levels of these cytokines have been reported within the mucosa, and clinical evidence from patients undergoing chemotherapy suggests that changes in the mucosa occur prior to the development of clinical manifestations such as ulceration. Serum levels of NF- κ B, TNF- α , IL-1 β , and IL-6 have also been shown to precede histological changes in the tissue of the alimentary tract. Because of the critical time constraints between detectable serum changes and the histological damage, this has been suggested to be a useful tool in predicting mucosal damage.²⁶⁶

In the whole study cohort, the GCF levels of IL-1 β tended to increase and IL-6 increased during the transplantation period. This may be due to damage to the oral

mucosa by conditioning, neutropenia, and immunosuppression including drugs such as methotrexate and rapamune. IL-1 β is produced by activated macrophages and is an effective and important mediator of the inflammatory response. IL-1 β is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.¹³

Also in serum IL-6 levels were elevated during the neutropenic phase after transplantation (T1) and in patients conditioned with MAC as opposed to RIC. This was expected, due to tissue damage and the high frequency of septicemia in the group under study. Serum IL-6 levels have been shown to be elevated during infection after HSCT.²⁶⁷ Our findings are also in agreement with the findings of Meierowitz et al., who showed that there was a positive correlation between increased levels of IL-6 in serum and mucositis and dysphagia.²⁶⁸ High levels of IL-6 after two weeks of treatment were correlated with the need for installation of a PEG tube.

Only serum IL-6 showed any correlation with OM score during HSCT, on day 8. Patients are often infected during transplantation, which may induce local inflammation. IL-6 acts both as a pro-inflammatory and an anti-inflammatory cytokine, and is secreted by T-cells and macrophages to stimulate the immune response to tissue damage, leading to inflammation. The role of IL-6 as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF- α and IL-1, and activation of IL-1ra and IL-10. IL-6 is the most important mediator of fever and of the acute-phase response.¹³

We also found reduced levels of IL-7 in serum at all time points in patients conditioned with MAC, when compared to those conditioned with RIC. IL-7 is a hematopoietic growth factor secreted by stromal cells in the red marrow and thymus. It is also produced by keratinocytes, dendritic cells, neurons, and endothelial cells, and may serve as a regulatory factor for intestinal mucosal lymphocytes. IL-7 is important for B- and T-cell development.¹³ The reduced values are probably due to the fact that MAC eradicates hematopoiesis and the IL-7-producing cells have not yet recovered.

IL-10 is an anti-inflammatory cytokine that is mainly produced by monocytes, and to a lesser extent by lymphocytes. It has pleiotropic effects in immunoregulation and inflammation.¹³ In serum, IL-10 levels were elevated one month after transplantation. This was probably due to T-cells from the donor producing IL-10, which is active in immunoregulation and inflammation and is consistent with IL-10's anti-inflammatory effect. It also reflects the clinical observation of healing one month after HSCT. Such production was not detected in GCF after HSCT, except in patients conditioned with MAC. This discrepancy may be due to the fact that at this early time point after transplantation, T-cells may not have invaded the gingiva in sufficient amounts to produce detectable cytokine levels. In patients conditioned with MAC, IL-10 levels were significantly elevated in GCF one month after HSCT relative to RIC. The increased production by IL-10 in these patients' GCF is difficult to understand because there is a general decrease in IL-10 in GCF after HSCT, which is thought to be due to lack of mature T-cells.

TNF- α is involved from early on in the inflammatory cascade. It is mainly produced by macrophages, but it is also produced by a broad variety of other cell types, for example lymphoid cells, mast cells, endothelial cells, and fibroblasts. Large amounts of TNF- α are released in response to bacterial products.¹³ The induction of TNF- α by microorganisms, for example *Streptococcus viridans*, may be important in the context

of mucositis development, particularly in the tissue damage process, resulting in further amplification of pro-inflammatory cytokine production.^{169,269} TNF- α together with IL-1 β , are normally both present at high levels also in serum during inflammation, and anti-inflammatory cytokines are at low levels. This cytokine balance is important during the inflammation process.²⁷⁰ Surprisingly, TNF- α levels were found to be reduced in serum during HSCT in patients conditioned with MAC. Using MAC, recipient cells producing TNF- α may be wiped out by the conditioning. It is possible that at time point 1 (7–14 days after HSCT), these cells were already eradicated, which would explain the reduced levels of TNF- α in serum. IL-6 production is induced by TNF- α , but TNF- α is also strongly inhibited by IL-6—forming an effective negative feedback loop that inhibits activation of the pro-inflammatory cytokine cascade.²⁷¹ It is also possible that the increased levels of IL-6 in serum and GCF inhibit TNF- α production or that the increased levels of the anti-inflammatory cytokine IL-10 in serum inhibit the production of TNF- α .

We found a concomitant increase in IL-6, but no other significant correlations between cytokine levels in GCF and serum at any time. This may be because GCF is not only a serum exudate but a complex mixture of substances derived from serum, leucocytes, structural cells of the periodontium and oral bacteria¹⁷³ which lead to different inflammatory response. Later on, immune cells from the donor will also have an influence.

MAIN FINDINGS AND CONCLUSIONS

- Fractionated total body irradiation resulted in less reduction of salivary secretion rate one year after allogeneic stem cell transplantation (HSCT) than single-dose total body irradiation, despite the higher total dose of radiation.
- Risk factors for a low stimulated salivary secretion rate one year after HSCT were (1) single-dose total body irradiation and (2) seropositivity of recipients for 3–4 herpesviruses. A cumulative increase in risk factors resulted in less salivary output.
- There was no difference in long-term salivary function after HSCT in adolescents at 15 years of age receiving conditioning with single dose total body irradiation, fractionated total body irradiation, or busulfan, but there was a negative correlation between age at conditioning with single-dose total body irradiation and salivary function. This correlation was not seen using fractionated total body irradiation or busulfan.
- There was a negative correlation between the total systemic exposure of busulfan and the stimulated whole salivary secretion.
- Female gender was a risk factor for salivary dysfunction at 15 years of age after HSCT, and girls had, at 15 years of age, a significantly lower salivary secretion than boys after HSCT.
- Myeloablative conditioning was associated with more acute oral complications such as oral mucositis, compared to reduced intensity conditioning. Severe oral mucositis prolonged stay in hospital.
- The oral hygiene protocol introduced was associated with a lower oral mucositis score. Other risk factors for oral mucositis were (1) all donor-recipient gender combinations except the female-donor-male-recipient situation and (2) year of transplantation, especially before 2011 when oral care was intensified. With a cumulative increase in risk factors, the oral mucositis score was higher.
- There was a good correlation between the toxicity grading according to WHO and OMAS when used in parallel.
- There was no difference in the volume of gingival crevicular fluid at any time, even between the groups conditioned with RIC or MAC.
- Patients conditioned with MAC or RIC had different patterns of cytokines.
- Significant correlations were found between severity of oral mucositis and septicemia as well as between increase in serum IL-6 levels and septicemia.

- Except for a concomitant increase in IL-6, there was no significant correlation between cytokine levels in GCF and serum at any time point.

The present thesis has clinical, retrospective and prospective cohort design. In the first two studies we related the outcome today after earlier exposure to different conditioning procedures; in study I, sTBI and fTBI and in study II, sTBI, fTBI and Bu. In those two studies, the patients were randomized after exposure. In the following two observational studies we followed the cohorts, RIC and MAC, forward from exposure to outcome. The patients were not randomized for the purpose of these studies.

In conclusion, we demonstrated that side effects affecting the salivary glands and oral mucosa are frequent after allogeneic HSCT. We provided evidence that all the conditioning regimens investigated have a long-term effect on salivary function after allogeneic HSCT in children. Moreover, we identified other risk factors for low salivary secretion after allogeneic HSCT in children. We demonstrated that different conditioning regimens in preparation for allogeneic HSCT result in different severity of OM and that an intensified standard of oral care reduces OM. Severe OM prolongs hospitalization. We also identified several other risk factors for the development of OM and showed that the WHO and the OMAS OM scoring systems are well correlated to each other. Furthermore, we demonstrated that cytokines in the GCF are activated during allogeneic HSCT and that patients who undergo MAC and RIC have different patterns of cytokines. Our findings also show that except for a concomitant increase in IL-6, there was no correlation between cytokine levels in GCF and serum in patients with OM. Furthermore, there was no difference in the volume of GCF at any time, even between the groups conditioned with RIC or MAC, which supports the clinical findings that OM caused by chemotherapy is uncommon in the gingival sulcus area.

CLINICAL IMPLICATIONS

The conditioning regimens used in the preparation for HSCT is continuously changing with subsequent influence on the oral side-effects. In order to establish recommendations for pre-, interim-, and post-cancer therapy management of oral complications in patients with hematological diseases who undergo a high-dose conditioning regimen and allogeneic stem cell transplantation, an understanding of the scope of oral complications must be established and be related to time after treatment and treatment regimen. Today, there is a lack of comprehensive and effective oral management regimens. With a deeper understanding of oral complications, oral care regimens to minimize such complications can be put forward and evaluated. Our findings suggest that it is important to study the effect of changes in conditioning regimens on oral health parameters such as salivary function. Long-term follow-up after allogeneic stem cell transplantation is required because children will have permanently reduced salivary function, and may require additional preventive measures throughout their lives in order to maintain oral health. By developing evidence-based recommendations that have the potential to enhance the appropriateness of clinical practice, the acute oral complications may be reduced, patient outcome will be better, and both cost-effectiveness and quality of life will be improved.

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