

# **Preclinical Studies for Cryoprevention of Oral Mucositis**

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Cover illustration: “The intraoral cooling device” by Sajjad Saffari

Preclinical Studies for Cryoprevention of Oral Mucositis

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*“Education is the most powerful weapon  
which you can use to change the world”*

Nelson Mandela, 2003

*To my beloved family*



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# ABSTRACT

Oral mucositis (OM) is a debilitating adverse effect, with a prevalence of up to 80% in patients with cancer who are conditioned with high-dose chemotherapy prior to hematopoietic stem cell transplantation. In its mildest form, OM is characterized by erythema. However, as it worsens, it can give rise to painful ulcerations in the oral mucosa. This may lead to an increased need for analgesics (and sometimes intravenous morphine) for pain relief, and there is an increased risk of systemic infections. Overall, OM-related complications entail increased health care costs. In fact, despite its frequency, impact on patients, and healthcare and economic burdens, current literature indicates few evidence-based interventions with confirmed efficacy for the prevention of OM. In response to this gap in the knowledge, a novel intraoral cooling device has been developed.

The long-term goal of the research described in this thesis is to establish an effective and well-tolerated method for cryoprevention of OM. The specific aims of this thesis were to: (i) investigate whether cooling, using a constant low temperature, is effective for oral tissue preservation; (ii) develop an animal model to study the early events which precede OM; (iii) assess the effectiveness of a novel intraoral cooling device as a cryopreventive method; (iv) evaluate whether local cooling affects the oral hemodynamics; and (v) establish how a research protocol should be designed for a randomized controlled trial to evaluate cryoprevention of OM.

Oral tissue preservation was better at lower temperatures (*Study I*). Proinflammatory cytokines were significantly upregulated (*Study II*). Several promising results were obtained with the intraoral cooling device (*Study III*). Local cooling may elicit other mechanisms in the oral mucosa than previously suggested that may be of importance for the prevention of OM (*Study IV*). A research protocol, to assess cryoprevention of OM, should be established through multidisciplinary collaborations and should include both objective- and subjective assessments (*Study V*).

In conclusion, this thesis is the first to focus on the prevention of OM using an alternative cryotherapeutic technique. The work is mainly concerned with preclinical studies to identify the ideal temperature for the prevention of OM. Furthermore, this thesis demonstrates the tolerability and cooling efficacy of the intraoral cooling device and shows that cooling may elicit other mechanisms in the oral mucosa than previously suggested. However, despite its promising capacity, the randomized controlled trial will elucidate the definite role of the intraoral cooling device in cryoprevention.

**Keywords:** Animal model, chemotherapy, cryotherapy, hematopoietic stem cell transplantation, ice chips, intraoral cooling device, microcirculation, randomized controlled trial, tissue-engineered oral mucosa, tissue oxygen saturation, tolerability





# SAMMANFATTNING PÅ SVENSKA

Oral mukositis (OM) är en allvarlig biverkning som drabbar cirka 80% av alla cancerpatienter som erhåller höga doser av cellgifter inför en hematopoetisk stamcellstransplantation. OM kännetecknas initialt av rodnad men kan i svårare fall även ge upphov till omfattande smärtsamma sår i munslemhinnan. Detta kan leda till ett ökat behov av analgetika, bl.a. i form av intravenöst morfin, för att uppnå smärtlindring, samt medföra en ökad risk för systemiska infektioner. Sammantaget så medför de komplikationer som uppstår till följd av OM till ökade sjukvårdskostnader. Trots att OM är vanligt förekommande och har en negativ inverkan på såväl patienter som på sjukvården, så finns det för närvarande få evidensbaserade åtgärder för att effektiva motverka uppkomsten av OM. Mot bakgrund av detta har en ny intraoral kylanordning utvecklats.

Det långsiktiga målet med forskningen som beskrivs i denna avhandling är att etablera en effektiv och vältolererad metod för kryoprevention av OM. De specifika målsättningarna var emellertid att: (i) undersöka om kylning med en konstant låg temperatur är effektiv för att bibehålla den orala slemhinnans integritet; (ii) utveckla en djurmodell för att studera tidiga händelser som föregår OM; (iii) utvärdera effekten av en ny intraoral kylanordning som en kryopreventiv metod; (iv) utvärdera om lokal kylning påverkar den orala hemodynamiken; samt (v) fastställa hur ett forskningsprotokoll bör utformas för att genomföra en randomiserad kontrollerad studie av kryoprevention vid OM.

Den orala slemhinnans integritet bevarades bättre vid lägre temperaturer (*Studie I*). Proinflammatoriska cytokiner ökade signifikant (*Studie II*). Flertalet intressanta resultat erhöles med den intraoral kylanordningen (*Studie III*). Lokal kylning utlöser eventuellt andra processer i den orala slemhinnan än vad som har föreslagits tidigare och som kan visa sig vara av betydelse för att förebygga OM (*Studie IV*). Ett forskningsprotokoll för att utvärdera kryoprevention av OM bör utformas utifrån tvärvetenskapliga samarbeten och innefatta både objektiva och subjektiva bedömningar (*Studie V*).

Sammanfattningsvis så är denna avhandling den första som fokuserar på att förebygga uppkomsten av OM med en alternativ kryoterapeutisk metod. Avhandlingen avser främst prekliniska studier i ett försök att identifiera den ideala temperaturen för att förebygga OM. Den påvisar även tolerabiliteten och kylningseffekten av den intraoral kylanordningen, samt att kylning eventuellt utlöser andra mekanismer i den orala slemhinnan än vad som har föreslagits tidigare. Trots att den intraoral kylanordningen hittills har uppvisat en lovande potential, så kommer dess effekt med avseende på kryoprevention först att kunna tillstrykas efter den pågående kliniska studien.



# LIST OF PAPERS

This thesis is based on the following studies, which are referred to in the text by their Roman numerals.

- I. **Walladbegi J.**, Smith S. A., Grayson A. K., Murdoch C., Jontell M., Colley, H. E. Cooling of the oral mucosa to prevent adverse effects of chemotherapeutic agents: An in vitro study. *J Oral Pathol Med.* 2018 May;47(5):477–483.
- II. **Walladbegi J.**, Johnsson M., Aydogdu Ö., Jontell M., Winder M. Early events in the oral mucosa affected by chemotherapeutic agents: An in vivo study. *In manuscript.*
- III. **Walladbegi J.**, Gellerstedt M., Svanberg A., Jontell M. Innovative intraoral cooling device better tolerated and equally effective as ice cooling. *Cancer Chemother Pharmacol.* 2017 Nov;80(5):965-972.
- IV. **Walladbegi J.**, Raber-Durlacher J.E., George R., Jontell M., Milstein D.M.J. Hemodynamics of the oral mucosa during cooling. *In manuscript.*
- V. **Walladbegi J.**, Svanberg A., Gellerstedt M. Protocol for a randomised controlled trial to study cryoprevention of chemotherapy-induced oral mucositis after autologous stem cell transplantation. *BMJ Open.* 2018 Oct 24;8(10):e021993.



# ABBREVIATIONS

<b>5-FU</b>	5-fluorouracil
<b>aHSCT</b>	Allogenic hematopoietic stem cell transplantation
<b>AHSCT</b>	Autologous hematopoietic stem cell transplantation
<b>CMT</b>	Chemotherapy
<b>COX-2</b>	Cyclooxygenase-2
<b>CT</b>	Cryotherapy
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>FCD</b>	Functional capillary density
<b>GVT</b>	Graft versus tumor
<b>H-DC</b>	High-dose chemotherapy
<b>HSCT</b>	Hematopoietic stem cell transplantation
<b>IC</b>	Ice chips
<b>ICD</b>	Intraoral cooling device
<b>IHC</b>	Immunohistochemistry
<b>IL-1<math>\beta</math></b>	Interleukin-1 $\beta$
<b>IL-6</b>	Interleukin-6
<b>ISOO</b>	International Society of Oral Oncology
<b>KGF</b>	Keratinocyte growth factor
<b>LLLT</b>	Low-level laser therapy
<b>MASCC</b>	Multinational Association of Supportive Care in Cancer
<b>NF-<math>\kappa</math>B</b>	Nuclear factor Kappa B
<b>OC</b>	Oral cavity
<b>OD</b>	Optical density
<b>OM</b>	Oral mucositis
<b>OMAS</b>	Oral Mucositis Assessment Scale
<b>P.P.</b>	Percentage points

## ABBREVIATIONS

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<b>PBS</b>	Phosphate-buffered saline
<b>QoL</b>	Quality of life
<b>RCT</b>	Randomized controlled trial
<b>RT</b>	Radiation therapy
<b>S-DC</b>	Standard-dose chemotherapy
<b>StO<sub>2</sub></b>	Tissue oxygen saturation
<b>TEOM</b>	Tissue-engineered oral mucosa
<b>TNF-<math>\alpha</math></b>	Tumor necrosis factor alpha
<b>WHO</b>	World Health Organization

# 1. INTRODUCTION

## 1.1 Background

The past decades have witnessed the emergence of a number of modalities for the management of oral mucositis (OM). Despite the frequency of OM, its impact on patients, and the associated healthcare and economic burdens, there are currently a few evidence-based interventions with confirmed efficacy. To date, mucositis management has largely been palliative, mainly aimed at reducing the symptoms of already established ulcers, and preventing systemic complications. However, although palliative strategies may be relevant once OM has become established clinically, the primary goal is prevention.

Based on expert opinions and a comprehensive review of the literature by The Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO), three strategies are currently recommended for the prevention of chemotherapy-induced OM (Lalla *et al.*, 2014). Cryotherapy (CT) using ice, which is the most extensively researched strategy, has been proven to be effective in a number of clinical trials (Worthington *et al.*, 2011; Riley *et al.*, 2015). However, despite well-substantiated documentation, the use of ice cooling as a preventive method in clinical practice is limited. The reason for this may be that ice has a detrimental effect on the comfort level of the patients by causing cold sensations, chills and shooting pain in the teeth, thereby leading to poorer adherence. Therefore, there have been calls in the literature to improve the use of cooling devices for cryoprevention (Kadokia *et al.*, 2014). In response to this call, an intraoral cooling device (ICD; Fig. 1), that can operate at different temperatures, has been successfully developed and was one of the rationales for this thesis.



**Figure 1.** Schematic illustration of the intraoral cooling device. Reprinted with permission from Walladbegi J., Gellerstedt M., Svanberg A., Jontell M. *Innovative intraoral cooling device better tolerated and equally effective as ice cooling.* *Cancer Chemother Pharmacol.* 2017 Nov; 80(5):965-72. (<http://creativecommons.org/licenses/by/4.0/>).

## 1.2 Chemotherapy

Chemotherapy (CMT), which involves the administration of cytostatic/cytotoxic drugs, has since its introduction in the 1960s contributed significantly to improving cure- and survival rates for patients with a wide range of malignancies. The different chemotherapeutic agents possess characteristics that enable them to target tumors at all anatomic locations. They exert their effects by inducing growth arrest and inducing cellular apoptosis, usually through inhibiting microtubule function, protein functions, or DNA synthesis. The mechanism of action of the agent may be cell cycle-dependent, arresting cancer cell growth at specific phases, or cell-cycle independent, crosslinking nucleobases in the DNA. Chemotherapeutic agents can be grouped according to mechanism of action, chemical structure or their relationships to other drugs. In general, they are categorized as alkylating agents, anti-metabolites, anti-tumor antibiotics, topoisomerase inhibitors, and mitotic inhibitors. In addition, proteasome inhibitors and other agents, e.g., L-asparaginase, which is an enzyme, act in different ways and do not fit into any of the main categories (Nakamura & Uchida, 1988; Seiwert *et al.*, 2007).

### 1.2.1 High-dose chemotherapy

High-dose chemotherapy (H-DC), which is also termed ‘myeloablative therapy’, involves the administration of chemotherapeutic agents at doses that are several-fold higher than the standard therapeutic dose. These conditioning regimens are administered to achieve two goals: (i) the eradication of all cancerous cells; and (ii) the provision of sufficient immunosuppression to allow engraftment of hematopoietic stem cells (Bacigalupo *et al.*, 2009; Locatelli *et al.*, 2014). Many anti-cancer agents operate according to a steep dose-response curve, i.e., small increments in the dosage result in relatively high-level destruction of cancer cells (Porrata & Adjei, 2001). As a consequence, there is an increased morbidity following H-DC compared to treatment with conventional CMT. In fact, the indiscriminate nature of cytotoxic agents, in failing to distinguish between rapidly dividing healthy cells and their malignant counterparts, makes it impossible to treat effectively the cancer without exposing the patient to various adverse effects. These adverse effects may include infections, hemorrhaging and impaired bone marrow function (Tao *et al.*, 2015; Pearce *et al.*, 2017).

While standard-dose chemotherapy (S-DC) causes a transient damage to the bone marrow, the corresponding myeloablative treatment is more hazardous and suppresses the bone marrow for longer periods. This condition is potentially life-threatening and requires intensive medical interventions to avoid mortality (Rodriguez *et al.*, 2007).



### 1.3 Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is the medical procedure used to replace the bone marrow. Stem cells from the bone marrow, umbilical cord or most commonly, the bloodstream (peripheral stem cells) of donors are prepared in advance (pre-conditioning), and are transplanted following the conditioning therapy (Felfly & Haddad, 2014; Park *et al.*, 2015). In theory, HSCT offers two major advantages. First, it enables administration of cytotoxic agents at doses several-fold higher than the standard dose, which would otherwise be lethal to the bone marrow. Second, in recipients, the donor transplant and its associated immune system allows for the potential development of a graft versus tumor effect (GVT), which accounts in part for successful treatments (Moore & Sakamoto, 2005; Porter, 2011).

In the present era, S-DC has proven to be beneficial in palliation but insufficient for cure in some hematologic malignancies (Imrie *et al.*, 2002). H-DC supported by HSCT has therefore been established as the standard of care for patients with multiple myeloma and lymphoma, as these patients are not considered to benefit from prolonged conventional CMT. Furthermore, it is also applied to patients with acute leukemia in addition to standard treatment, to achieve complete remission. HSCT is either autologous (AHSCT) or allogenic (aHSCT), and is selected based on several factors, such as type of malignancy, CMT sensitivity, comorbidity, availability of a suitable donor, age, and the malignancy's susceptibility to GVT effects (Majhail *et al.*, 2015). Annually, more than 55,000 HSCTs are performed worldwide. Of these, ~ 30,000 are AHSCT and ~ 25,000 aHSCT (Henig & Zuckerman, 2014). The corresponding annual figures in Sweden are approximately 400 AHSCT and 300 aHSCT (Passweg *et al.*, 2016).

Chemotherapies in general, and H-DC followed by HSCT in particular, continue to be the mainstay treatment for the aforementioned malignancies. However, due to the toxic effects of chemotherapies on highly proliferative tissues, this type of therapy remains a dangerous and resource-intensive process associated with various potential adverse effects and reactions. Mucositis, and more specifically OM, along with its associated symptoms, have been frequently reported in the literature as adverse effects of CMT (Epstein *et al.*, 2012; Migliorati *et al.*, 2015).

### 1.4 Oral mucosa and mucositis

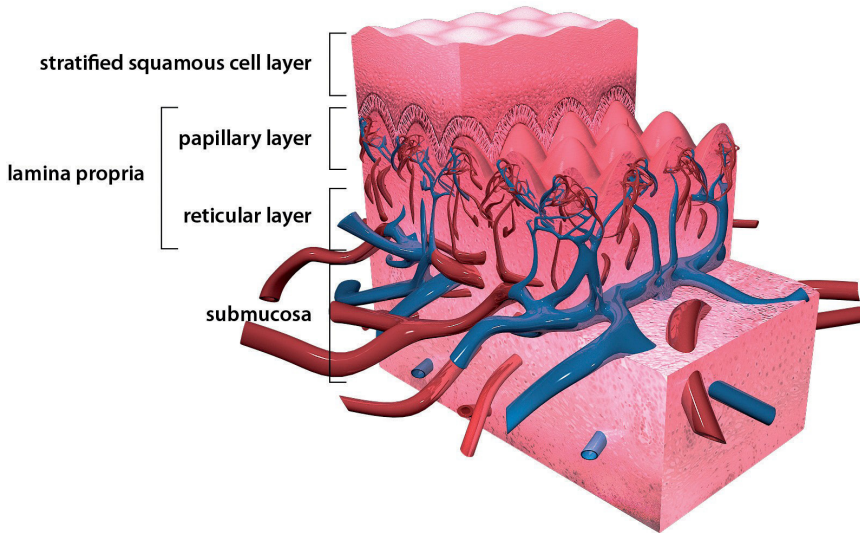
The oral cavity (OC), which is sometimes referred to as a mirror of general health or disease (Islam *et al.*, 2011), functions as a site for sound modification, communication, and initiation of the digestive process by salivation. It also plays

a significant role in the protection of the internal body integrity, through the immune cells residing in the mucosal compartments (Sloan *et al.*, 1991). It has been stated that “*The peculiarities of the OC are unique. No other body cavity shares such a close relationship to the external environment, represents as many varied functional anatomical entities, or contains a micro flora in the amount or variety encountered in the human mouth*” (Walker, 1990). Furthermore, the OC is constantly challenged and has a high functional activity. Thus, an intact and functioning OC is crucial for the individual’s well-being and even minor disruptions of the functions or constitution of the OC can seriously impair the quality of life (QoL) of the individual.

### 1.4.1 Structure of the oral mucosa

The OC is composed of sophisticated anatomical structures and extends from the vermilion border of the lips to the junction of the hard and soft palates superiorly and the circumvallate papillae of the tongue inferiorly. It is further divided into several anatomical landmarks, which presents with regional variations in mucosal architecture that tend to react differently to exogenous harms, e.g., non-surgical cancer therapy (Wu *et al.*, 2014; Montero & Patel, 2015).

The mucosa of the OC consists of two layers, an outer layer of stratified squamous epithelium (with thickness varying in the range of ~ 80–350  $\mu\text{m}$ ); and a deeper layer of *lamina propria* separated by a basement membrane (Prestin *et al.*, 2012). Being derived from the embryonic ectoderm and ectomesenchyme, the oral epithelium and underlying *lamina propria* display structural modifications in different regions of the OC, generating three histological types. In the areas that are subjected to mechanical forces (e.g., the attached gingiva and hard palate), there is a keratinized stratified squamous epithelium, which is firm and tightly attached to the underlying structures by collagenous connective tissue. In contrast, non-keratinized stratified squamous epithelium, is characterized as pliable and elastic and lines those regions which require flexibility to accommodate chewing, swallowing, and speech (e.g., the lips, cheeks, vestibule, floor of the mouth, inferior surface of the tongue, and the soft palate). The dorsum of the tongue is covered by a specialized epithelium, which is a mosaic of the other two mucosal types and is attached tightly to the muscles (Winning & Townsend, 2000; Squier & Kremer, 2001). In the keratinized mucosa, the epithelium is composed, from the deepest to the most superficial layer, of *stratum basale* (basal layer), *spinosum* (prickle layer), *granulosum* (granular layer), and *corneum* (keratinized layer), whereas in non-keratinized mucosa, the *stratum basale* and *spinosum* are the same but the two superficial layers are termed *stratum intermedium* and *stratum superficiale* (Fig. 2).



**Figure 2.** Three-dimensional model of the human oral mucosa. The oral mucosa is composed of an outer, non-vascularized, stratified, squamous epithelium (i.e., keratinized or non-keratinized), which lines the entire oral cavity. The vascular plexus in the reticular layer of the *lamina propria* provides a horizontal branching network of blood vessels from which ascending arterioles project into the papillary layer, forming capillary networks, and subsequently drain back into the descending venules. The epithelium and underlying *lamina propria* are separated by a basement membrane. Reprinted with permission from Dr. D. M. J. Milstein, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

The structural integrity and tissue functions of the oral mucosa are mediated by several different cell populations. The majority are amplifying cells, keratinocytes, the roles of which are to maintain the epithelial volume by differentiating from the basal layer to the superficial layer and to replace the desquamated cells. This process, for a cell to divide and pass through the entire epithelium, is referred to as ‘cellular turnover’, which varies from 2 to 3 weeks depending on location. The turnover rate is highest for the cells in the non-keratinized regions. Other cells, such as Merkel cells, can also be found in the basal layer. These are situated adjacent to nerve fibers, therefore it has been suggested that they are sensory cells that respond to touch. In addition, melanocytes are present and produce pigment (melanin), which contribute to the color of the oral mucosa. The immunological activities of the epithelium are usually mediated by Langerhans’ cells, which are dendritic cells that originate from the bone marrow and are sometimes seen in the suprabasal layers of the oral epithelium. Langerhans’ cells possess specific characteristics in that they recognize and process antigenic materials derived from the external environment and present them to helper T-lymphocytes, hence the presence of T-lymphocytes in the nucleated epithelial layers, when specimens of normal mucosa are examined microscopically (Sloan *et al.*, 1991; Squier & Kremer, 2001).

The *lamina propria* is a connective tissue layer that exhibits regional variations in its constituent elements (elastin, collagen types I and III). It is generally divided into an upper papillary layer and a lower reticular layer with a capillary plexus in-between. The papillary layer, which consists of loose connective tissue with equal amounts of fibers, cells, and intracellular substances, forms finger-like projections into the epithelium. In contrast, the reticular layer is a dense connective tissue that is dominated by fibers. The most commonly found cells in the *lamina propria* are fibroblasts, although various immune cells are also present (Sloan *et al.*, 1991; Squier & Kremer, 2001).

The submucosa adheres to the lower reticular layer and is anchored firmly in the underlying muscle or bone by collagen and elastic fibers. It contains loose connective tissue, adipose tissue, larger blood- and lymphatic vessels, nerve fibers, and minor salivary glands. In the gingiva and hard palate, the submucosa is absent and the oral mucosa attaches directly to the underlying periosteum, constituting the mucoperiosteum (Qin *et al.*, 2017). The various forms of oral mucosa possess unique characteristics and histologic structures that are linked to their particular functions.

### 1.4.2 Basic principal functions of the oral mucosa

While the oral mucosa has several important functions, the primary purpose is to protect the deeper layers and provide a barrier against exogenous toxic substances, microorganisms, and mechanical insults during chewing. In addition, the oral mucosa is extensively innervated, which allows the reception of signals related to touch, pain, and temperature. The salivary glands and taste buds responsible for tissue moisture/lubrication and taste recognition, respectively, are also present in the oral mucosa (Zweifach, 1957; Sloan *et al.*, 1991).

The equilibrium between tissue homeostasis and the potent metabolic requirement for repair and regeneration in the oral mucosa is supported by a highly vascularized network of capillaries (arterioles, venules, and short vascular segments known as arteriovenous shunts). Thus, an uninterrupted and well-functioning microcirculation, delivering nutritive factors constituents from the blood, is essential to maintaining the structural integrity of the oral mucosa (Scardina & Messina, 2003). Interestingly, the oral mucosa has a degree of permeability, which allows for rapid absorption of different factors and, in certain circumstances, enables the microcirculation to function as a drug delivery system (Harris & Robinson, 1992).

### 1.4.3 General mucositis

The term mucositis is derived from the Italian word *mucosite* and was first described in the 1950s. However, until the early 1980s, these lesions were often referred to in

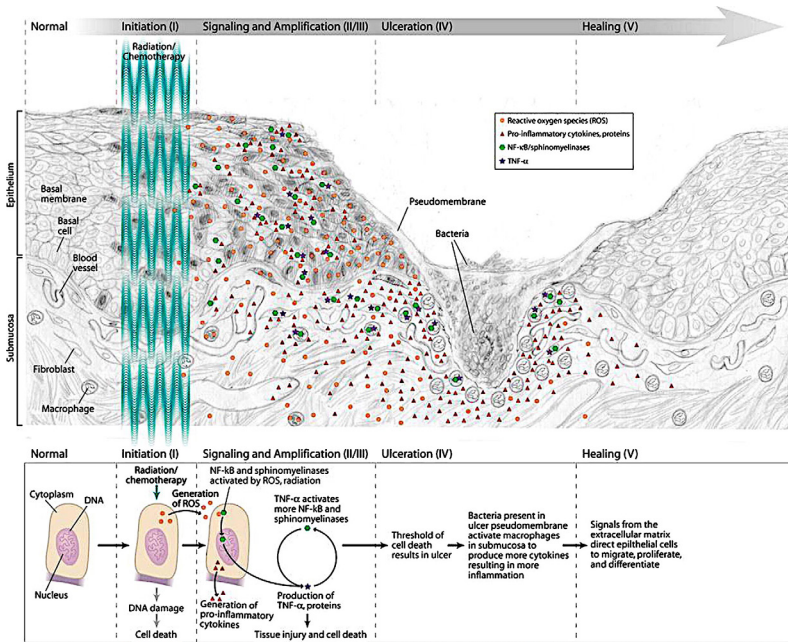
the literature as *stomatitis*. Unfortunately, *stomatitis* is an unspecific term that can include a variety of other complications in patients with cancer, e.g., infections. The currently accepted definition of mucositis is more specific, referring to “*the painful inflammation and ulceration of the mucous membranes lining the alimentary tract, as an adverse effect of chemo- and/or radiation therapy for cancer*”. This definition better reflects the association between its clinical appearance and the cancer therapy-mediated injury (Peterson, 1999).

The history of mucositis is probably as old as the above-mentioned cancer therapies. Despite this, the complexity of the pathogenesis of mucositis was poorly appreciated until relatively recently. Mucositis was initially considered to be exclusively a consequence of indiscriminate destruction of rapidly dividing basal epithelial stem cells, i.e., clonogenic cell death. In 1998, an initial hypothesis, including four principal phases through which mucositis develops and resolves, was put forward (Sonis, 1998). New evidence has, however, led to revisions being made to the initial hypothesis (Sonis, 2004, 2007, 2009), generating a new model that has been accepted as the explanatory for the condition. This model describes mainly the pathological events that occur in the oral mucosa. Nonetheless, a similar scenario can be assumed to arise throughout the gastrointestinal tract mucosa as it is covered with a similar tissue, although there are regional differences in structure and epithelial turnover rate.

The contemporary model of mucositis development comprises a number of vascular-, cellular-, and immune-mediated reactions within the submucosa, together with both direct- and indirect destruction of epithelial stem cells. The pathobiologic process associated with the development of mucositis is multifactorial but can be arbitrarily described by the following five stages: initiation <sup>1</sup>, primary damage response <sup>2</sup>, signal amplification <sup>3</sup>, ulceration <sup>4</sup>, and healing <sup>5</sup> (Sonis, 2009) (Fig. 3). Briefly, following several submucosal events, tissue injury is initiated in the basal epithelial cells either directly through DNA damage or indirectly through reactive oxygen species. This damage provokes a complex series of events that involve enzymatic activities and the activation of transcription factors, e.g., Nuclear factor Kappa B (NF- $\kappa$ B), Wnt, and P53, and their associated canonical pathways. Consequently, the NF- $\kappa$ B pathway, which is the most studied relative to mucositis development, governs the upregulation of genes that encode molecules that have demonstrated activities in the pathogenesis of mucositis. These molecules include the proinflammatory cytokines, Tumor necrosis factor alpha (TNF- $\alpha$ ); Interleukin-6 (IL-6); Interleukin-1 $\beta$  (IL-1 $\beta$ ); and stress responders such as cyclooxygenase-2 (COX-2). Furthermore, as cytotoxic agents percolate into the underlying *lamina propria*, fibrinolysis occurs, which stimulates macrophages to produce damaging metalloproteinases. These series of events eventually lead to atrophy and ulcerations. The final healing phase is mediated by signaling molecules from the extracellular matrix, which direct the migration, proliferation, and differentiation of



the epithelium bordering the ulcerative areas (Sonis, 2009). The complex process of mucositis is initiated within seconds of the administration of CMT, although there is a time-lag between the molecular and cellular insult and the clinical manifestation of mucositis.



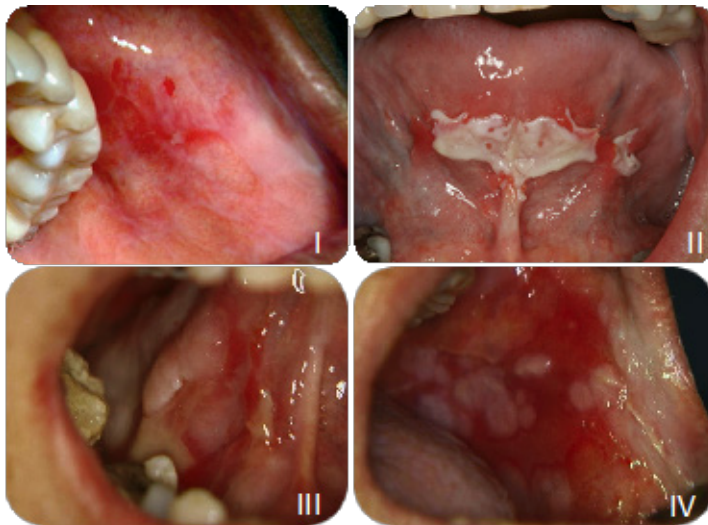
**Figure 3.** The pathobiology of mucositis as a five-stage process: (I) initiation; (II) primary damage response; (III) signal amplification; (IV) ulceration; and (V) healing. Reprinted from *Oral Oncology*, 45(12), Sonis S. T., *Mucositis: The impact, biology and therapeutic opportunities of oral mucositis*, pp. 1015-20., © (2009), with permission from Elsevier.

### 1.4.4 Oral mucositis

OM refers to the painful erythematous and ulcerative lesions that appear in the oropharyngeal mucosa. The course of OM is generally predictable and reflects the associated cancer treatment (Lalla *et al.*, 2008; Peterson *et al.*, 2011). This adverse reaction is frequently encountered within a week post-CMT and is characterized in its mildest form by erythema. Thereafter, the mucosal alterations progress, increase in intensity, and manifest themselves as erosions and/or ulcers that are covered with a white pseudomembrane, which usually resolve within 2 weeks. However, the healing period may be longer when secondary infections with bacteria, virus or fungi occur (Chaveli-Lopez, 2014) (Fig. 4). The ulcerations tend to be irregular and shallow and can consolidate into larger wounds. OM can affect both the keratinized and non-keratinized mucosa. However, in general, it is limited to the non-keratinized mucosa

(lips, cheeks, vestibule, floor of the mouth, ventral surface of the tongue, and the soft palate).

OM is a highly significant and sometimes dose-limiting condition, that has been reported as the single most-debilitating complication of cancer therapy (Bellm *et al.*, 2000). It affects up to 40% of patients who receive S-DC and almost 80% of patients who are undergoing H-DC in preparation for HSCT (Peterson *et al.*, 2010, 2011). OM can be present in combination with a variety of debilitating symptoms that may comprise the ability of the patients to maintain oral hygiene practices (Gandhi *et al.*, 2017). For example, intractable oral pain, which may lead to an increased need for analgesics and, on occasions, opioids that are administered intravenously (Harris, 2006). OM is further associated with undernourishment, weight loss, the use of feeding tubes or total parenteral nutrition, and impaired QoL, and it can represent a portal of entry for systemic infections that can lead to sepsis and death (Biswal, 2008). Taken together, these symptoms along with their related sequelae can result in hospitalization and may incur increased costs for healthcare systems (Elting *et al.*, 2003; Elting *et al.*, 2007; Epstein *et al.*, 2012).



**Figure 4.** Oral mucositis, Grades I-IV, according to the World Health Organization scale. I, Soreness with or without erythema; II, erythema, ulcers, whereby patients can swallow solid food; III, ulcers with extensive erythema, whereby patients cannot swallow solid food; and IV, mucositis to the extent that alimentation is not possible. Grades III and IV are considered as severe oral mucositis. Photographs courtesy of Dr. D. Öhman, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden.

### 1.4.4.1 Risk factors

Several risk factors have been proposed as being significant for the degree and duration of OM. These factors are mainly divided into those that are inherent to the patient and those that are related to the treatment regimen. In addition, genetics, host environment, and the tumor itself have been considered as factors that may contribute to the manifestation of OM (Sonis & Clark, 1991; Barasch & Peterson, 2003; Lalla *et al.*, 2008; Sonis, 2012b; Allen *et al.*, 2018)

Proposed patient-related risk factors include: age, gender, low body mass, nutritional status, oral microflora, pre-existing medical conditions, salivary function, oral and dental health status, and maintenance of liver and kidney functions. For example, patients with pre-existing medical conditions, such as psoriasis, might be at lower risk of developing OM compared to controls. Conversely, patients with Addison's disease develop OM more frequently than controls. Both of these diseases seem to influence the propensity of cells to undergo apoptosis, albeit in opposite ways. Psoriasis is characterized by high anti-apoptotic activity, whereas Addison's disease is characterized by increased pro-apoptotic activity and higher levels of pre-existing proinflammatory cytokines (Sonis, 2004, 2009). However, in general, these risk factors are complex, poorly investigated, and sometimes conflicting.

Regarding the treatment-related risk factors, patients who are diagnosed with hematologic malignancies are generally at greater risk of developing OM, compared to those with solid tumors, except for tumors of the head and neck, in which a majority of all patients suffer from side-effects caused by the radiation therapy (RT) (Peterson & Sonis, 1982; Peterson *et al.*, 2011). The risk of OM for common solid tumors, such as prostate, breast, colorectal, and lung cancer, is approximately 20% during the first CMT cycle, with the risk increasing to >60% in subsequent cycles (Sonis, 2009). The type of cytostatic agent, dose, frequency, schedule of administration and concomitant treatment with RT are other treatment-related factors that may be of importance for the development of OM. Alkylating agents (e.g., melphalan), especially when given at high doses, and antimetabolites (e.g., 5-fluorouracil; 5-FU), often predispose patients to the development of OM. Furthermore, certain agents given systemically (e.g., methotrexate and etoposide) may be secreted in the saliva and further increase the risk of cellular toxicity in the OC and thereby increase the risk of OM development (Wilkes, 1998; Barasch & Peterson, 2003).

### 1.4.4.2 Diagnosis

OM is diagnosed based on clinical characteristics, primarily its appearance, localization, and debut of symptoms. There are several validated instruments for recording the extent and severity of OM. A frequently used scale is that of the World Health Organization



(WHO), which comprises Grades 0–4 (0 indicates “no changes” and 4 indicates “ulcers, alimentation not possible” due to OM). The WHO scale combines both objective and subjective measures of OM and is easy to use in clinical practice (Fig. 5). The Oral Mucositis Assessment Scale (OMAS) is an objective scale, suitable for research purposes, which measures erythema and ulcerations on scales of 0–3 for ulcers and 0–2 for erythema (Grade 0 corresponds to “normal”, and Grades 2 and 3 relate to “severe erythema” and “ulcers greater than 3 cm<sup>2</sup>”, respectively). This assessment generates mean scores for OMAS ulcers (0–3) and OMAS erythema (0–2) and a total mean, the OMAS-total (0–5) (Fig. 6) (WHO, 1979; Sonis *et al.*, 1999; Lalla *et al.*, 2008; Maria *et al.*, 2017).

Toxicity grading according to World Health Organization

**Pain:**

VAS (0-10) Oral Cavity: \_\_\_\_\_

VAS (0-10) Throat: \_\_\_\_\_

**Ability to swallow:**

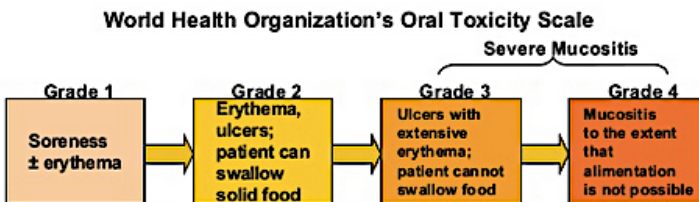
Question: Can you eat? \_\_\_\_\_

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 5.** World Health Organization’s oral toxicity scale for oral mucositis. Reprinted with permission from Maria O.M., Eliopoulos N., Muanza T. *Radiation-Induced Oral Mucositis. Front Oncol.* 2017 May; 7:89. Originally reprinted with permission from Dr. Patrick Stiff, Loyola University Medical Center, Maywood, IL, USA. (<http://creativecommons.org/licenses/by/4.0/>).

**Oral Mucositis Assessment Scale**

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

<p><b>Ulceration/pseudomembrane:</b></p> <p>0 = no lesion</p> <p>1 = &lt; 1 cm<sup>2</sup></p> <p>2 = 1 – 3 cm<sup>2</sup></p> <p>3 = &gt; 3 cm<sup>2</sup></p>	<p><b>Erythema:</b></p> <p>0 = None</p> <p>1 = Not severe</p> <p>2 = Severe</p>
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**Figure 6.** Oral Mucositis Assessment Scale. Reprinted with permission from Sonis, S. T., Eilers, J. P., Epstein, J. B., LeVeque, F. G., Liggett, W. H., Mulagha, M. T., Peterson, D. E., Rose, A. H., Schubert, M. M., Spijkervet, F. K., Wittes, J. P. *Validation of a new scoring system for the assessment of clinical trial research of oral mucositis induced by radiation or chemotherapy.* *Cancer*, 85: 2103-2113., © 1999 John Wiley & Sons, Inc.

#### 1.4.4.3 Management

Mucositis in general, and OM in particular, are perhaps the most widely researched side-effects of cancer therapy. Nevertheless, this morbid condition remains a major challenge. The ultimate goal of mucositis research is to identify the most appropriate target for interventions (Al-Dasooqi *et al.*, 2013). Based on an improved understanding of the pathobiology of OM, a multitude of studies directed at the prevention and treatment of OM have emerged. However, inconsistency and sometimes conflicting results have led to clinicians still searching for a unified, evidence-based approach. To date, various strategies and agents have been described. These approaches encompass a diversity of mechanisms, although the results obtained in various clinical trials have been controversial, and in most cases not sufficiently conclusive to merit their recommendation. The following section of this thesis will summarize the current guidelines for management of OM. These can be grouped as follows: (i) basic oral care and good clinical practice; (ii) diet and pain management; (iii) palliation, and (iv) prevention (Harris, 2006; McGuire *et al.*, 2006; Lalla *et al.*, 2008; Peterson *et al.*, 2011; Lalla *et al.*, 2014).

### *Basic oral care and good clinical practice*

Maintaining appropriate oral hygiene appears to be advantageous in terms of reducing the risk of infections in the OC and, consequently, reducing the severity and duration of OM (Djuric *et al.*, 2006). The OC harbors numerous microorganisms that colonize the teeth and mucous membranes. Although OM is not considered to be an infectious disease, secondary colonization of ulcerated areas by microorganisms can contribute to impaired healing. Furthermore, many cancer modalities adversely affect the salivary flow and the patient's ability to physiologically clear microorganisms, which may increase further the risk of secondary infections (Avsar *et al.*, 2007; Chaveli-Lopez, 2014). Therefore, standardized protocols, executed by a multidisciplinary team that combines nurses and dental professionals, are recommended (Rubenstein *et al.*, 2004; Scully *et al.*, 2004; Keefe *et al.*, 2007; Lalla *et al.*, 2014; Saito *et al.*, 2014).

The current consensus is to have a complete oral examination conducted by professional staff prior to cancer treatment, in order to identify and subsequently eliminate any pre-existing dental pathology or source of mucosal irritation. As part of the protocol, instructions and oral hygiene information, the use of a soft tooth brush that is replaced frequently, interdental flossing (individual assessment), and regular mouth rinsing with non-alcoholic rinses (e.g., saline mouth rinses) are also recommended (Keefe *et al.*, 2007; Lalla *et al.*, 2008). During hospitalization, regular monitoring of the patient for signs and early detection of oral manifestations is essential, and should be documented when observed. In ambulant patients for whom follow-up is not possible, antimicrobial rinses should be prescribed (Elad *et al.*, 2015). Mechanical plaque control may reduce the risk of OM progression, and several studies have demonstrated a reduction of oral complications following the implementation of oral hygiene procedures (Borowski *et al.*, 1994). It has further been reported that oral hygiene procedures should be carried out several times/day and that feedback to the patients regarding their oral hygiene efforts is helpful and should be provided regularly (Elad *et al.*, 2015).

### *Diet and pain management*

The severe pain associated with OM can cause modifications to the appetite and lead to dysphagia and malnutrition. Therefore, pain management and food choices that promote oral health are essential and should be advised upon by health care providers (Scully *et al.*, 2004). In moderate to severe OM, topical anesthetics can provide short-term pain relief, although paracetamol alone, or in combination with opioids is the recommended strategy to manage effectively the pain sensations. Sour and spicy foods can aggravate the symptoms of OM and should therefore be avoided (Scully *et al.*, 2004).

Unfortunately, studies have shown that a daily oral examination is rarely carried out among nurses, advice pertaining to oral management is inconsistent (Raber-Durlacher, 1999), and routines for basic oral care are not taught (Larson *et al.*, 1998).

### *Palliation*

In recent years, various therapeutic options have become available for the management of an already established ulcer in the OC. These can generally be divided into three main categories: (i) “magic” mouthwashes; (ii) mucoadhesive gels/liquids; and (iii) electrolyte solutions. Magic mouthwashes refer to a collection of formulations with varying contents (lidocaine, topical steroids, antifungal agents and/or antibiotics), which aim to relieve OM symptoms (Keefe *et al.*, 2007; Bensinger *et al.*, 2008). Mucoadhesive formulations (Gelclair, MuGuard, Mucotrol and Episil), coat the OC and provide a barrier to protect the injured mucosa (Naidu *et al.*, 2005). Electrolyte solutions (Caphosol and NeutraSal), which were originally developed for the treatment of hyposalivation- or for xerostomia-related complications, have also been used to manage OM (Hashemi *et al.*, 2015). However, they have failed to demonstrate substantial benefit compared to standard therapies, and statistically powered, randomized, controlled studies are needed to better evaluate the true potential of these agents (Sonis, 2012a).

### *Prevention*

The MASCC/ISOO, has following comprehensive literature reviews published guidelines for the management of OM. The first guidelines were published in 2004 (Rubenstein *et al.*, 2004). These were updated in 2007 (Keefe *et al.*, 2007). The most recent updates, which were published in 2014, recommend the following three strategies for prevention of CMT-induced OM: (i) recombinant human keratinocyte growth factor-1 (KGF-1/palifermin); (ii) low-level laser therapy (LLLTT); and (iii) cryotherapy (CT) (Lalla *et al.*, 2014).

(i) Palifermin is a keratinocyte growth factor-1 (KGF-1) that is produced by recombinant DNA technology in *Escherichia coli*. Palifermin mimics the endogenous KGF in that it binds to the KGF receptor which is expressed in epithelial cells in a variety of tissues, and activates the Ras-MapK (mitogen-activated protein kinases) signaling pathway. Subsequent activation of the transcription factor will lead to the synthesis of several proteins that are important for the proliferation, differentiation and migration of keratinocytes, which are responsible for maintaining the structural integrity of the mucosa (Rubin *et al.*, 1995; Blijlevens & Sonis, 2007). MASCC/ISOO recommends that palifermin (at a dose of 60 µg/kg per day) to be used for three consecutive days prior to conditioning and for three days post-HSCT in patients receiving H-DC for hematologic disorders (Radtke & Kolesar, 2005; Sonis, 2010;

Lalla *et al.*, 2014). Data from a Phase III study comparing palifermin to placebo reveal a significant reduction in the incidence and duration of severe OM and the costs for morphine and parenteral nutrition in the experimental group, as compared to the control group. However, despite statistically significant differences, about 60% of the patients treated with palifermin still experienced severe OM (Spielberger *et al.*, 2004). A single health economics study concluded that palifermin comes at a considerable price, currently USD 8,250 per patient in the HSCT setting (Elting *et al.*, 2007). In addition, palifermin is associated with several adverse effects, including pruritis, erythema, taste alterations, and paresthesia (Radtke & Kolesar, 2005). Ultimately, the marketing authorization for palifermin has been withdrawn by the European Medicines Agency at the request of the marketing authorization holder.

(ii) Photobiomodulation therapy, also referred to as low-level laser therapy or LLLT, entails the local application of non-ionizing, monochromatic, coherent light onto biologic tissues to reduce inflammation and pain, as well as to improve tissue repair. The mechanism of action of LLLT is a subject of some debate. However, the current general consensus is that visible red near-infrared light energy is absorbed by the cellular mitochondria and converted into energy that is used in cellular repair, healing and improved blood circulation (Ottaviani *et al.*, 2013; Bensadoun, 2018).

The biologic effects vary in relation to the wavelength of light used. Currently, a wavelength of 650 nm, a power of 40 mW, and each square centimeter applied for the required time to achieve a tissue energy dose of 2 J/cm<sup>2</sup>, is the recommended strategy by MASCC/ISOO to prevent OM in patients who are receiving H-DC followed by HSCT (Lalla *et al.*, 2014). The recommendations are based on the results of a Phase III randomized, double-blind, placebo-controlled trial, in which statistically significant reductions in OM and pain were observed in the arm that applied 650-nm LLLT compared to the arm with 780-nm LLLT and placebo (Schubert *et al.*, 2007). These findings were consistent with previous studies that evaluated the OM preventive effects of LLLT (Barasch *et al.*, 1995; Cowen *et al.*, 1997). However, despite the encouraging effects reported for LLLT, two systematic literature reviews deviate in conclusion with regards to the benefits of LLLT for preventing severe OM (Bjordal *et al.*, 2011; Worthington *et al.*, 2011). Moreover, LLLT is a time-consuming procedure that requires daily visits by the patient for up to 14 days. There is one published study in which the efficacy of CT/LLLT combined was compared to LLLT alone, for the reduction of OM severity (de Paula Eduardo *et al.*, 2015). The authors of that study concluded that the combination of CT with LLLT was superior to LLLT alone in reducing the severity of OM. However, it is noteworthy that a study arm with only CT was not included in that study. Thus, most likely, the positive results related to CT/LLLT may be attributable to the CT.

(iii) The term “cryotherapy” is derived from the Greek *cryo* (κρύο), which denotes ‘cold’, and *therapeía* (θεραπεία) meaning ‘cure’. CT refers to the local or general application of low temperatures as a medical therapy. The analgesic and anti-inflammatory properties of CT have been exploited for several thousand years, initially by the Egyptians and later by the Greek physician Hippocrates. The Edwin Smith Papyrus, an ancient Egyptian medical thesis (c. 1600 BC) that is believed to be a copy of work dating from c. 3000 BC, makes numerous references to the use of cold in medicine (Cooper & Dawber, 2001; Wang *et al.*, 2006). Over the past centuries, CT has evolved from generalized applications to treatment for a variety of specific conditions, including major stroke, severe traumatic brain injury, and newborn hypoxic-ischemic encephalopathy (Bernard & Buist, 2003). CT has over the past decades become established as a standard therapy within the field of hematology/oncology to ameliorate the incidence and severity of CMT-induced OM.

Oral CT refers to the application of ice chips (IC), crushed ice or popsicles. The protective mechanisms behind this intervention have not been studied in detail. However, the promotion of vasoconstriction, resulting in reduced delivery of chemotherapeutic agents to at-risk tissues (e.g., the mucous membrane), continues to be viewed as the most likely mechanism (Mahood *et al.*, 1991). Numerous clinical trials have been performed in subsequent years for various solid and hematologic malignancies (Peterson *et al.*, 2013; Riley *et al.*, 2015). The MASCC/ISOO recommendation regarding the use of oral CT for the prevention of CMT-induced OM is based on a systematic literature review, which included 22 original articles and two meta-analyses (Peterson *et al.*, 2013). The panel supports 30 minutes of oral CT in patients who are receiving bolus 5-FU (recommended) and high-dose melphalan, with or without RT, as conditioning for HSCT (supported) (Lalla *et al.*, 2014). The evidence is in accordance with the updated guidelines published in 2007 (Keefe *et al.*, 2007) and is largely in agreement with the Cochrane collaboration review (Riley *et al.*, 2015). CT is applicable to these regimens, as both drugs are characterized by a relatively short half-life. Depending on the dosage and the individual metabolism, melphalan and 5-FU have a plasma half-life elimination times of 8-12 minutes and 16 minutes, respectively (Peterson *et al.*, 2013).

The conducted studies have been consistent in favoring the use of oral CT for prevention of CMT-induced OM. Only a few percent of the patients develop OM following cryoprevention (Johansson *et al.*, 2019). It is considered well-tolerated (Lilleby *et al.*, 2006) and a readily applicable, cost-effective intervention (Wang *et al.*, 2015). In fact, concerns have been raised as to why CT is not part of a global routine clinical practice for patients who are receiving H-DC prior to HSCT (Sharifi *et al.*, 2017) In addition, CT has been shown to reduce: the number of days with total parenteral nutrition (Lilleby *et al.*, 2006; Svanberg *et al.*, 2010); the need for analgesics (Lilleby

*et al.*, 2006; Svanberg *et al.*, 2007; Salvador *et al.*, 2012); the length of hospitalization (Svanberg *et al.*, 2010); and the level of oral pain (Lilleby *et al.*, 2006). Furthermore, in support of CT, in a 5-year follow-up study, a significantly higher survival rate was observed for patients who were assigned to ice therapy, as compared with the controls (Svanberg *et al.*, 2012).

CT continues to be the best alternative of the three aforementioned preventive strategies. However, for some patients, the use of ice may be confounded by the physically uncomfortable sensation that they experience (Gori *et al.*, 2007) (e.g., shooting pain in the teeth and cold feeling). In a subset of patients, a conditioned aversion to using ice in relation to CMT has been noted (Peterson *et al.*, 2013). In addition, the use of ice requires that it is made from water of good quality, so that there is no risk of contamination by microorganisms, with the consequent risk of infections in already immunosuppressed patients.





## 2. SCIENTIFIC QUESTIONS

The long-term goal of the research described in this thesis is to establish an effective and well-tolerated method for cryoprevention of OM in patients who are receiving chemotherapy. The specific aims of this thesis were to: (i) investigate whether cooling, using a constant low temperature, is effective for oral tissue preservation when exposed to chemotherapeutic agents; (ii) develop an animal model to study the early events which precede the clinically established OM; (iii) assess if a novel intraoral cooling device is better tolerated, and as effective as a cryopreventive method, compared to ice chips; (iv) evaluate whether local cooling affects oral microcirculation and tissue oxygen saturation; and (v) establish how an adequate research protocol should be designed, for a randomized controlled trial, to evaluate cryoprevention of chemotherapy-induced OM. These aims are addressed to answer the following scientific questions:

1. Is cell viability better preserved using a constant low temperature when the oral mucosa is exposed to chemotherapeutic agents? (*Study I*)
2. Are the levels of proinflammatory cytokines reduced when a constant low temperature is used and the oral mucosa is exposed to chemotherapeutic agents? (*Study I*)
3. What are the early events in the oral mucosa which precede a clinical established oral mucositis? (*Study II*)
4. Is an intraoral cooling device better tolerated than ice chips as a cryopreventive method? (*Study III*)
5. Is an intraoral cooling device as effective as ice chips in terms of temperature reduction and cooling distribution? (*Study III*)
6. Does local cooling affect the oral microcirculation? (*Study IV*)
7. Does local cooling affect oxygen saturation in oral tissues? (*Study IV*)
8. How should an adequate research protocol be designed for a randomized controlled trial to evaluate cryoprevention of chemotherapy-induced OM? (*Study V*)



## 3. MATERIALS & METHODS

Detailed descriptions of the materials and methodologies used in the different experiments can be found in the respective papers (I–V). Presented below are the most important details of the study designs, procedures and protocols, data collection, and statistical analyses employed.

### 3.1 Study designs

*Study I* was designed to assess the effect of CMT-induced cell damage on oral mucosa cooled to different temperatures, *in vitro* using an artificial model. *Study II* was considered to assess the early events in the oral mucosa after exposure to CMT. *Studies III* and *IV* involved experiments in healthy volunteers to compare IC and an ICD with regards to tolerability and cooling efficacy, as well as the effects on the oral microcirculation and tissue oxygen saturation (StO<sub>2</sub>). *Study V* comprises a research protocol in which the experiences acquired in *Studies I–IV* have been employed in our ongoing randomized, controlled trial (RCT). This research protocol is extensive and therefore worthy of being published.

### 3.2 Procedures and Protocols

#### 3.2.1 Studies I and II

##### *Tissue-engineered oral mucosa (Study I)*

The tissue-engineered oral mucosa (TEOM) models were produced as previously described (Colley *et al.*, 2011). Briefly, normal oral keratinocytes and normal oral fibroblasts were isolated from biopsies obtained from the buccal and gingival oral mucosa of patients during routine dental procedures. De-epithelized dermis (1 cm<sup>2</sup>) was placed in each well of a 6-well plate, an 8-mm-diameter steel ring was placed on the upper surface, and  $1 \times 10^6$  normal oral keratinocytes and  $5 \times 10^5$  normal oral fibroblasts were added in 500  $\mu$ l of Green's medium. The medium was changed twice daily for 48 hours, after which the rings were removed and the models incubated at the air-to-liquid interface on steel grids for a further 10 days at 37°C in 5% CO<sub>2</sub>.

##### *Presto Blue (Study I)*

Presto Blue is a resazurin-based indicator that is frequently used to determine cytotoxicity *in vitro*. In viable cells, resazurin is reduced to resorufin by mitochondrial activity. This

reduction causes Presto Blue to change from its blue non-fluorescent form to the fluorescent pink resorufin. The conversion rate is proportional to the number of metabolically active cells and can be evaluated using fluorescence measurements (Xu *et al.*, 2015).

#### *Enzyme-linked immunosorbent assay (Study I)*

An enzyme-linked immunosorbent assay (ELISA) is a plate-based assay that detects and quantifies specific molecules (peptides/proteins, hormones and vitamins) in a liquid sample using an antigen-antibody reaction (Aydin, 2015). In general, an ELISA comprises at least one antibody with a high affinity for a particular antigen, an enzyme-linked conjugate, and a substrate that upon reaction produces a color change. There are three different types of ELISA: direct assay, indirect assay, and capture assay (sandwich).

Direct and indirect ELISAs differ depending on the context of the experiment. The direct assay is the simplest form of ELISA and is used for high-molecular-weight antigens. A primary antibody, labeled with an enzyme, is added to the antigen to form an antigen-antibody-enzyme complex. Subsequent addition of an appropriate enzymatic substrate to the medium produces a signal, involving a change in coloration. The indirect assay applies to a setting with two antibodies, in which an unlabeled primary antibody is used to bind to the antigen, followed by the addition of a labeled secondary antibody. This results in the formation of an antigen-antibody-antibody-enzyme complex.

The capture assay is reported to be 2–5-times more sensitive than all other ELISAs. For this reason, it was employed in *Study I* for the detection and quantification of the proinflammatory cytokines IL-6 and TNF- $\alpha$ . In contrast to the previously described assays, this method uses wells that are coated with a capture antibody, which binds to the antigen in the sample, followed by the addition of a primary labeled antibody, which allows for detection of the antigen-antibody-enzyme complex. The antigen concentration can then be quantified spectrophotometrically using the optical density (OD) or fluorescence detection.

#### *Spectrophotometry (Study I)*

Spectrophotometry, which is a branch of spectroscopy, is used to measure the reflection, absorbance and transmission of light by a sample, as a function of wavelength. However, for most biological applications, the OD, i.e., the sum of the light absorbed, scattered or reflected by a solution, is measured. A higher OD indicates how much slower the light travels through the material. Absorbance is related logarithmically to transmission ( $A = -\log T$ ) and there exists a relationship between absorbance and concentration: The

Lambert-Beer law. The Beer's law states that the absorptive capacity of a dissolved substance is directly proportional to its concentration in a solution.

### *Animals (Study II)*

The male Sprague-Dawley rats that were used in *Study II* were bred and housed under standard conditions in the animal facility at University of Gothenburg according to the institutional guidelines. This breed of rat was first raised by the Sprague-Dawley farms in Madison, Wisconsin, in 1925, later to become the Sprague-Dawley Animal Company. This laboratory rat is a multipurpose breed of albino rat used extensively in medical research. Furthermore, compared to other breeds male Sprague-Dawley rats have a longer tail in proportion to their body length. As a consequence, the tail vein is larger and secure an easy injection. Therefore, this breed was particularly well suited to conduct the present study.

### *Immunohistochemistry (Study II)*

Tissue sections were deparaffinized in xylene, rehydrated stepwise in solutions containing high ethanol concentration to high milli-Q water concentration and then immersed in citrate buffer, which was heated to boiling temperature (95-100°C) in a microwave oven for 10 minutes. The sections were kept in the citrate solution in room temperature for 20 minutes. When the solution reached 40°C the sections were removed from the solution, followed by 10 minutes incubation in a solution containing copper sulphate (5 mM) and ammonium acetate (50 mM; pH 5.0). Nonspecific background staining was blocked and subsequently the sections were incubated at 4°C overnight in phosphate-buffered saline (PBS) containing normal goat serum, Triton x-100 and a cocktail of primary antibodies; monoclonal mouse anti IL-6 and polyclonal rabbit anti TNF- $\alpha$ . The following day the sections were incubated at room temperature for 1 hour in a PBS cocktail containing secondary antibodies Alexa fluor 488 goat anti rabbit and Texas Red goat anti mouse together with 1% normal goat serum and 0.1% Triton x-100. Finally, the sections were dehydrated stepwise in solutions containing high milli-Q water concentration to high ethanol concentration and mounted with coverslips and prolong gold antifade reagent containing DAPI.

All immunostainings were visualized using a Nikon 90i brightfield and fluorescence microscope with appropriate filters for FITC, Texas Red and DAPI, fitted with a DS-Fi camera and the NIS element imaging Software v.4.40. To compare the staining intensities between the treatment groups, all images were captured using the same settings, including exposure time, contrast settings and digital gain.

#### 3.2.2 Studies III, IV and V

##### *Subjects*

In *Study III*, a total of 20 subjects, 17 females and 3 males, with mean age of  $23.9 \pm 6$  years was recruited to the study from the Institute of Odontology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden to participate in the study. All the subjects were healthy and had no medical conditions or were not using any drug with substantial impact on the cardiovascular system. None of the participants had mucosal lesions or were smokers or users of oral tobacco products.

In *Study IV*, a total of 10 students or employees, 4 females and 6 males, with mean age of  $29 \pm 6$  years was recruited to the study from the Academic Medical Center, Amsterdam, The Netherlands. Inclusion was arbitrary and based on the availability of the participants. Subjects were considered eligible to participate in the study if they met all of the following criteria: no medical diagnosis established by a physician, no taking of drugs with an impact on the cardiovascular system; no mucosal lesions; and no use of tobacco or snuff.

##### *Questionnaires (Studies III and V)*

A questionnaire that was specially designed for the purpose of the study was employed to evaluate the primary endpoint in *Study III*. The questionnaire included a total of 15 questions, primarily aimed at evaluating which of the two methods (IC or ICD) that was preferred by the subjects. Additional questions addressing adverse events were included, and space to share other comments in the running text was provided. Prior to the study, the questionnaire was face-validated to ensure that the questions and answers were interpreted as intended. A tailored version of this questionnaire was used in *Study V*, to enable a tolerability assessment and the subsequent comparison of the two cooling interventions in the RCT. Furthermore, *Study V* included a validated QoL instrument (FACT-G v. 4), and a study-specific diary. The QoL instrument considers a broad range of life domains categorized within four dimensions of well-being: physical, social, emotional, and functional. The diary collates information about any side-effects caused by the conditioning therapy.

##### *Thermographic imaging (Study III)*

The FLIR E60bx and its associated FLIR tools software, along with a free software tool (BioPix; [www.biopix.se](http://www.biopix.se)) were used to analyze the secondary endpoints in this study. The FLIR E60bx is a high-resolution (320×240 pixels) infrared thermal imaging camera that can detect temperatures between  $-20^{\circ}\text{C}$  and  $120^{\circ}\text{C}$ . The camera operates with plenty of tools to fine-tune and analyze images quickly, and to transfer data to specific free software packages (FLIR Tools) for editing and further detailed analysis.

BioPix is an easy-to-use free software that facilitates automatic quantifications of images and biological preparations.

#### *Microcirculatory imaging and tissue oxygen saturation (Study IV)*

Microcirculation measurements were performed using the CytoCam Video Microscope System. Briefly, this commercially available system comprises a lightweight, hand-held instrument that operates by epi-illuminating the tissue of interest with green light at a wavelength of 530 nm. Since the hemoglobin from erythrocytes absorbs the green light, the remaining light scatters into the surrounding tissue and a high-resolution image of dark circulating erythrocytes in the lumen of blood vessels, contrasted by a bright background in a field of view, is generated. The hand-held instrument is connected to a fan-less, medical-grade panel personal computer equipped with the software for camera operation and video data processing. Counting of objects in the captured images was performed using the Adobe Photoshop counting tool.

StO<sub>2</sub> levels, were measured using the InSpectra™ StO<sub>2</sub> Tissue Oxygen Model monitor. This system operates with near-infrared spectroscopy (NIRS), using a 15-mm optical sensor that emits and detects reflected near-infrared wavelengths (700–1000 nm). The amount of light that is reflected back to the sensor following tissue transillumination is dependent upon the oxygen saturation of chromophores, e.g., hemoglobin and myoglobin. The monitor operates non-invasively with a rapid response that estimates continuously the approximate value of StO<sub>2</sub> by calculating the overall oxy- and deoxy-hemoglobin content in the circulating red blood cells.

#### *Ice (Studies III, IV and V)*

In *Studies III* and *IV*, the IC were produced from tap-water in a commercial ice maker that fulfilled the strict hygiene regulations of a hospital environment. Prior to cooling, the temperature of the IC was measured as -0.5°C. The ice was stored in a metal container at room temperature during the cooling procedures. In *Study V*, it was determined that in the RCT, popsicles will be provided in addition to IC, if this is preferred by the patients.

#### *Cooling device (Studies III, IV and V)*

The ICD is composed of a soft-plastic material that is designed to cool the cheeks, lips, floor of the mouth, tongue, gums, and hard palate. The ICD consists of conduits for water, which is delivered *via* a portable cooling and thermostat unit. The unit, which produces water at temperatures that can be set at between 6°C and 22°C, is connected to the ICD by tubes that allows a flow rate of 0.25 mL/min. A water temperature of 8°C with a flow rate of 0.25 mL/min was used throughout the cooling procedures in each

of the studies. In *Study III*, only one medium-size of the ICD was available, whereas both, small-and medium-sized ICDs, were available in *Study IV* and were designated for use in the RCT in *Study V*.

## 3.3 Data collection

### 3.3.1 Study I

Several pilot experiments were carried out to determine: the ability of the model to function in a cold environment; the exposure time/optimal concentration of 5-FU; and the appropriate follow-up time. Consequently, for the TEOM, treatment with 162 µg/mL of 5-FU for 2 hours and with a follow-up period of 48 hours, was established as the optimal conditions to be used in the main study. The TEOM models were pre-incubated at 20°C, 25°C, 30°C or 35°C for 30 minutes before treatment with 5-FU (162 µg/mL) for 2 hours or incubated at 35°C with no drug for the control. Following incubation, the TEOM models were rinsed in PBS at the specified temperatures to eliminate superficial cytostatic agents and fresh medium was added. The models were incubated for a further 30 minutes at the specified temperatures, washed again with PBS to eliminate profound cytostatic agents, replaced with fresh medium, and incubated for 48 hours at 37°C before assessments were made of viability and proinflammatory cytokine release using Presto Blue and ELISAs, respectively.

### 3.3.2 Study II

The animals were anaesthetized and injected in the tail vein with either saline or a cytostatic drug, 5-FU, in order to induce OM. One-hour post injection, the animals were injected with buprenorphine for analgesic purposes.

Three or five days after treatment the animals were euthanized with an overdose of pentobarbital. The buccal mucosa was excised and stored in phosphate-buffered paraformaldehyde solution for one day. The fixated tissue was then further dissected if necessary and stored in phosphate-buffered sucrose solution. Sucrose immersed samples were sent to a company specialized in tissue sectioning. The specimens were embedded in paraffin and sectioned into 8 µm transversal sections before histological analysis, including immunohistochemistry (IHC).

### 3.3.3 Studies III, IV and V

In *Studies III* and *IV*, the subjects were provided with detailed information related to the respective study protocol and were asked to complete a form with information



about their medical history. Informed consent was obtained upon understanding of the facts, implications, and consequences of the cooling procedures. Eligible subjects were enrolled and randomized in the order in which the two procedures were to be commenced, and they were asked to refrain from alcoholic or caffeinated beverages, food or any medication for at least 1 hour prior to the study start. The subjects were examined in a dental office (ambient temperature, 22°C) and acclimatized for 30 minutes prior to measurements. Basic hemodynamics were measured prior to and after cooling in *Study III* and only prior to cooling in *Study IV*. These measurements were carried out to obtain an indication of any systemic effects of the cooling and to exclude unknown cardiovascular diseases, respectively. In *Study V*, the following sequence was chosen to be used in the RCT: written informed consent, enrollment/inclusion, and randomization at a ratio of 1:1 to cooling with IC/popsicles or cooling with the ICD.

In *Studies III* and *IV*, prior to using IC, the subjects were informed and instructed to insert an ounce of IC in the mouth and swirl it in the OC, so as to cool as much of the mucosa as possible. In addition, to achieve cooling of the hind-most part of the throat, the subjects were instructed to gurgle the melted slurry for a few seconds before swallowing or spitting out. In the sessions using the ICD, the device was self-inserted by the subjects under supervision, and adjusted until it felt comfortable. A staff member verified that there was good adherence to the oral mucosa before cooling was started. The participants used the IC and ICD until they interrupted the cooling or for a maximum of either 60 minutes in *Study III* or 30 minutes in *Study IV*. In *Study V*, a slightly modified protocol was decided upon for the RCT, with adaption to the half-life values of the chemotherapeutic drugs administered for each individual diagnosis. The cooling time to be employed in the RCT was estimated as 1.5 hours for multiple myeloma and 3–6 hours for lymphoma.

In *Study III*, the baseline temperatures were measured using the FLIR E60bx camera prior to and immediately after the cooling sessions, in each of the following intraoral locations: right buccal mucosa, left buccal mucosa, upper labial mucosa, lower labial mucosa, anterior and posterior dorsal tongue, anterior ventral tongue, and hard palate. Following each cooling session, the subjects completed the questionnaire related to tolerability and adverse events. In *Study IV*, the microcirculation and StO<sub>2</sub> were measured in the right buccal mucosa, right upper lip, and right lower lip, prior to and immediately after each cooling session with the two cooling methods. This was followed by declarations by the subjects as to which of the two interventions they preferred. In *Study V*, it was determined that all the measurements in the RCT (for details, see the article), with the exception of the patient-reported assessment of the cooling method, will be registered beginning at admission and continue until discharge or until Day +28.

All the data obtained from the experiments in *Studies I–IV* were collected and stored in data-sheets designed in Microsoft Office Excel for Mac 2016 (Microsoft Inc., Redmond, WA, USA) before statistical analysis. In *Study V*, a case report form, referred to as a “checklist”, was designed for data collection in the RCT. The checklist can be identified by a preprinted trial number that is assigned at registration. The investigator or an authorized staff member will throughout the RCT complete the checklist onsite. The checklist is dated and signed by the investigator upon completion. The PheedIt system will be used for the capture of clinical data and will serve as the clinical database for the study. Data from the checklist will be entered, cleaned, and validated by the sponsor-appointed person before being entered in the PheedIt system.

## 3.4 Statistical analyses

In *Study I*, all the data were quantitative and presented in a descriptive manner as mean  $\pm$  SD. One-way analysis of variance (ANOVA) was used to determine any statistically significant differences between the experimental and control TEOM models. However, to limit type I errors (false-positive results), a *post hoc* test, Dunnett’s test for multiple comparisons, was performed. This statistical tool, also referred to as “many-to-one comparison”, compared each of the experiments with the single control. A *p*-value  $< 0.05$  was considered statistically significant. The statistical analysis was performed using the GraphPad Prism ver. 6.00 software (GraphPad Software Inc., La Jolla, CA, USA).

In *Study II*, all values were expressed as mean  $\pm$  SD. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple comparisons. A *p*-value  $< 0.05$  was regarded as statistically significant. All statistical calculations were performed in the GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA).

In *Study III*, the primary endpoint was analyzed using McNemars’s sign test, which is a non-parametric statistical test employed to compare paired nominal data, with a dichotomous trait in dependent samples. Data on adverse events were extracted from the questionnaires and presented in a descriptive manner. The secondary endpoints were analyzed using a two-sided paired samples Student’s *t*-test. The *t*-test is a parametric test used to determine whether the mean difference between two data sets is zero. ANOVA was further used to test for period and sequence effects. A significance level of 5% was used, i.e., a *p*-value  $< 0.05$  was regarded as statistically significant. The statistical analysis was performed using the SPSS ver. 23 statistical analysis software package (IBM, Armonk, NY, USA).

In *Study IV*, normality distributions for all the study variables were assessed with a Shapiro-Wilk test, and a Gaussian distribution was confirmed for all the quantitative variables. Microcirculation parameters and StO<sub>2</sub> variables were assessed with a paired samples Student's *t*-test. In the assessment of tolerability, i.e., "which of the two cooling methods did you prefer?", a sign test (McNemar's test) was used. A *p*-value < 0.05 was regarded as statistically significant. The calculations were conducted using the IBM SPSS Statistics ver. 24 software package (IBM Inc., Armonk, NY, USA).

In *Study V*, it was calculated that a sample size of a least 90 patients per arm will give a power of 80% to discover an average difference of at least 0.42 OMAS, based on previously published data (Peterson *et al.*, 2009). The analysis is based on the SD for OMAS being 1 in both groups and the use of an independent Student's *t*-test with a significance level of 5%. The sample size was calculated (power [1- $\beta$ ]=0.8;  $\alpha$ =0.05) using the G\*power ver. 3.1.9.2 software (University of Düsseldorf, Germany).

The statistical analyses intended for use in the RCT are at the population level, on an intention-to-treat basis. The variables are grouped into primary, secondary, and tertiary according to the level of significance. Analysis of the primary variables will be conducted in a multiple regression model, to describe the relationship between the continuous dependent variable (OMAS-total) and the independent categorical variables (treatment group, diagnosis, and center). The secondary variables of OMAS-ulceration and OMAS-erythema will be analyzed in the same way as the primary variables, whereas the incidence of OM (according to the WHO) and the tolerability of the cooling methods will be analyzed with a logistic regression model (see the detailed descriptions in the article). A *p*-value < 0.05 will be regarded as statistically significant. The statistical analyses will be performed using the SPSS ver. 23 statistical analysis software package (IBM).

### 3.5 Ethical approvals

*Study I*, was approved by the Sheffield Research Ethics Committee (09/H1308/66).

*Study II*, was approved by the local Animal Ethics Committee at the University of Gothenburg (permit #1603/18).

For *Study III*, the regional Ethical Review Board in Gothenburg did not consider an ethical application necessary.

*Study IV*, will be approved after minor corrections (chairman action) by the Institutional Medical Ethics Committee of the Academic Medical Center of the University of Amsterdam (reference no. NL69365.018.19).

*Study V*, was approved by the regional Ethical Review Board in Gothenburg (Dnr: 586-15).

# 4. RESULTS

This section lists the main findings from *Studies I–V*, followed by a more detailed description of the results obtained from the respective studies.

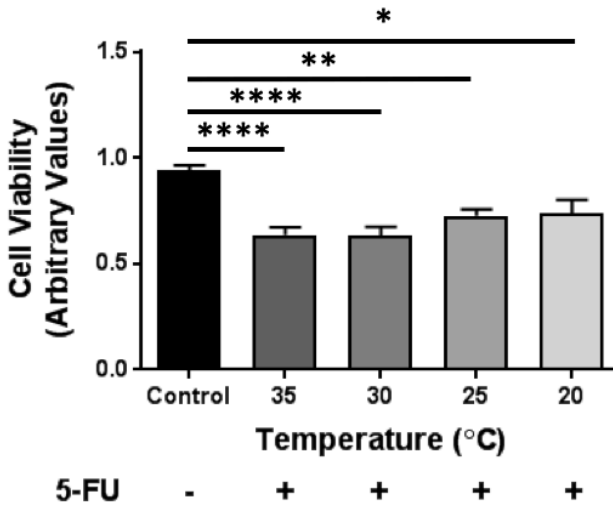
## Main findings

1. A higher cell viability was observed at lower temperatures (*Study I*).
2. TNF- $\alpha$  levels were statistically significantly lower in TEOM models incubated at 20°C compared to treated controls that were incubated at 35°C (*Study I*).
3. The proinflammatory cytokines (IL-6 and TNF- $\alpha$ ) were significantly upregulated in the oral mucosa five days after treatment with chemotherapy (*Study II*).
4. The ICD was significantly better tolerated than IC (*Study III*).
5. The ICD was as effective as IC in terms of temperature reduction and cooling distribution (*Study III*).
6. The oral microcirculation was reduced following cooling with the ICD (*Study IV*).
7. The oral tissue oxygen saturation level was reduced following cooling with IC or the ICD (*Study IV*).
8. A research protocol to assess cryoprevention of chemotherapy-induced OM should be established through multidisciplinary collaborations and should include both objective and subjective assessments (*Study V*).

## 4.1 Study I

### Is cell viability better preserved using a constant low temperature when the oral mucosa is exposed to chemotherapeutic agents?

TEOM models, incubated at 20°C, 25°C, 30°C or 35°C and treated with 5-FU, compared to untreated controls at 35°C, revealed a statistically significant decrease in viability for all the treated models at all of the temperatures tested, as compared to the untreated controls. This occurred in a temperature-dependent manner, i.e., a lower temperature showed higher cell viability. However, no statistically significant difference was observed when the treated TEOM models incubated at different temperatures were compared with each other (Fig. 7).

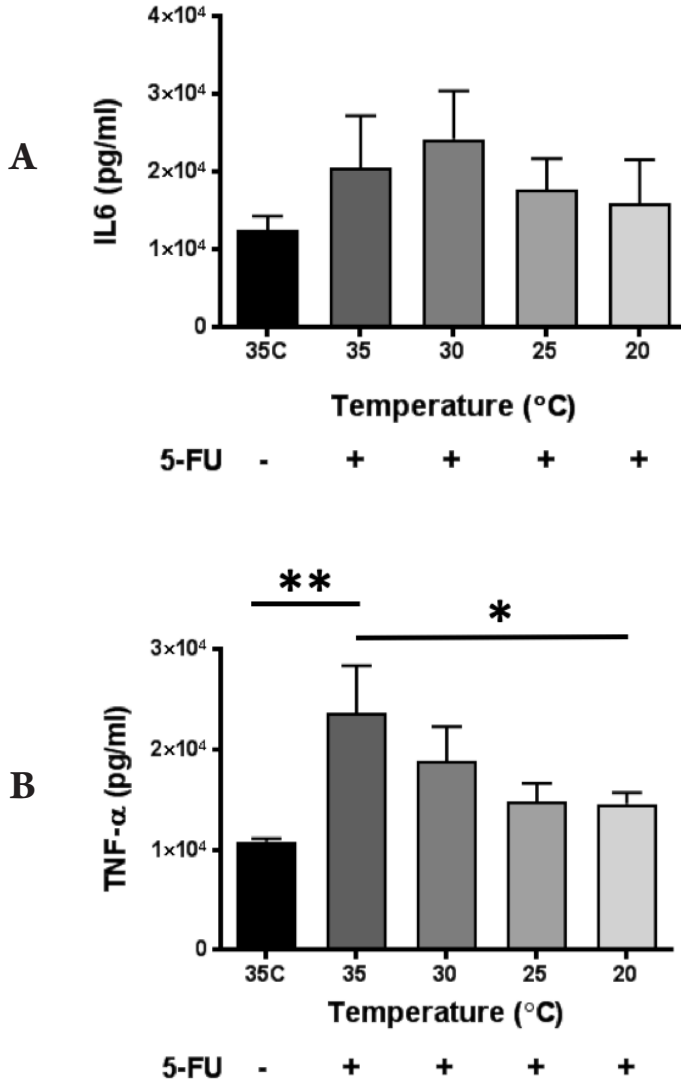


**Figure 7.** The effects of cooling on cell viability in the tissue-engineered oral mucosa model. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ . Reprinted with permission from Walladbeigi J., Smith S. A., Grayson A. K., Murdoch C., Jontell M., Colley, H. E. *Cooling of the oral mucosa to prevent adverse effects of chemotherapeutic agents: An in vitro study.* *J Oral Pathol Med.* 2018; 47: 477–483., © 2018 John Wiley & Sons Ltd.

### Are the levels of proinflammatory cytokines reduced when a constant low temperature is used and the oral mucosa is exposed to chemotherapeutic agents?

In general, the 5-FU-treated TEOM models displayed increased levels of IL-6 and TNF- $\alpha$  at all the temperatures tested, as compared to the untreated controls. The levels of IL-6 were markedly higher at 30°C and 35°C than at 20°C or 25°C. However, the difference was not statistically significant. In contrast, the TNF- $\alpha$  levels were

significantly higher in the 5-FU-treated TEOM models incubated at 35°C than in the untreated controls incubated at the same temperature ( $p < 0.01$ ) or the treated models incubated at 20°C ( $p < 0.05$ ) (Fig. 8).

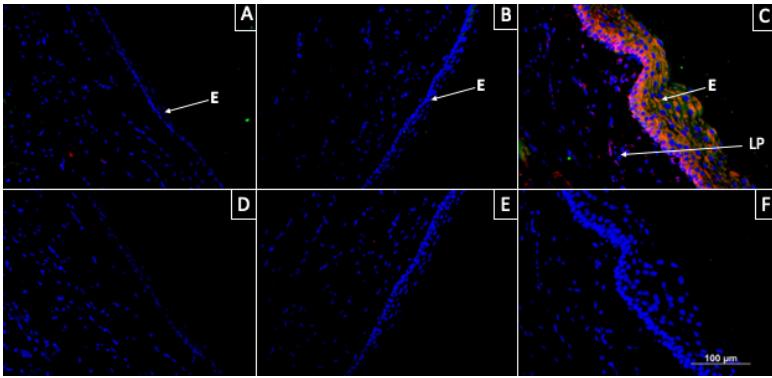


**Figure 8.** The effects of cooling on the release of (A) IL-6 and (B) TNF- $\alpha$  in the tissue-engineered oral mucosa model. \* $p < 0.05$ , \*\* $p < 0.01$ . Reprinted with permission from Walladbegi J., Smith S. A., Grayson A. K., Murdoch C., Jontell M., Colley, H. E. *Cooling of the oral mucosa to prevent adverse effects of chemotherapeutic agents: An in vitro study.* *J Oral Pathol Med.* 2018; 47: 477–483., © 2018 John Wiley & Sons Ltd.

## 4.2 Study II

### What are the early events in the oral mucosa which precede a clinical established oral mucositis?

The IHC staining for IL-6 and TNF- $\alpha$  revealed that the oral epithelium in control tissue displayed little or no expression of these cytokines (Fig. 9A). Three days following treatment with 5-FU, there was still no detectable expression (Fig. 9B). However, five days after treatment with 5-FU, in a hypertrophic and hyperplastic state, the oral epithelium displayed distinct expression of both IL-6 and TNF- $\alpha$  (Fig. 9C). IL-6 was predominantly seen in the basal cell layer, while the TNF- $\alpha$  staining was confined to the suprabasal area. In addition, five days following 5-FU treatment revealed detectable levels of IL-6 in the fibroblasts present in the underlying *lamina propria* (Fig. 9C).



**Figure 9.** Expression of IL-6 and TNF- $\alpha$  after 5-fluorouracil (5-FU) treatment. Representative images showing (A) absence of expression of IL-6 and TNF- $\alpha$  in the oral epithelium of saline-treated (control) rats. Three days after treatment with 5-FU (B) there was still no detectable expression of IL-6 or TNF- $\alpha$ . Five days after treatment with 5-FU (C) significant expression of both IL-6 and TNF- $\alpha$  was seen in the hypertrophic and hyperplastic epithelium and levels of IL-6 was also seen in the *lamina propria*. The lower panel (D-F) shows the corresponding negative controls. Red = IL-6. Green = TNF- $\alpha$ . Blue = nucleus stain with DAPI. E = Epithelium. LP = *Lamina Propria*. Scale bar = 100  $\mu$ m.

## 4.3 Study III

### Is an intraoral cooling device better tolerated than ice chips as a cryopreventive method?

The majority of the subjects, 16 out of 20 (80%), favored the ICD over IC ( $p = 0.0118$ ). When the cooling sequence was analyzed, i.e., the order in which the cooling procedures were undertaken, 9/10 subjects who started with IC followed by the ICD and 7/10 who followed the opposite sequence stated a preference for the ICD



( $p = 0.291$ ). The ANOVA showed no statistically significant sequence effects. There were, however, two significant period effects, for temperature reduction ( $p = 0.014$ ) and change in systolic blood pressure ( $p = 0.048$ ). Regarding adverse events, there was a clear trend towards design-related problems for the ICD, with difficulties with swallowing ( $n = 15$ ), rubbing discomfort ( $n = 12$ ), and poor fit ( $n = 7$ ) being the most commonly reported. In contrast, the problems linked to IC were low temperature-related and included: cold ( $n = 12$ ); numbness ( $n = 11$ ); teeth sensations ( $n = 8$ ); and pain ( $n = 5$ ).

### Is an intraoral cooling device as effective as ice chips in terms of temperature reduction and cooling distribution?

No statistically significant differences were observed with regards to temperature reduction and cooling distribution between the two cryotherapies. The mean temperature reductions were  $8.08^{\circ}\text{C}$  and  $7.91^{\circ}\text{C}$  for the IC and ICD, respectively, giving a mean difference of  $0.17^{\circ}\text{C}$  ( $p = 0.795$ ). The corresponding values for cooling distribution were 48.44% for IC and 47.33% for the ICD, i.e., a difference of 1.11% ( $p = 0.457$ ) (Table 1).

**Table 1.** Mean temperature reductions and cooling distributions in the oral mucosa following cooling with ice chips and the intraoral cooling device.

Subj.	Temperature reduction ( $^{\circ}\text{C}$ )		Cooling distribution (%)	
	Ice chips	Cooling device	Ice chips	Cooling device
1*	8.50	8.90	51.48	46.76
2	10.80	10.80	54.81	55.81
3	4.90	9.60	48.41	52.49
4	11.00	11.10	45.69	50.96
5	6.10	8.90	52.23	49.14
6*	7.90	6.30	48.06	43.33
7	6.40	6.60	53.58	39.44
8	10.80	9.00	50.29	43.45
9	9.10	6.90	43.66	50.59
10	10.60	9.20	46.63	50.59
11	10.70	8.80	45.28	49.01
12	8.10	5.90	45.76	49.30
13	9.40	9.50	46.13	48.00
14	3.10	8.70	47.35	45.45
15	10.30	8.90	49.28	49.68
16	9.20	9.40	43.58	52.05
17	7.00	7.60	48.23	41.36
18*	8.00	1.60	41.48	39.39
19	5.20	1.60	59.25	42.36
20*	4.40	8.80	47.69	47.41
<b>Mean</b>	<b>8.08</b>	<b>7.91</b>	<b>48.44</b>	<b>47.33</b>
<b>SD</b>	<b>2.41</b>	<b>2.54</b>	<b>4.24</b>	<b>4.54</b>

The subjects marked in red started cooling with ice chips and the subjects marked in blue started cooling with the intraoral cooling device. Subjects who tolerated ice chips better than the intraoral cooling device are marked with an asterisk (\*). Modified and reprinted with permission from Walladbegi J, Gellerstedt M, Svanberg A, Jontell M. *Innovative intraoral cooling device better tolerated and equally effective as ice cooling.* *Cancer Chemother Pharmacol.* 2017 Nov; 80(5):965-72. (<http://creativecommons.org/licenses/by/4.0/>).

## 4.4 Study IV

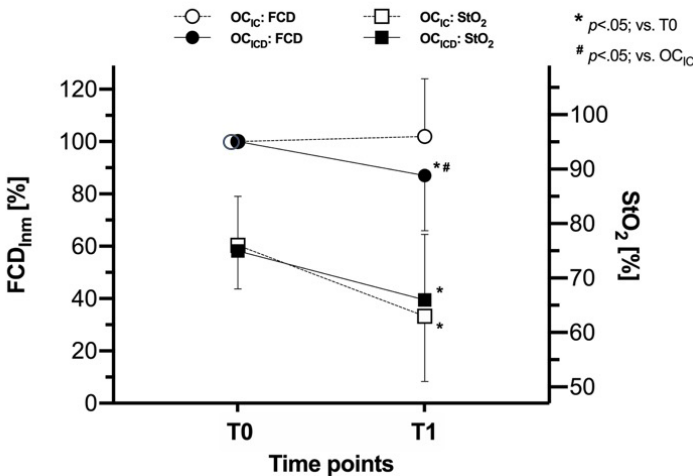
### Does local cooling affect the oral microcirculation?

Analysis of the microcirculation in the oral-lining mucosa, interpreted by functional capillary density (FCD), revealed no significant difference between the two interventions at baseline. In the sessions using IC, a mean increase of two percentage points (P.P.) was detected following 30 minutes of cooling. However, this slight increase was not statistically significant compared to the baseline measurements. In contrast, a mean decrease of 13 P.P. was observed when the same comparison was made with the ICD ( $p < 0.05$ ). A subsequent comparison of the two cryotherapies after 30 minutes of cooling showed a statistically significant difference for FCD, with a mean difference 15 P.P. ( $p < 0.05$ ) (Fig. 10).

### Does local cooling affect oxygen saturation in oral tissues?

Concomitant measurements of tissue oxygen saturation ( $StO_2$ ) revealed a statistically significant decrease with both cooling methods when baseline was compared with the follow-up data. A mean difference in P.P. was registered for both IC (13;  $p < 0.05$ ) and for the ICD (10;  $p < 0.05$ ). No statistically significant difference was observed between the two cryotherapies following 30 minutes of cooling (Fig. 10).

**Oral lining mucosa functional capillary density ( $FCD_{Inm}$ ) and tissue oxygen saturation ( $StO_2$ ) before and 30 min after cooling**



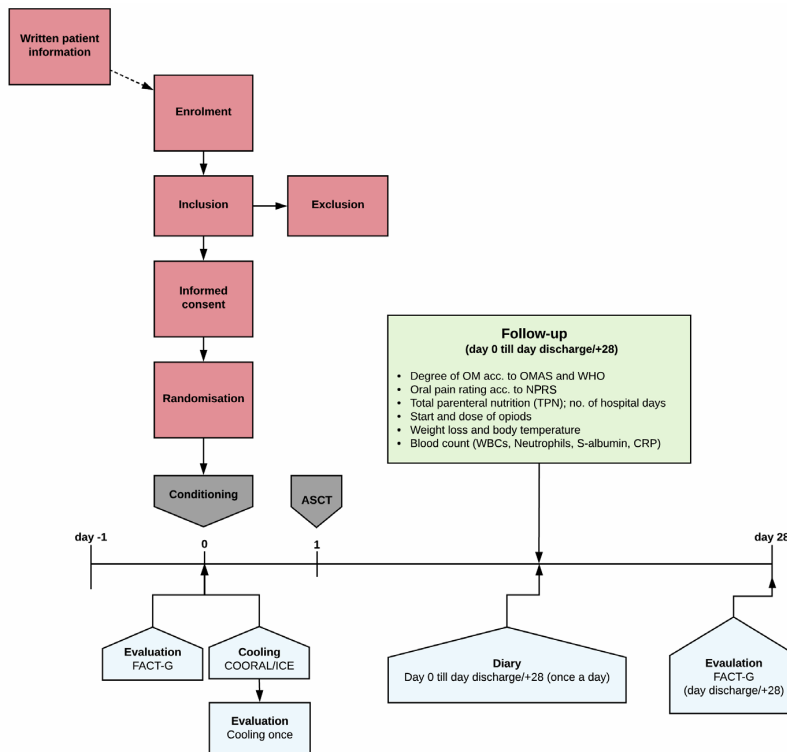
**Figure 10.** A composite graph illustrating the results of pooling all the oral microcirculatory functional capillary density (FCD) and tissue oxygen saturation ( $StO_2$ ) measurements before and 30 minutes after cooling with ice chips and intraoral cooling device. T0 baseline time point, T1 follow-up time point,  $OC_{IC}$  oral cryotherapy with ice chips,  $OC_{ICD}$  oral cryotherapy with intraoral cooling device. \* $p < 0.05$ ; vs. T0, # $p < 0.05$ ; vs.  $OC_{IC}$ .

## 4.5 Study V

### **How should an adequate research protocol be designed for a randomized controlled trial to evaluate cryoprevention of chemotherapy-induced OM?**

The preparation of this study protocol for the RCT was initiated nearly 2 years in advance of the RCT. Appropriate research questions, aims, endpoints, and statistical analyses, including a sample size calculation, were addressed and discussed with a statistician. Frequently measured variables within this field, e.g., the severity and duration of OM, were included in a preliminary draft, along with patient-reported outcomes, e.g., oral pain and QoL. An inquiry regarding participation in the clinical study was sent to all university hospitals in Sweden. The hospitals that expressed interest ( $n = 4$ ) were provided with the preliminary protocol and were given the opportunity to make suggestions and requests. This was followed by a multi-professional interdisciplinary gathering, in which the preliminary draft was discussed and a decision was made regarding the final version of the clinical protocol. An appropriate study design was adopted for each of the participating hospitals and sub-diagnosis, respectively. An example of the flowcharts used is illustrated in Fig. 11.

The research question(s), aims, and endpoints were ranked according to the degree of clinical significance. OM given as OMAS-total was designated as the primary endpoint, followed by several secondary endpoints, e.g., OMAS-ulcers and OMAS-erythema. As patient-reported evaluations were also of great importance to assess, diaries, questionnaires and validated QoL instruments were applied.



**Figure 11.** Flowchart for patients with multiple myeloma at Karolinska University Hospital. **Day 0:** Admission, chemotherapy conditioning, oral mucosal cooling, along with completion of the quality of life (QoL) questionnaire (FACT-G) and evaluation of the cooling method. **Day 1:** Autologous stem cell transplantation (ASCT). Follow-up (green box) and perception of oral problems using a diary begins at admission and continues until discharge or **Day +28**. QoL (FACT-G) is evaluated again at discharge. CRP, C-reactive protein; NPRS, Numeric Pain Rating Scale; OM, oral mucositis; OMAS, Oral Mucositis Assessment Scale; WBC, white blood cell. Reprinted with permission from Walladbegi J., Svanberg A., Gellerstedt M. *Protocol for a randomized controlled trial to study cryoprevention of chemotherapy-induced oral mucositis after autologous stem cell transplantation*. *BMJ Open*. 2018 Oct; 8(10):e021993. (<http://creativecommons.org/licenses/by-nc/4.0/>).

## 5. GENERAL DISCUSSION

Various interventions have been evaluated for the management of OM. These include, mucosal surface protectants, anti-inflammatory formulations, antimicrobials, growth factors, photobiomodulation therapy, CT and a plethora of miscellaneous agents (Stokman *et al.*, 2006; Lalla *et al.*, 2008; Worthington *et al.*, 2011). However, despite the numerous options, CT using ice remains the best intervention to alleviate OM following conditioning therapy with cytotoxic drugs (Rubenstein *et al.*, 2004; Keefe *et al.*, 2007; Peterson *et al.*, 2013; Kadakia *et al.*, 2014; Lalla *et al.*, 2014; Riley *et al.*, 2015). Therefore, it is reasonable to assume that CT is the most appropriate method for the prevention of CMT-induced OM. Unfortunately, due to several shortcomings, there is limited use of ice cooling as a preventive method in clinical practice. This is the rationale for the development of the ICD. In addition, as the ICD can be operated at different temperatures, novel research questions were addressed as part of this thesis.

While the treatment of cancers with cytotoxic therapies is becoming increasingly effective, it continues to be responsible for the onset and duration of OM. Although, ice has been shown to be effective in reducing this adverse effect (Mahood *et al.*, 1991; Roche *et al.*, 1993; Cascinu *et al.*, 1994; Dumontet *et al.*, 1994; Meloni *et al.*, 1996; Baydar *et al.*, 2005; Karagozoglul & Filiz Ulusoy, 2005; Nikoletti *et al.*, 2005; Lilleby *et al.*, 2006; Mori *et al.*, 2006; Papadeas *et al.*, 2007; Svanberg *et al.*, 2007; Svanberg *et al.*, 2010), no previous studies have investigated whether the use of a constant low temperature better preserves the integrity of oral tissues exposed to chemotherapeutic agents.

Therefore, in *Study I*, TEOM models (Colley *et al.*, 2011; Colley *et al.*, 2013) were incubated at different clinically relevant temperatures and treated with 5-FU. In general, our findings demonstrated a better tissue preservation when models were incubated at lower temperatures, suggesting that a constant temperature of 20°C is the most beneficial for the prevention of cytotoxic tissue damage. Interestingly, these results were obtained in the absence of blood vessels, which is noteworthy given that vasoconstriction secondary to cooling is the generally accepted preventive mechanism by which CT prevents tissue damage (Mahood *et al.*, 1991). The absence of a microcirculatory system supports the notion that CT elicits other muco-protective events, in addition to impaired microcirculation, and this may be of relevance for the alleviation of OM in clinical settings. One such muco-protective event may be a lower level of metabolic activity in the basal epithelial cell layer (Lilleby *et al.*, 2006). However, as the TEOM model does not completely replicate the complex structure of the oral mucosa seen *in vivo*, it was of interest to further elucidate this matter in a more physiologically relevant environment (e.g., in the presence of microcirculation and immune components).

Therefore, to determine more precisely at which ideal temperature OM can be prevented clinically, as a first step we developed an animal model where the early events which preceded the clinically established OM were identified.

The increased expression of IL-6 and TNF- $\alpha$  in *Study II* and in the previous *in vitro* study both confirm the previously suggested characteristics of the early events leading to OM (Sonis, 2009). However, considering the absence of any inflammatory infiltrate in the oral mucosa it is likely to assume that IL-6, and probably also TNF- $\alpha$  are initially produced by the parenchymal cells of the oral mucosa. It is well-known that both IL-6 and TNF- $\alpha$  can act as proinflammatory mediators and a large number of studies have shown that IL-6 and TNF- $\alpha$  often act in concordance, including in the oral mucosa (Mostefaoui *et al.*, 2004; Jennings *et al.*, 2016). Likewise, both IL-6 and TNF- $\alpha$  have been shown to be expressed in human oral keratinocytes (Formanek *et al.*, 1998). Further, several studies have shown that IL-6 stimulates various cell types to proliferate, including fibroblasts and keratinocytes (Mihara *et al.*, 1995; Sawamura *et al.*, 1998). However, when the concentrations of these mediators become too high, a cell-toxic effect emerge that causes clinically manifested OM. Since our observations are largely in agreement with the pervious suggestions about the early events which precede clinically established OM (Sonis, 2009) this animal model may be suitable for future studies to identify the ideal temperature for cryoprevention of OM.

Given that our ultimate goal is to implement the ICD in hospital settings, it was of the outmost importance to carry out an initial evaluation of the tolerability and cooling efficacy of the ICD. The results from *Study III*, when comparing the IC and ICD, revealed a significantly better tolerability in healthy volunteers for the ICD. This clinically relevant difference was related to the higher cooling temperature provided by the ICD compared with IC. Since this was the first study to assess an alternative cryotherapeutic method, no evidence was available in the literature to either support or contradict the findings. Nonetheless, when this outcome was reassessed in *Study IV*, 9 out of 10 subjects preferred the ICD over IC, confirming the tolerability findings in *Study III*.

Many studies over the years have utilized ice, in various forms, for the prevention of OM. Most of the studies, included in a comprehensive literature review by the Cochrane collaboration, reported no or few adverse events (Riley *et al.*, 2015). In contrast to those reports, cooling with IC in our study resulted in several adverse events, mainly attributed to the cold, numbness, and tooth sensations. The discrepancy may be explained by the fact that tolerability was the primary endpoint in our study, whereas in most of the other clinical studies tolerability was a minor concern.

In addition to tolerability, the results from *Study III* displayed no statistically significant differences for the secondary endpoints. This was interesting, as the ICD operated at

higher temperatures than the IC, and was available only in one size. Given the inter-individual variations in oral anatomy, one can speculate as to whether the availability of only one size impaired the cooling efficacy of the ICD and whether better cooling effects can be expected with several different sizes of the ICD.

The protective role of CT has for many years been conceptually linked to vasoconstriction, although the mechanism has to the best of our knowledge not been previously evaluated, neither in the first study assessing CT in a clinical trial (Mahood *et al.*, 1991) or in any of the follow-up studies covering this topic (Peterson *et al.*, 2013; Kadakia *et al.*, 2014). In contrast to the previously proposed theory, *Study IV* in general demonstrated no vasoconstrictive effect, interpreted by FCD, following 30 minutes of cooling. This observation was particularly true for the sessions using IC, which was surprising, since the theory of vasoconstriction following CT emerged from trials using ice. In similarity to the other studies described in this thesis, no evidence exists in the literature to either support or contradict our findings. Furthermore, although no effect on the microcirculation was observed in the three investigated oral mucosal regions with IC and in the buccal mucosa with the ICD, it is not realistic to assume that the microcirculation remained unaffected throughout the cooling procedures. There is probably a vasoconstrictive effect at some point. However, due to compensatory mechanisms that are likely present in the oral mucosa to counteract the negative effects of very low temperatures, e.g., shivering (Kulandavelu *et al.*, 2015; Haman & Blondin, 2017), vasoconstriction could not be demonstrated with the methods employed.

Interestingly, the concomitant measurements of  $StO_2$  in *Study IV* showed a statistically significant decrease following 30 minutes of cooling with both IC and ICD. However, it remains unclear as to what caused the decline in  $StO_2$  and several questions arise: (i) Could the decline *per se*, regardless of what may have caused it, lead to sequential mechanisms of relevance for tissue preservation. For example, a lower level of metabolism as a consequence of reduced access to tissue oxygen, resulting in reduced absorption of chemotherapeutic agents?; (ii) Could the obtained results be attributed to the above-mentioned shivering mechanism, as the cells would probably need more oxygen to generate heat after cold exposure?; and (iii) Are there other cellular mechanisms (Bidaux *et al.*, 2015; Bidaux *et al.*, 2016) that consume oxygen in response to cold stimuli and are these of relevance for tissue preservation when exposed to chemotherapy? Further research to answer these questions is warranted.

Several promising results emerged from *Studies I–IV*. However, those regarding tolerability and cooling efficacy at higher cooling temperatures (*Study III*) and those for tolerability (*Study IV*) are of particular interest to carry forward and assess in the RCT. In order to address fully our findings in the RCT, we were prompted to design an extensive research protocol as part of this thesis.

The preparation of this study protocol was initiated almost 2 years in advance of the RCT, indicating that the derivation of a well-designed protocol is time-consuming. This is particularly true in a multicenter study, with several sub-diagnoses and different treatment regimens. In comparison to the numerous trials performed to prevent OM in cancer settings, our protocol includes several pivotal aspects that are unique. First, a sample size of 180 patients in total needs to be enrolled, with 90 patients in each arm. This would make the study the largest ever performed within the field. Only one trial has included more patients (Sorensen *et al.*, 2008), although that study had a three-arm design, resulting in smaller subgroups. Second, no previous study has assessed an alternative cryotherapeutic method, in which a higher cooling temperature than that provided by ice upon exposure, can be assessed for the prevention of OM. In addition, no previous study has examined whether a higher cooling temperature would be better tolerated by patients, as compared to using ice.

Furthermore, assessments of OM using two different diagnostic tools (WHO and OMAS), combined with a blinded evaluation of OM by dentists specialized in orofacial medicine, has previously been applied albeit to a very small extent. In total, 11 out of 14 studies included in a systematic review (Riley *et al.*, 2015) used the WHO scale or a scale based on this scale to assess OM. Three studies used the highly comparable National Cancer Institute Common Toxicity Criteria, and one study used a modified OMAS but provided data according to the WHO scale. This review revealed that: only two studies reported on oral pain; no study reported QoL data; two studies reported on the normalcy of diet; three studies reported the number of days in hospital; two studies reported the number of days with opioid analgesics; and no study reported the number of days during which the patients were unable to take medicine orally. Following careful deliberation among the disciplines in the multi-professional gathering, the majority of these outcomes were included in *Study V*, along with QoL instruments, diaries, and study-specific questionnaires, to address thoroughly a wide range of parameters in the RCT. Objective assessments supplemented with patient-reported evaluations are of the utmost importance to include in clinical studies of patients with cancer who are at high risk of developing OM. This is to enable observations from different perspectives of any OM-related problems. Furthermore, it serves to reduce the risk of overlooking impediments that may be of relevance for the patient's well-being, especially in outpatient settings. The realization that OM is a complex, multifactorial condition that significantly affects patient welfare has convinced us that any effort to reduce the morbidity associated with of OM will help to avoid unwanted dose-reductions or unscheduled breaks in the cancer therapy protocol.

In conclusion, this thesis is the first to focus on the prevention of OM using an alternative cryotherapeutic method. It is mainly concerned with preclinical studies that are designed



to identify the ideal oral mucosal temperature for the prevention of OM. Furthermore, this thesis establishes the tolerability and cooling efficacy of the novel ICD in healthy volunteers, prior to clinical enrollment. Looking to the future, the proposed RCT is likely to elucidate the definitive role of the ICD in cryoprevention of OM.



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# APPENDIX

- I. **Walladbegi J.**, Smith S. A., Grayson A. K., Murdoch C., Jontell M., Colley, H. E. Cooling of the oral mucosa to prevent adverse effects of chemotherapeutic agents: An in vitro study. *J Oral Pathol Med.* 2018 May;47(5):477–483.
  
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