Chapter 1

General introduction, aims and outline
GENERAL INTRODUCTION

The survival of children with cancer has increased substantially in the last decades [1,2]. This increased survival is the consequence of different aspects like improved health care systems with specialized centers and multidisciplinary teams [1]. Furthermore, there is increased knowledge concerning the importance of supportive care during treatment in the pediatric cancer patients [3,4]. However, the most important contributing factors increasing survival are the more intensive treatment protocols including chemotherapy, radiotherapy and surgery. Due to these more intensive treatment protocols there is an increase in the frequency and intensity of side effects. Therefore, improvement in supportive care is needed in order to fight against these side effects during treatment in pediatric cancer patients. One of the most important targets of damage as side effect of chemotherapy is the gastrointestinal tract.

Gastrointestinal tract

The treatment of cancer in children includes many protocols with high doses of chemotherapy to attack rapidly proliferating cells, like cancer cells. However, the gastrointestinal tract, especially the small intestinal epithelium is one of the most rapidly proliferating tissues in the body and is therefore unfortunately vulnerable for chemotherapeutic treatment [5].

The small intestine can be divided in three parts from proximal to distal; the duodenum, jejunum and ileum. In all these parts of the small intestine the mucosa consists of three layers; the epithelium, the lamina propria and the muscularis mucosa [6]. The epithelium in the small intestine is columnar and consists of two different parts, namely the crypts of Lieberkühn and the villi. The crypts are the proliferation area. The villi, fingerlike protrusions, are the surface area of absorption [7] and consist of differentiated epithelial cells.

Stem cells are situated in the crypts and produce and renew cells continuously [8,9]. A stem cell has by nature two main properties; the ability to maintain itself throughout long periods of time, the so called self-renewal ability, and the ability to produce all the differentiated cells of the target tissue, the so called multipotency [10]. The intestinal epithelium is, like skin and bone marrow, constantly renewing; the entire intestinal epithelium is renewed every 3-5 days [7,9,11]. Each crypt produces cells, the transit-amplifying cells, and distributes the cells to the villi. The cells move from the crypt to the villus, and each villus receives cells from various crypts [12]. During the upward migration, the transit amplifying cells differentiate into three main cell types forming the epithelium of the villus [8,9]. The majority of the cells differentiate into enterocytes and represent the primary absorptive cells [8,9]. Their brush border contains many different transporter proteins [8]. Other cells differentiate into goblet cells which secrete mucus, mostly in the distal small intestine. The third group of cells differentiates into enteroendocrine cells which
synthesize a variety of macromolecules and produce a variety of peptide hormones [8,11]. Besides upward migration, some cells migrate to the base of the crypt and differentiate into the fourth cell type, the Paneth cells [8,9,13]. Paneth cells produce antimicrobial products; they protect the small intestine from bacterial translocation [8,14].

Cells are constantly being produced within the crypts and there is a comparable rate of cell loss from the villus tip and luminal border of colonic crypts [5]. This cell loss is probably a combination of programmed cell death and cell sloughing [5]. In the healthy intestine there is a balance between the cell production in the crypts and apoptosis at the tips of the villi [12]. As mentioned before, the small intestine is especially vulnerable for chemotherapy. Every chemotherapeutic agent probably affects different levels of the crypt cell hierarchy [15-17]. When chemotherapy is administered the balance between production in the crypts and loss of cells at the villus tip is disturbed, resulting in villus atrophy which is one of the features of mucositis.

The different parts of the small intestine have several functions like nutrient digestion and absorption, barrier function and immune function [6]. Therefore, disturbance of the dynamic small intestinal epithelium has major consequences; alteration of digestion and absorption of nutrients, disturbance of barrier function resulting in entrance of microorganisms causing infections, and affecting microbiota and the immune function.

One of the most debilitating side effects of both chemotherapy and radiotherapy is mucositis. Mucositis is a complex inflammatory reaction of the mucosa of the complete alimentary tract, and can be divided in oral and gastrointestinal (GI) mucositis. This thesis focuses on GI mucositis, further referred to as mucositis. The incidence of mucositis is estimated with a broad range of 40-100% of the patients, dependent on several patient- and treatment related factors [15]. Patients suffering from mucositis have symptoms like abdominal pain, nausea, vomiting and diarrhea, leading to a decreased quality of life during treatment. Furthermore, these symptoms can lead to a lowering of the chemotherapy dose or a delay of the next chemotherapy course. Thus, mucositis is a dose-limiting toxicity influencing the quality of life and possibly influencing the survival and is therefore a major problem in the pediatric oncology setting.

Pathophysiology of mucositis

Previously, the mechanism of mucositis was proposed to be a consequence of DNA damage caused by radiotherapy and chemotherapy inflicting injury to the rapidly proliferating cells of the basal epithelium [18]. However, in the last decades this topic has been investigated in detail and more information has become available, which led to the insight that the mechanism of mucositis is much more complicated. Clinically, the most important damage is the result of epithelial injury, but mucositis seems to be a condition which is not solely happening in the epithelium; it
comprises several compartments of the mucosa, submucosa and connective tissue [18]. All these insights and information led to the development of a 5-phase pathophysiological model of oral mucositis by Sonis et al., in 2004 [18,19]. In this model, the different phases do not exactly follow each other; some parts overlap or happen simultaneously; it is a dynamic process.

In the first phase, *the initiation phase*, there is direct DNA damage, halting proliferation which results in damage to the cellular lining in the basal epithelium and in the submucosa. At the same time during this initiation phase, there is generation of Reactive Oxygen Species (ROS), molecules or ions consisting of radical and non-radical oxygen species, formed by incomplete reduction of oxygen [20]. ROS are toxic to cells, starting a biological cascade. In this stage the most important destruction of cells is localized into the submucosa. Although there is already apoptosis in the epithelium, the clinical appearance of the mucosa seems normal [18,21].

The second phase is *the primary damage response*, a cascade of occurrences in the submucosa that eventually results in death of basal epithelial cells [21]. Transcription factors, like p53 and nuclear factor-kappa B (NF-κB), are activated by chemotherapy and by ROS [18]. Transcription factors control gene expression [21]. NF-κB is important in inflammatory and immune responses, but also in the regulation of haematopoietic cells, keratinocytes and lymphoid structures [22,23]. Activation of NF-κB can have both pro-apoptotic and anti-apoptotic effects [22,23]. Upon activation by chemotherapy or radiotherapy, NF-κB has the capacity to upregulate genes inducing the production of pro-inflammatory cytokines, like tumor necrosis factor alpha (TNF-α), IL1β, IL-6, as well as adhesion molecules and cyclooxygenase-2 (COX2) [5,18]. These pro-inflammatory cytokines eventually cause cell death and injury in all cells and tissues that comprise the mucosa, not solely the epithelium [18]. Simultaneously, chemotherapy and ROS are associated with the activation of ceramide synthase and the cell-membrane lipid sphingomyelin, causing an increase in ceramide levels resulting in apoptosis [18,24]. Transcription factor AP-1 stimulates the production of metalloproteinases, thereby disrupting and injuring fibroblasts within the submucosa [18,21].

In the third phase there is *the signal amplification*. Due to the activation of pro-inflammatory cytokines by transcription factors, not only the tissue is damaged, but also a positive-feedback system is activated [18]. Besides being destructive for the tissue, TNF-α is also an activator of NF-κB, sphingomyelinase and metalloproteinases [18,21]. At the same time COX2, a pro-inflammatory mediator, is upregulated and activates metalloproteinases which will further augment the tissue damage [23]. This feedback system amplifies the primary damage initiated by chemotherapy [18].
The fourth phase, the ulceration phase, is the most symptomatic stage and it is not until this stage that mucositis becomes clinically relevant [18,21,23]. Patients have ulcers, which are portal entries for bacteria and fungi, making them more prone to develop a bacteraemia or sepsis [18,25]. Bacteria colonize the ulcers and penetrate into the submucosa, stimulate infiltration of macrophages to produce pro-inflammatory cytokines [18,24,25].

Finally, in the final (healing) phase, when the chemotherapy or radiotherapy has ended mucositis is a self-healing condition.

This 5-phase model has been developed years ago for oral mucositis and has been adapted for GI mucositis [26,27]. However, the pathophysiology is probably more complex in the small intestine and the complete mechanism has not been elucidated yet. In the small intestine there are several functions in different parts, there is columnar epithelium, there are tight junctions and microbiota, which are complex aspects not included in the model so far. It has been suggested that the microbiota influence the pathophysiology of mucositis, but till now, there is no scientific evidence for this assertion [28-32]. In the past few years a number of reviews have been published in literature, unfortunately no new insights were added or altered the 5-phase model [18,21,23,25,32]. Therefore, to date the pathophysiology of mucositis is still not elucidated.

Chemotherapy-induced mucositis

Mucositis can be induced by different chemotherapeutic agents. In several studies it was suggested that there are differences in the pathophysiology of mucositis if induced by different chemotherapeutic agents [23,33,34]. There are differences in timing of histological changes and pro-inflammatory cytokine levels in intestinal tissues and serum [23,33,34].

One of the chemotherapeutic agents which causes mucositis is Methotrexate (MTX), often used in high dose in treatment protocols for children with cancer, like the Euramos protocol for osteosarcoma in pediatric cancer patients [35]. MTX is an antifolate chemotherapeutic agent and targets the enzyme dihydrofolate reductase (DHFR) [36]. MTX is a competitive inhibitor of this enzyme [36]. Consequently, this will lead to a decreased formation of tetrahydrofolate from dihydrofolate, resulting in a deficiency of tetrahydrofolate. Eventually this results in decreased purine and thymidine synthesis, therefore an inhibition of DNA replication causing cell death [36,37]. Normally MTX enters cells via a transporter, the so-called reduced folate carrier 1 (RFC-1) or solute carrier family 19 member 1 (SLC19A1) [37,38]. However, MTX in high dose can enter cells via passive diffusion as well [38]. Therefore, the effect of MTX on cell death is dependent on many factors, like the time of enzyme inhibition and the degree of reduction of folates in the cell. Furthermore, concentration and influx and efflux are determinants for the effect of MTX.
Models to study chemotherapy-induced mucositis

Animal models have been used in many studies concerning mucositis. Bowen et al. gave an overview of several animal models providing extensive information about mucositis [39]. Both mouse and rat models have been used with different chemotherapeutic agents like 5-FU, irinotecan and methotrexate, to induce mucositis. Furthermore, several dosages and route of administration have been used. Based on several rodent mucositis models a validated mucositis rat model has been developed in our laboratory [40]. The optimal dosage of MTX and the best time interval to study mucositis was determined. In this model mucositis is induced with a single injection of MTX intravenously in young male Wistar rats [40]. This single dosage and route of administration induces mucositis in most rats, without causing mortality. The rats are 4-6 weeks old when receiving the MTX injection; therefore, it is a model with growing rats. After MTX injection the rat shows typical signs of mucositis. They lose weight from two days after MTX injection, and start to gain weight from day five. From day two till day five the rats have a decreased food intake and may develop diarrhea. In this rat model the most severe day of mucositis is four days after MTX injection. Histological signs of mucositis at day four after MTX injection are villus atrophy and increased crypt length [40]. In this rat model it was established that plasma citrulline serves as a noninvasive marker for mucositis. Plasma citrulline is an amino acid synthesized almost exclusively by the enterocytes of the small intestine; it is therefore a marker of the enterocyte mass [41-44]. It correlates in this rat model with the severity of mucositis as measured by villus length of the jejunum [40]. Moreover, it was shown in clinical studies that plasma citrulline correlates with the severity of mucositis in adults and children [41,44-47]. In this thesis we use this MTX-induced mucositis rat model in chapter 2, 3, 4 and 7. In these chapters we use plasma citrulline sequentially to determine the severity of mucositis over time, without the need to terminate animals at several time points, thereby minimizing the number of animals used in the studies.

Prevention and treatment of mucositis

In 2004 the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO) made the first version of a clinical practice guideline for the management of mucositis, with an update in 2007 and 2014 [48-52]. Unfortunately, thorough review of the literature found no evidence for an intervention, except for two recommendations concerning the consequences of mucositis [52,53]. First, intravenous administration of amifostine is recommended to prevent radiation proctitis in patients receiving radiation therapy, and second octreotide is recommended to treat diarrhea induced by chemotherapy associated with haematopoietic stem cell transplantation if loperamide is ineffective [52]. However, for other agents there was no guideline possible due to inadequate, conflicting or limited evidence [52].
Therefore, to date there is no adequate prevention or treatment for mucositis and more research is needed.

Clinical care of mucositis

Patients with mucositis experience symptoms like nausea, vomiting, abdominal pain, diarrhea and decreased intake [15,18,49]. However, these symptoms are not very specific for mucositis, therefore the diagnosis and determination of the severity of mucositis are difficult in clinical practice. Currently, there is no gold standard diagnostic tool and both assessment scales and biomarkers have been suggested for the diagnosis of mucositis [44,54-57]. Another problem during mucositis is that most patients decrease their food intake, lose weight and are in need of nutritional support. However, till now the optimal feeding strategy during mucositis is unknown. Both enteral- and parenteral nutrition are currently used in the care of children with cancer. In previous experiments in a methotrexate-induced mucositis rat model it was shown that amino acids and glucose were only absorbed if continuously enterally administered [40,58,59]. However, fatty acids and lactose were not digested and absorbed, even if administered continuously [40,60]. The overall conclusion was that parenteral nutrition would be preferable to maintain weight, although tube feeding continuously with elemental nutrients can be absorbed by the intestine in the rat. However, parenteral nutrition also has disadvantages. Therefore, more research is needed concerning risk, diagnosis, and nutritional support to improve the clinical care of mucositis.

AIMS AND OUTLINE

The general aim of the research described in this thesis is to improve the management of chemotherapy-induced mucositis in pediatric cancer patients. This aim can be subdivided in two sub aims. The first sub aim was to find new insights in preventive and therapeutic agents for gastrointestinal mucositis, for which research is described in chapter 2, 3 and 4. The second sub aim was to find novel information about the clinical care concerning risk, diagnosis and feeding strategy during gastrointestinal mucositis, this is given in chapter 5, 6, 7 and 8.

In the first part of the project, we used our mucositis rat model to investigate the effect of several interventions for possible prevention or treatment of MTX-induced mucositis, thereby focusing on the first sub aim. Each phase of the pathophysiological model of mucositis offers a potential target to influence the severity or accelerate the recovery.
First of all, the crypts of the small intestine renew cells every 3-5 days and are therefore more vulnerable to chemotherapy. Intestinal growth factors might be an option as intervention to either preserve the crypt cells during chemotherapy to decrease the severity, or to increase proliferation and thereby accelerate recovery. Therefore, in chapter 2 we aimed to determine the effect of oral insulin as possible intestinal growth factor.

Second, in the 5-phase pathophysiological model the pro-inflammatory cytokines, like TNF-α, are thought to be important factors. Therefore, we aimed to determine the effect of a TNF-α inhibitor, Etanercept, on the severity and recovery of mucositis in the MTX-induced mucositis rat model as shown in chapter 3.

Finally, as possible intervention to alter the severity of mucositis the Farnesoid X receptor (FXR), which is a member of the nuclear hormone receptor superfamily, is an interesting target. FXR-agonists have been shown to inhibit the bile salt synthesis, decrease inflammatory cytokines, improve histological features and microbiota, thereby possibly targeting more than one phase of the 5-phase mucositis model in a beneficial way [61]. Therefore, in chapter 4 we aimed to determine the effect of an FXR-agonist on the severity and recovery of mucositis in the rat.

To answer the second sub aim, to find new insights in the clinical care concerning risk, diagnosis and feeding strategy during gastrointestinal mucositis, first in chapter 5 we reviewed the current literature on the knowledge of risk, diagnosis and supportive care of mucositis in the pediatric cancer patients. We outlined the risk analysis in children where possible and gave an overview of possible risk factors in adults. Furthermore, we evaluated scoring scales and biomarkers as a possible diagnostic tool. Finally, we summarized the possible supportive care focusing on pain management, diarrhea and nutritional support. Furthermore, in chapter 6 we outlined the current knowledge how to diagnose mucositis with the use of biomarkers or non-invasive tests.

Second, we determined the effect of a feeding strategy in the mucositis rat model. Based on a previous experiment in which total enteral tube feeding was not tolerated in our mucositis rat model, we aimed to determine the feasibility of minimal enteral feeding during MTX-induced mucositis in the rat in chapter 7. Besides the feasibility we aimed to determine the effect of minimal enteral feeding on the recovery phase in comparison with normal own food intake.

To translate the preclinical findings of nutrient digestion and absorption and nutritional support in the rat model to the clinical setting, we wanted to determine the current practice of nutritional support during mucositis in the pediatric clinical setting. Therefore, in chapter 8 we performed a survey to determine the current concordances and discordances in the clinical practice about feeding strategy during mucositis internationally. Moreover, we performed a multicenter
prospective observational study to see the current clinical practice of feeding strategy and the effect on several parameters, including plasma citrulline as biomarker.

Finally, we discussed the results from all chapters, the conclusions and future perspectives in chapter 9.
REFERENCES


Prevention and treatment