CHAPTER 1

GENERAL INTRODUCTION, AIMS AND OUTLINE OF THE THESIS
Chapter 1

GENERAL INTRODUCTION

Mucositis
Mucositis is a severe side effect of chemotherapy and radiotherapy, affecting both children and adults. It is defined as damage to the mucous membranes throughout the entire alimentary tract caused by anti-cancer treatment [1]. Of all the side effects that can result from chemotherapy, mucositis is mentioned by patients as the most debilitating one [2]. Mucositis is often subdivided into ‘oral mucositis’ and ‘gastrointestinal (GI) mucositis’. Although aspects of GI mucositis have been studied in all segments of the lower alimentary tract, investigations have been focussing on the small intestine [1]. Oral mucositis is visible in the oropharynx as highly red, easily bleeding tissue, with or without the presence of ulcers. Patients with oral mucositis suffer from pain and dysphagia [1]. GI mucositis can only be visualized when a biopsy of the mucosa is taken via endoscopy, which is rather invasive. Patients with GI mucositis suffer from anorexia, nausea, vomiting, abdominal pain, diarrhea and weight loss [1, 3]. Oral and GI mucositis do not always coincide. Research conducted on mucositis mainly focuses on oral mucositis in adult patients. Less attention is paid to GI mucositis, especially in children, although GI mucositis forms a major complication in patients [4, 5].

The pathobiology of mucositis
Mucositis is a transient condition, involving 5 (artificially determined) consecutive phases according to Sonis [6, 7]. Although Sonis’ model describes the development of oral mucositis, the pathobiology of GI mucositis is thought to be similar. However, local differences due to specialized differentiation cause both morphological and functional differences [1].

1. Initiation. Upon anti-cancer treatment, both DNA and non-DNA damage occur. DNA strand breaks cause direct injury in cells of the basal epithelium (mucosa) as well as in the submucosa. At the same time, reactive oxygen species (ROS) are formed, starting downstream biological events.

2. Primary damage response. DNA and non-DNA damage (including ROS) initiate a cascade of events. Several transduction pathways are activated, activating transcription factors like protein 53 (p53) and nuclear factor-kappaB (NF-κB). As a result, pro-inflammatory cytokines like tumor necrosis factor alpha and interleukins IL-1β and IL-6 are produced, resulting in basal-cell death and injury. Furthermore, hydrolyzation of the cell-membrane lipid sphingomyelin can lead to an increase of ceramide, causing apoptosis. Via activation of activator protein 1 (AP-1), the secretion of metalloproteinases (MMPs) is stimulated, thereby damaging fibroblasts within the submucosa.
3. **Signal amplification.** Upon initial activation of transcription factors, genes get upregulated and biologically active proteins accumulate and target the submucosa. Apart from tissue damage, pro-inflammatory cytokines cause a positive feed-back loop which amplifies the primary damage that is initiated by chemotherapy and radiation.

4. **Ulceration.** This is the most debilitating phase for patients, causing the symptoms of mucositis. The mucosal integrity gets lost, causing painful lesions that are prone to bacterial colonization. These portals of entry can cause bacteremia and sepsis especially in neutropenic cancer patients. Cell-wall products from bacteria can penetrate the submucosa, thereby stimulating the formation of more pro-inflammatory cytokines by mononuclear cells. This process is thought to promote the expression of pro-apoptotic genes and enables tissue injury. Via chemotaxis, inflammatory cells migrate to the lesion where they cause local inflammation.

5. **Healing.** Mostly, mucositis is an acute disease that resolves by itself shortly after anti-cancer treatment ends. Healing of the mucosa is thought to be roughly similar to healing of other types of mucosal injury. However, the sequence of events that leads to mucositis probably influences the healing process. Signals from the submucosa influence the rate of epithelial cell migration and proliferation, and the differentiation of healing tissue. Factors of influence on tissue behavior are probably the type of anti-cancer treatment (chemotherapy versus radiation), the selected agents and the dose and timing of therapy.

In Sonis’ model, the resident intestinal microbiota hardly play a role in the pathogenesis of mucositis. However, recent research has shown that anti-cancer treatment is associated with a decrease in the number of anaerobic bacteria and a decrease in microbial diversity [8, 9]. Moreover, these changes in intestinal microbiota coincide in time with an increase in the severity of mucositis. Van Vliet et al. hypothesized that the deregulation of microbial homeostasis upon chemotherapy treatment causes decreased microbial protection of enterocytes against harmful stimuli. They suggest that the intestinal commensal bacteria could influence all phases of Sonis’ mucositis model [10]. Future research on this topic is needed.

In patients, mucositis presents with clinical symptoms as early as day 3-5 after starting chemotherapy with a peak around day 7-14. In mice and rat models, mucositis follows a similar pattern over a shorter time course [1].

**Mucositis and the small intestine**

Since many kinds of anti-cancer treatment kill rapidly dividing cells, the GI tract is highly sensitive to these treatments, especially the small intestine. The small intestine can be divided into 3 regions: the duodenum (proximal part), jejunum (middle part) and ileum (distal part). There are morphological and functional differences along the
proximal-to-distal axis. For instance, towards the distal end of the small intestine, the length of villi as well as the proliferation rate in crypts decreases [11-13].

The mucosa of the small intestine consists of 3 layers: the epithelium (innermost layer, facing the intestinal lumen), the lamina propria mucosae (connective tissue) and the muscularis mucosae (smooth muscle) [14]. Small intestinal epithelium is made of a monolayer of cells with numerous invaginations (crypts of Lieberkühn) and finger-like protrusions (villi). Figure 1 shows a longitudinal section of the rat jejunal mucosa with its characteristic crypt-villus organization under normal conditions and after treatment with the chemotherapeutic agent methotrexate (MTX).

![Figure 1. Hematoxylin and eosin staining of the rat jejunum (Fijsstra).](image)

The small intestinal epithelium forms a highly folded structure (plicae of Kerckring) that separates the exterior (intestinal lumen) from the interior. It forms a highly selective barrier that prevents the entrance of toxins and noxes into the body and at the same time enables nutrient digestion and absorption. However, upon mucositis, pathogenic bacteria can invade the host and cause severe infections [8].

Among mammalian tissues, the small intestinal epithelium has one of the most rapid cell turnover rates. Stem cells in the lower half of the crypts give rise to daughter cells, thereby producing all the cells of the epithelium. Newly produced cells migrate out of the crypts up to the villus or migrate downward into the base of the crypts and reside under or between the stem cells. During migration up to the villus, most cells differentiate into functional enterocytes (±88%, facilitating digestion and absorption of nutrients), Goblet cells (±4%, production of mucus) and entero-endocrine cells
(±0.5%, production of hormones) [15]. Cells migrating into the crypts form Paneth cells (±7.5%, production of defensins that are important for immunity and host defense, and providing a niche for intestinal stem cells [16]). Migration takes about 3 days in rodents and 5 in humans. Within a few days, cells are deleted from the villus tips by apoptosis or shedding into the lumen [17].

Stem cells in the crypts are capable of proliferation (giving progeny to all epithelial cells) and allow regeneration of tissue after injury [18]. They are organized in a hierarchic manner and 3 distinct categories can be distinguished. The actual stem cells, that are the least differentiated ones, are very sensitive to DNA damage and can’t repair such damage. The second category is formed by clonogenic or daughter cells, derived after division of actual stem cells and becoming dividing transit cells that ultimately differentiate. However, in an early stage, they can repair their DNA damage and retain stem cell properties [19]. These cells may repopulate the crypt when actual stem cells are killed by chemotherapy or radiation. A third category is also formed by clonogenic or daughter cells, but is only recruited when the level of damage increases and the first 2 stem cell types have been killed, to ensure crypt survival [18].

**Nutrient digestion and absorption**

One of the major functions of the intestinal mucosa is nutrient digestion and absorption [14]. Enterocytes, which are highly specialized and polarized, are responsible for the degradation and absorption of the major food constituents (i.e. carbohydrates, fats and proteins) after ingestion [14]. This process is facilitated by several enzymes and transporters that are present in the highly folded apical membrane domain of villus enterocytes, named the brush border. Intracellular binding proteins are needed for the transport of specific substrates from the apical to the basolateral membrane of enterocytes. At the basolateral side, these substrates are transported to the blood (carbohydrates and proteins) or lymph (fat). Apart from transcellular transport by enterocytes, carbohydrates and proteins can also be absorbed via paracellular transport [20].

**Carbohydrates.** The digestion of dietary polysaccharides starts with salivary and pancreatic amylase. Then, resulting disaccharides need to be digested by glycohydrolizing enzymes (i.e. lactase, sucrase, isomaltase and maltase) that are present on the epithelial brush border, before absorption of their derived monosaccharides takes place. Absorption of monosaccharides glucose, galactose and fructose occurs by active and passive transport across the epithelial border by Sodium-dependent Glucose Transporter 1 (SGLT1), Glucose Transporter 2 (GLUT2) and Sodium-dependent Glucose Transporter 5 (SGLT5) [21].

**Fats.** Dietary lipids undergo a number of intraluminal physicochemical alterations (emulsification of bulk fat, lipolysis of triglycerides into di- and monoacylglycerol and unesterified or free fatty acids (medium- or long-chain), solubilization of lipolytic
products to form mixed micelles) before lipolytic products are translocated across the enterocyte membrane [22]. After translocation, absorbed fatty acids undergo reacylation and assembly into chylomicrons before they end up in mesenteric lymph and finally in the blood [23, 24]. The exact mechanism of fatty acid uptake from the intestinal lumen by enterocytes is still a matter of debate [22, 24].

Proteins. After protein digestion by gastric (i.e. pepsin) and pancreatic (i.e. trypsin, chymotrypsin and carboxypeptidases) enzymes, the resulting oligopeptides are hydrolyzed by peptidases on the brush border membrane. Then, the resulting small peptides (tri- and dipeptides) and amino acids are absorbed by enterocytes via highly regulated transporter systems that are present in their apical and basolateral membrane. These systems are defined by the kinetic properties of the specific amino acids they transport; i.e. neutral, cationic or anionic. A unique feature of intestinal enterocytes is that they do not only absorb amino acids directly from the lumen by their apical membrane: they can also take up amino acids from the mesenteric arterial circulation by their basolateral membrane after such amino acids have become systemically available [25-28].

A number of studies showed that protein- and mRNA expression of brush border enzymes and transporters involved in nutrient digestion and absorption are decreased during mucositis, suggesting maldigestion and malabsorption [29-31]. Also, a few functional digestion and absorption studies during mucositis have been performed [32, 33]. However, the way mucositis functionally affects nutrient digestion and absorption is thus far not well understood, and is the focus of this thesis.

The clinical burden of mucositis
The incidence of GI mucositis is not exactly known because accurate evaluation by intestinal biopsies is problematic in patients. Therefore, it is scored by more subjective symptoms in clinic, which are not very accurate [1]. With chemotherapy, 40-100% of patients report GI mucositis, depending on the chemotherapeutic agent that is used and the given dose [1]. Little is known about the incidence in children. However, mucositis seems to be observed more frequently in children with cancer than in adults, probably because of a higher mitotic rate of the GI mucosa in children [5]. In children with acute myeloid leukemia (receiving high doses of chemotherapy), mucositis was found to be present in 55% of chemotherapy cycles [34]. The complications of mucositis (i.e. anorexia, diarrhea, weight loss etc.) are associated with an increased use of injectable analgesics, nutritional problems and longer hospitalizations [1]. Moreover, since mucositis and its associated complications lead to a dose-reduction of chemotherapy, mucositis compromises overall survival in cancer patients [35].
Mucositis scoring systems
Mucositis is typically scored via rather subjective symptoms like pain and diarrhea as described by the ‘Common Terminology Criteria for Adverse Events’ from the National Cancer Institute (NCI) [34, 36]. However, these criteria were never designed to score mucositis on a day to day basis, and have not been validated in children. Moreover, symptoms of GI mucositis correlate poorly with the severity of mucositis, especially in young children who are less capable of localizing pain and are often incontinent for feces due to their developmental stage [34]. Thus, a more objective, easy measurable parameter to score GI mucositis is needed, in order to diagnose this (sometimes subclinical) disease and to offer patients optimal treatment. Diverse parameters reflecting inflammation, loss of enterocytes and intestinal permeability have been tested to score mucositis [34]. Of these, plasma citrulline (a nonprotein amino acid made by enterocytes [37]) was found to be a promising marker [34, 38-40]. The value of plasma citrulline as an objective marker for the level of mucositis and the respective intestinal function is another focus of this thesis.

Methotrexate (MTX)
MTX is a folate antagonist which is widely used in adult and pediatric cancer patients, alone or in combination with other chemotherapeutic agents [1, 4, 41]. After entering the cell, MTX inhibits the enzyme dihydrofolate reductase (DHFR, catalyzing the conversion of dihydrofolate to tetrahydrofolate). Thereby, it interferes with folate synthesis and indirectly inhibits the synthesis of thymidine monophosphate which is a nucleotide (pyrimidine) required for DNA synthesis. In addition, MTX, its metabolites and folate byproducts that are formed during binding of MTX to DHFR can also directly inhibit folate-dependant enzymes of nucleotide (pyrimidine and purine) synthesis. As a result, MTX leads to the inhibition of DNA synthesis, and subsequently to the inhibition of RNA and protein synthesis. MTX is active during the S-phase of the cell cycle (the synthesis phase during which DNA is replicated), and therefore has a large toxic effect on rapidly dividing cells such as malignant cells and cells of the GI mucosa [42, 43].

The treatment of mucositis
Well-designed studies regarding treatment regimens for GI mucositis are scarce due to the relative inaccessibility of the intestine and the obvious difficulty in obtaining biopsies at multiple time points after cytotoxic therapy. Nevertheless, evidence-based guidelines for the prevention and treatment of GI mucositis have been formed by the Mucositis Study Group of the Multinational Association of Supportive Care and Cancer/International Society for Oral Oncology (MASCC/ISOO), using symptoms of mucositis as clinical endpoints [1, 3, 44]. Guidelines for patients with chemotherapy-induced mucositis (+/- radiotherapy) recommend basic bowel care, including the maintenance of adequate hydration. Also, consideration should be given to the potential for transient lactose intolerance and the presence of bacterial
pathogens. When loperamide fails to control chemotherapy-induced diarrhea, octreotide (>100 µg subcutaneously, twice daily) is recommended [3].

AIMS AND OUTLINE OF THE THESIS

As stated earlier, patients with mucositis suffer from weight loss, which is associated with a reduced overall survival in cancer patients [35]. Weight loss during mucositis seems primarily the result of a reduced food intake [45], which suggests that (force-) feeding might be able to prevent weight loss during mucositis. Apart from a reduced food intake, alterations in nutrient digestion and absorption and energy metabolism might also play a role. There are indications that nutritional support might not only improve the nutritional state, but also accelerate recuperation and increase survival of mucositis patients [46-49].

It is unknown how to optimally feed patients with mucositis, because their capacity to digest and absorb nutrients is hardly known. Normally, enteral nutrition, which is the physiological way of feeding, is preferred to total parenteral nutrition (TPN) because the latter carries an increased risk of infection and, upon prolonged administration, may cause liver disease [50, 51]. However, when the absorptive function of the intestine is compromised, TPN offers a useful feeding alternative. In contrast to pediatric patients, adult patients with mucositis regularly receive TPN [52]. Both pediatric and adult patients with mucositis sometimes receive enteral tube feeding, although there is no consensus about the optimal mode of enteral feeding (bolus versus continuous), or the composition of enteral formulas. We aimed to determine the capacity to digest and absorb nutrients during GI mucositis, to ultimately design a rational feeding strategy for mucositis patients.

Accurate evaluation of mucositis via intestinal biopsies is rather invasive and potentially dangerous in patients because of the risk for infection and intestinal perforation. Also, there is no objective, easy measurable parameter to score GI mucositis in patients. Therefore, we chose to determine nutrient digestion and absorption in a chemotherapy-induced mucositis rat model. Since plasma citrulline seems to be a promising marker for mucositis in patients [34, 38-40], we also aimed to determine the value of plasma citrulline as an objective marker for the level of GI mucositis and the respective intestinal function, in a rat model.

In Chapter 2, we describe the clinical and histological characteristics of the MTX-induced mucositis rat model that we developed in our lab. Regarding nutrient digestion and absorption in this model, we first focused on carbohydrates because of their major role in dietary energy supply. Especially lactose is an important carbohydrate in breast milk and Western pediatric diets and formulas. During mucositis, we determined lactose digestion and absorption of its derivative glucose by
using stable isotope labeled lactose and glucose in trace amounts. Enzyme activity and/or expression of glycohydrolases (lactase, sucrose, isomaltase and maltase) and epithelial glucose transporters (SGLT1, GLUT2 and GLUT5) were also determined. Furthermore, we describe the value of plasma citrulline as an objective marker for mucositis and lactose digestion/glucose absorption during mucositis.

To determine whether glucose could be a useful source of energy during mucositis, we performed a follow-up experiment. As described in Chapter 3, we determined the quantitative capacity to absorb glucose in rats with mucositis, relative to that in controls. Therefore, we administered a physiologically relevant amount (meal size) of stable isotope labelled glucose as a bolus by oral gavage (resembling the physiological situation of consuming meals [53]) or continuously by intraduodenal infusion (improving nutrient absorption during another form of intestinal failure; short bowel syndrome [54]). We also describe the value of plasma citrulline as a marker for glucose absorption during mucositis.

Since long-chain fatty acids serve several important functions in the body, and provide twice as much energy as carbohydrates and proteins on weight basis, we next determined the absorptive capacity of long-chain fatty acids during mucositis, as described in Chapter 4. Therefore, rats with and without mucositis received a physiologically relevant amount (meal size) of fat containing stable isotope labeled palmitic acid and linoleic acid, either as a bolus by oral gavage or continuously by intraduodenal infusion. Furthermore, we assessed whether either plasma citrulline or the presence or absence of diarrhea would be a better marker for the long-chain fatty acid absorption capacity during mucositis.

There are indications that intestinal absorption of amino acids might be intact during mucositis [33], in contrast to absorption of di- and tripeptides [55]. Therefore, we at last aimed to determine the capacity to absorb enterally administered amino acids during mucositis as described in Chapter 5. After rats with and without MTX-induced mucositis received a physiologically relevant amount (meal size) of stable isotope labeled amino acids (leucine, lysine, phenylalanine, threonine and methionine) via continuous intraduodenal infusion, we determined the plasma availability of amino acids, their utilization for protein synthesis, and the preferential side of the intestine for amino acid uptake. We also describe the value of plasma citrulline as a marker for amino acid absorption during mucositis.

Based on our findings on nutrient digestion and absorption during mucositis in the mucositis rat model, we finally determined the effects of 4 different (par)enteral feeding strategies during mucositis on body weight, as described in Chapter 6. Rats with MTX-induced mucositis continued ad libitum purified diet (AIN-93G, strategy 1), received continuous enteral force-feeding with glucose and amino acids (Nutriflex®, strategy 2) or with standard tube-feeding (Nutrini®, strategy 3), or received standard
parenteral feeding (NuTRIflex® Lipid, strategy 4) for 3 days. Control rats continued ad libitum purified diet. We also describe the effects of these feeding strategies on intestinal recovery from mucositis, as measured by plasma citrulline concentration and jejunal histology.

Although significant progress has been made in understanding the pathobiology of GI mucositis, progress is difficult due to the relative inaccessibility of the small and large intestine. Because of the difficulty in obtaining biopsies at multiple time points after cytotoxic therapy, new agents for the management of mucositis are being tested in patients by using clinical symptoms of mucositis as endpoints. As part of the Mucositis Study Group of MASCC/ISOO, we reviewed the literature and updated evidence-based guidelines for the prevention and treatment of GI mucositis, as described in Chapter 7.

In Chapter 8, we summarize and discuss the most relevant findings in this thesis, draw conclusions and describe perspectives for future mucositis related research.

REFERENCES
