CHAPTER 8

SUMMARY AND GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES
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SUMMARY AND GENERAL DISCUSSION

Patients with mucositis suffer from weight loss, which is associated with a reduced overall survival in cancer patients [1]. This weight loss seems primarily the result of a reduced food intake [15], which suggests that (force-) feeding might be able to prevent weight loss during mucositis. 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 a high risk of infection and, upon prolonged administration, may cause liver disease [2-4]. However, when the absorptive function of the intestine is compromised, TPN offers a useful feeding alternative. The experiments described in this thesis aimed to determine the digestive and absorptive capacity of nutrients during gastrointestinal (GI) mucositis in the rat, to ultimately design a rational feeding strategy for mucositis patients.

Since accurate evaluation of mucositis via intestinal biopsies is rather invasive and potentially dangerous in patients, and there is no objective, easy measurable parameter to score GI mucositis in patients, we chose to determine nutrient digestion and absorption in a chemotherapy-induced mucositis rat model. Because plasma citrulline seemed to be a promising marker for mucositis in patients [5-8], 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.

The mucositis rat model

We developed a rat model with methotrexate (MTX)-induced GI mucositis based on other rodent mucositis models [9-11], as described in chapter 2. To find the optimal dosage of MTX (i.e. development of mucositis without causing mortality), and the best time interval to study nutrient digestion and absorption during mucositis (i.e. when histological symptoms of mucositis were most severe), we performed several pilot studies with varying dosages of MTX (30-150 mg/kg). Rats were killed at different days after intravenous MTX injection (day 2-10) to determine the level of mucositis by histology. Histological symptoms of mucositis were comparable between all parts of the small intestine, i.e. the duodenum, jejunum and ileum. The optimal dosage of MTX turned out to be 60 mg/kg. Based on these pilot studies, nutrient digestion and absorption experiments were performed in rats that received 60 mg/kg MTX or saline (controls), 4 days after their intravenous injection. Jejunal histology was used as a representative for small intestinal damage.

In the model, rats transiently develop mucositis upon MTX injection, thereby showing the typical subsequent phases as described by Sonis [12, 13]. At day 2 after injection with MTX, crypt loss is seen while villi still appear normal. At day 4, typical histological symptoms of mucositis like villus atrophy and blunting, enterocyte damage, infiltration of inflammatory cells and accumulation of Goblet cells at villus tops is
seen. By this time, crypts tend to be elongated, indicating crypt regeneration. From day 6 on, villi of rats start to recover. Typical clinical symptoms of mucositis like a decreased intake of food and water, diarrhea and weight loss are present from day 2 until day 5, after which rats recover.

Histological and clinical symptoms of mucositis differed substantially between individual MTX-treated rats. We hypothesized that this variance in observed individual symptoms of mucositis, as also seen in patients, could be a result of genetic variability between outbred Wistar rats [14]. Moreover, the mucositis rat model is based on a single intravenous injection of MTX, leaving the period of epithelial crypt cell susceptibility to MTX shorter than in models where multiple MTX injections are administered [12, 13, 15-17]. The amount of subsequent crypt loss by apoptosis, crypt atrophy and ultimately villus atrophy [18] per MTX-injected rat might therefore be explained by these factors. The variance in individual symptoms of mucositis in the rat model allowed us to determine intestinal function during mild to severe mucositis.

**Nutrient digestion and absorption during GI mucositis in the rat**

After establishing a stable and reproducible mucositis rat model, we subsequently determined the digestive and absorptive capacity of the major food constituents (i.e. carbohydrates, fats, proteins) during mucositis in the rat model. Therefore, we used a stable isotope dilution technique; a technique that has successfully been applied for many years in our lab [19-26]. Stable isotope labeled nutrients were enterally administered as a bolus by oral gavage (since intermittent bolus administration resembles the physiological situation of consuming meals [27]) or continuously by intraduodenal infusion (since continuous enteral nutrient administration has been shown to improve nutrient absorption during other forms of intestinal failure [3]).

**Carbohydrates**

In our first experiment, as described in chapter 2, rats with and without MTX-induced mucositis orally received trace amounts of \([1^{-13}C]\)lactose and \([U^{-13}C]\)glucose to determine carbohydrate digestion and absorption. The digestion of lactose (the sole disaccharide in breast milk and an important disaccharide in Western pediatric diets and formulas [28]) was severely decreased during mucositis, and we showed that this was due to a decreased jejunal lactase activity and immunohistochemical protein- and mRNA expression after MTX treatment. The enzyme activity of other glycohydrolases (i.e. sucrase, isomaltase and maltase) was also decreased during mucositis, indicating that all disaccharides, as well as polysaccharides, will probably not be hydrolyzed and its derivatives not be absorbed during mucositis. It therefore seems wise to omit disaccharides and polysaccharides from the diet of patients with mucositis to prevent possible negative side effects of carbohydrate maldigestion, like lactose intolerance.

In contrast to lactose digestion, glucose absorption was still intact during mucositis, when supplied in trace amounts, in spite of decreased immunohistochemical
protein- and/or mRNA expression of glucose transporters SGLT1 and GLUT2. To determine whether glucose could be a useful source of energy during mucositis, we studied the quantitative capacity to absorb [1-\(^{13}\)C]glucose during mucositis when enterally administered in physiologically relevant amounts (meal size) as a bolus by oral gavage or continuously by intraduodenal infusion, as described in chapter 3. When glucose was administered as a bolus, rats with mucositis only absorbed 15% of the administered glucose, compared with 85% in controls. However, upon continuous intraduodenal glucose infusion, the median absorptive capacity for glucose in rats with mucositis did not differ from controls (80 versus 93% of administered glucose respectively), although glucose absorption varied from severely reduced to completely normal between individual MTX-treated rats (range 21-95%). Apparently, continuous enteral administration could completely overcome the reduced absorptive capacity for glucose in about half of rats with mucositis. Our findings can be explained by the concept that by continuous enteral nutrient infusion, saturation of the (residual) carrier proteins is maximized and the intestinal function increased, when compared with bolus administration of nutrients. Apart from absorption via transporters on the epithelial membrane, glucose absorption could also have been possible via paracellular absorption [29] or via leakage through damaged tight junctions since gut permeability is increased during mucositis [30, 31]. Our findings suggest that enteral glucose is preferably administered continuously to patients with mucositis, to optimize glucose absorption.

**Fats**

To determine the absorptive capacity of long-chain fatty acids (LCFA) during mucositis, rats with and without MTX-induced mucositis enterally received a physiologically relevant amount (meal size) of saturated ([U-\(^{13}\)C]palmitic acid) and unsaturated ([U-\(^{13}\)C]linoleic acid) fatty acids dissolved in oil, either as a bolus by oral gavage or continuously by intraduodenal infusion, as described in chapter 4. MTX treatment severely reduced the appearance of [U-\(^{13}\)C]palmitic- and [U-\(^{13}\)C]linoleic acid in plasma and liver, compared with controls, either when administered as a bolus or continuously (all at least -63%). It is remarkable that continuous enteral fat administration could not overcome the reduced absorptive capacity for LCFA during mucositis, in contrast to glucose absorption. Differences in absorption might be explained by the fact that intestinal absorption of fatty acids is much more complicated than that of carbohydrates (and proteins). In fact, the exact molecular mechanism of fatty acid translocation across the epithelial membrane is still a matter of debate [32-34]. Apparently, the mucosal damage during mucositis is too prominent to allow for normal absorption of LCFA, even when administered continuously. Parenteral administration of LCFA might therefore be a rational alternative for enteral LCFA administration in patients with mucositis. Furthermore, plasma citrulline turned out to be a better marker than diarrhea to detect LCFA malabsorption during mucositis.
Proteins
Next, we determined the capacity to absorb amino acids during mucositis. Rats with and without MTX-induced mucositis enterally received a physiologically relevant amount (meal size) of $^{13}$C- or $^{15}$N-labeled amino acids (leucine, lysine, phenylalanine, threonine and methionine), as described in chapter 5. Since glucose absorption during mucositis improved upon continuous enteral administration, as compared with bolus administration, amino acids were enterally administered by continuous intraduodenal infusion. The median systemic availability of all amino acids except for leucine was similar in MTX-treated rats and in controls. However, individual availability of all amino acids differed substantially within the group of MTX-treated rats, ranging from severely reduced (<10% intake) to not different from controls (>40% intake in 5 of 9 rats). Since absolute amino acid utilization was mostly reduced or similar in MTX-treated rats, compared with controls, we concluded that continuous enteral administration enabled normal amino acid absorption in about half of rats with mucositis. These findings were in line with our findings regarding glucose absorption. Like glucose absorption, amino acid absorption could have been possible via transporters on the epithelial membrane, via paracellular absorption [29] or via leakage through damaged tight junctions [30, 31]. Our findings suggest that enteral amino acids are preferably administered continuously to patients with mucositis, to optimize amino acid absorption. Furthermore, we showed that the intestine prefers basolateral instead of apical amino acid uptake to meet its need for amino acids for protein synthesis during mucositis.

We aimed to determine the digestive and absorptive capacity of nutrients during GI mucositis in the rat. We found the digestion of disaccharides to be severely reduced during mucositis, as was the absorption of LCFA. In contrast, the absorption of glucose and amino acids could be normal during mucositis, when enterally administered continuously by intraduodenal infusion. Of note, large interindividual differences in glucose and amino acid absorption were seen during mucositis.

Continuous (par)enteral feeding during mucositis in the rat
Now that we concluded that the absorption of glucose and amino acids could be normal during mucositis, when enterally administered continuously, we set out to determine if continuous enteral feeding with these nutrients could prevent weight loss during mucositis. In this experiment, we 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 (Nutrin®i, strategy 3), or received standard parenteral feeding (NuTRIflex® Lipid, strategy 4) for 3 days. Control rats continued ad libitum purified diet. We found that both enteral feeding strategies were poorly tolerated by rats with mucositis. Most rats had to be killed
early because of severe watery diarrhea, abdominal distention, lethargy and hyperglycemia after 1-2 days of feeding. Only a few rats tolerated enteral feeding with glucose and amino acids. We hypothesized that the reduced spontaneous intake during mucositis (causing the loss of body weight), as seen in rats as well as in humans, might be a mechanism to protect the damaged intestine that apparently does not tolerate normal daily amounts of enteral nutrition. In contrast to enterally-fed rats, all parenterally-fed rats with mucositis grew similarly as saline-treated controls. This could be explained by the fact that via parenteral nutrition, nutrients are delivered directly into the blood and the damaged intestine is completely bypassed. **Our findings in the rat suggest that nutrition should be administered parenterally during GI mucositis to prevent weight loss, since daily amounts of enteral nutrition (even of glucose and amino acids) are badly tolerated during enteral nutrition.**

Apart from the effect on body weight, we also determined the effect of the mentioned feeding strategies on intestinal recovery as measured by plasma citrulline concentrations and jejunal histology. We found advantageous effects of enteral nutrition (including minimal intake in ad libitum-fed rats) during mucositis on intestinal citrulline synthesis (preferably glucose and amino acids) and histology, in comparison with solely parenteral nutrition. Advantageous effects of enteral nutrition have also been reported during other forms of intestinal failure [35], as may be explained by its stimulatory effect on intestinal epithelial cells and the production of trophic hormones [27, 35, 36]. **Taken together, our findings in the rat indicate that the optimal feeding strategy during GI mucositis consists of a combination of parenteral nutrition to prevent weight loss, and tolerated amounts of enteral glucose and amino acids via continuous infusion to optimize intestinal recovery from mucositis.**

**Plasma citrulline as a marker for (intestinal function during) mucositis**

An objective, easy measurable parameter to score GI mucositis in patients is needed in order to diagnose this (sometimes subclinical) disease and to offer patients the best treatment. Since plasma citrulline (a nonprotein amino acid made by enterocytes [37]) was earlier found to be a promising marker for mucositis in patients [5-8], we measured plasma citrulline in rats of all our previously described experiments. At day 4 after MTX injection (the day when symptoms of mucositis were most severe and nutrient digestion and absorption experiments were performed), plasma citrulline was always severely decreased in MTX-treated rats, as compared with controls. In individual rats, plasma citrulline level strongly correlated with villus length, which is the most accurate (but rather invasive) indicator of mucositis [5, 14]. However, at day 5 after MTX injection (when intestinal villi were mostly recovered from mucositis, as described in chapter 6) plasma citrulline was still decreased in most MTX-treated rats, as compared with controls. Our findings therefore suggest that plasma citrulline has
limited value as a marker for the level of mucositis; low plasma citrulline concentrations post chemotherapy might indicate that individuals suffer from mucositis or that they are in an early recovery phase from mucositis.

We related villus length and plasma citrulline concentrations with nutrient digestion and absorption in individual rats to determine if individual levels of mucositis correlated with individual levels of nutrient (mal)digestion and (mal)absorption. If so, plasma citrulline (as a relatively noninvasive marker for mucositis) might be used in clinic to adapt the feeding strategy of individual patients, regardless of their level of mucositis. Both villus length and plasma citrulline correlated strongly with lactose maldigestion and fat malabsorption during mucositis, but poorly with the absorption of glucose and amino acids when enterally administered continuously. About half of MTX-treated rats with severe mucositis (villus length <300 μm and plasma citrulline <30 μmol/L) showed a rather reduced absorption of continuously administered glucose and amino acids, while the other half absorbed glucose and amino acids efficiently.

The second aim of this thesis was to determine the value of plasma citrulline as an objective marker for the level of GI mucositis and the respective intestinal function, in the rat. We found plasma citrulline to be a marker with limited value for the level of GI mucositis. Since absorption of continuously administered glucose and amino acids did not correlate with the level of mucositis (as measured by villus length and plasma citrulline), citrulline seems not a usable marker to differentiate between individuals with intact or reduced glucose and amino acid absorption during mucositis.

The management of mucositis in cancer patients

Progress in understanding the pathobiology of mucositis is difficult due to the relative inaccessibility of the intestine and the obvious difficulty in obtaining biopsies at multiple time points after cytotoxic therapy. Nevertheless, new agents for the management of mucositis are being tested in patients using symptoms of mucositis as clinical endpoints. As part of the Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO), we reviewed the literature and updated the evidence-based guidelines for the prevention and treatment of GI mucositis [38, 39]. For our review, a literature search for relevant papers indexed in Medline on or before December 31, 2010 was conducted (including only human studies), as described in chapter 7. One new recommendation, two new suggestions and one change from previous guidelines could be made. Firstly, the panel recommends against the use of misoprostol suppositories for the prevention of acute radiation-induced proctitis. Secondly, the panel suggests probiotic treatment containing Lactobacillus spp. may be beneficial for prevention of chemotherapy and radiotherapy-induced diarrhea in patients with
malignancies of the pelvic region. Thirdly, the panel suggests the use of hyperbaric oxygen as an effective means in treating reducing radiation-induced proctitis. At last, new evidence has emerged which is in conflict with the previous guideline concerning the use of systemic glutamine, meaning that the panel is unable to form a guideline. No guideline was possible for any other agent, due to inadequate and/or conflicting evidence. Our updated review of the literature has allowed new recommendations and suggestions for clinical practice to be reached, highlighting the importance of regular updates.

CONCLUSIONS

The major aim of this thesis was to determine the capacity of nutrient digestion and absorption during GI mucositis in a rat model, to ultimately design a rational feeding strategy for mucositis patients. In the last 4.5 years, we were able to develop and characterize a stable MTX-induced mucositis rat model in which we determined the digestion and/or absorption capacity of carbohydrates, long-chain fatty acids and amino acids. Of these nutrients, only the absorption of glucose and amino acids could be normal during mucositis (but differed substantially between individual rats), when enterally administered continuously.

We performed the nutrient digestion and absorption tests at day 4 after injection with MTX or saline, while symptoms of MTX-induced mucositis were actually present from day 2 until day 5 in the rat. We therefore do not know when maldigestion and malabsorption of nutrients exactly starts or ends and, if we extrapolate our findings to the clinic, for how long the feeding strategy of mucositis patients should be adapted. Although plasma citrulline turned out to be to be a marker with limited value for the level of mucositis, it could still be used in clinic to partially adapt the feeding strategy (i.e. no enteral polysaccharides or LCFA), and more generally the treatment, of mucositis patients. Plasma citrulline could be useful in addition to the currently used, more subjective ‘National Cancer Institute Common Toxicity Criteria’ as a parameter for GI mucositis in patients [5, 40]. Since plasma citrulline was reduced from day 1 after MTX injection on, nutrient maldigestion and malabsorption may actually already start in an early phase of mucositis when clinical symptoms are still absent. Moreover, since plasma citrulline remained reduced until day 5 after MTX injection, when intestinal villi were mostly recovered from mucositis, nutrient maldigestion and malabsorption may even exist longer than expected upon intestinal histology. Therefore, the nutritional status of patients receiving chemotherapy should be carefully watched, and their plasma citrulline concentrations measured, from the start of anti-cancer treatment on, at least until plasma citrulline levels start getting back to normal.
Unfortunately, continuous enteral feeding with glucose and amino acids (as well as with standard formula) in normal daily amounts was often poorly tolerated by rats. Parenteral feeding on the other hand (which is quite invasive and carries an increased risk of infection [2-4]) prevented weight loss during mucositis, in contrast to ad libitum feeding, although ad libitum feeding caused accelerated intestinal recovery. Future research in mucositis patients is indicated to define their optimal feeding strategy in order to prevent weight loss during mucositis and optimize intestinal recovery from mucositis.

**FUTURE PERSPECTIVES**

Our findings in the rat indicate that during GI mucositis, the optimal feeding strategy consists of a combination of parenteral nutrition to prevent weight loss, and tolerated amounts of enteral glucose and amino acids via continuous infusion to optimize intestinal recovery from mucositis. However, we do not know to what extent enteral nutrition is tolerated in patients with mucositis. Further research in mucositis patients is needed to determine whether either continuous enteral administration with glucose and amino acids or TPN is indicated to prevent weight loss during mucositis. Since both enteral tube-feeding (in pediatric and adult mucositis patients) and parenteral feeding (only in adult mucositis patients [41]) are already commonly used in the clinic, studies with varying doses of enteral and parenteral nutrition in mucositis patients can now be undertaken.

*If continuous enteral administration of glucose and amino acids in normal daily amounts is well tolerated in mucositis patients,* but individual glucose and amino acid absorption differs substantially between patients (like in rats), plasma citrulline will probably not be a usable discriminatory marker between their reduced or intact absorption. Future research should then focus on finding another discriminatory marker in order to select patients that would benefit most from continuous enteral glucose and amino acids.

*If continuous enteral administration of glucose and amino acids in normal daily amounts is poorly tolerated in mucositis patients* (like in rats), additional research in patients on the role of enteral glucose and amino acids to improve intestinal recovery from mucositis seems attractive. The design should focus on titrating sufficient enteral nutrients to promote intestinal recovery from mucositis on the one hand, and on negative side effects of enteral feeding on the other hand.

To date, there is a considerable need for management interventions to prevent and/or treat GI mucositis. Unfortunately, well performed human studies in which new interventions are tested are scarce, due to the difficulty in obtaining biopsies at multiple time points after cytotoxic therapy. However, now that proof of citrulline to
be a useful marker for GI mucositis accumulates (in addition to the more subjective ‘National Cancer Institute Common Toxicity Criteria’), this might change. For now, the mucositis rat model seems ideal to test new strategies for the prevention and treatment of GI mucositis. The model allows for repeated scoring of mucositis (by plasma citrulline and/or intestinal histology) and absorptive function before, during and after mucositis, by using stable isotope-labeled nutrients. Promising agents could then be examined in patients in the clinic.

Theoretically, all agents that intervene in the pathobiology of GI mucositis, as described by Sonis [12, 13], could prevent or treat GI mucositis and should therefore be the focus of future research. Of these agents, especially R-spondin1 (a novel epithelial mitogen that stimulates the growth of mucosa in the small and large intestine [42]), seems attractive to test in the rat model. Prophylactic treatment with R-spondin1 has been shown to protect mice against chemotherapy- or radiation-induced oral mucositis [43]. Since the pathobiology of oral and GI mucositis is thought to be similar [12, 13], R-spondin1 might also prevent chemotherapy-induced GI mucositis.

Another important focus of future research in the rat model could be the role of intestinal microbiota during mucositis. 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 [44, 45]. We found similar changes in the mucositis rat model (preliminary data). Although not specifically mentioned in Sonis’ model, Van Vliet et al. suggest that the intestinal commensal bacteria could influence all phases of Sonis’ mucositis model [46]. A causal relationship between the intestinal microbiota and mucositis has been proposed but has not been demonstrated so far. Further research to understand the role of bacteria in the pathogenesis of mucositis is indicated, for instance by determining the effects of broad-spectrum antibiotics on mucositis. Since specific groups of bacteria might be able to modulate the severity of mucositis, research focusing on restoring dysbiosis during mucositis also becomes essential. For instance, the effects of prebiotics and/or probiotics (like Lactobacillus spp. [47]) on mucositis should be studied more extensively. Since administration of living bacteria to immunocompromised hosts (like cancer patients on anti-cancer therapy) can be life threatening [48, 49], alternatives like bacterial parts (for instance DNA) also deserve attention.

REFERENCES

Summary and General Discussion, Conclusions and Future Perspectives


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