Nutrient digestion and absorption during chemotherapy-induced intestinal mucositis in the rat
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LACTOSE MALDIGESTION DURING METHOTREXATE-INDUCED GASTROINTESTINAL MUCOSITIS IN A RAT MODEL


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Chapter 2

ABSTRACT

Background Patients with chemotherapy-induced gastrointestinal mucositis suffer from anorexia, diarrhea and stomach pain, often causing weight loss and malnutrition. When the intestinal function during mucositis would be known, a rational feeding strategy might improve the nutritional state, accelerate recuperation and increase survival of mucositis patients. Methods We developed a methotrexate (MTX)-induced mucositis rat model to study nutrient digestion and absorption. To determine lactose digestion and absorption of its derivative glucose during mucositis, we injected Wistar rats intravenously with MTX (60 mg/kg) or NaCl 0.9% (controls). Four days later, we orally administered trace amounts of [1-13C]lactose and [U-13C]glucose and quantified appearance of labeled glucose in the blood for 3 h. Finally, we determined plasma citrulline level and harvested the small intestine to assess histology, myeloperoxidase level, glycohydrolase activity, immunohistochemical protein and mRNA expression. Results MTX-treated rats showed profound villus atrophy and epithelial damage. During the experimental period, the absorption of lactose-derived [1-13C]glucose was 4.2-fold decreased in MTX-treated rats, as compared with controls (p<0.01). Lactose-derived [1-13C]glucose absorption correlated strongly with villus length (rho=0.86, p<0.001) and with plasma citrulline level (rho=0.81, p<0.001). MTX treatment decreased jejunal lactase activity (19.5-fold, p<0.01), immunohistochemical protein- and mRNA expression (39.7-fold, p<0.01), as compared with controls. Interestingly, MTX treatment did not affect the absorption of [U-13C]glucose during the experimental period. Conclusions We conclude that lactose digestion is severely decreased during mucositis while glucose absorption is still intact, when supplied in trace amounts. Plasma citrulline level might be a useful objective, noninvasive marker for lactose maldigestion during mucositis in clinic.

INTRODUCTION

Gastrointestinal mucositis (further referred to as ‘mucositis’) is a severe and debilitating side effect of chemotherapy, especially in children [23, 30]. Mucositis is a transient condition that consists of different stages of inflammation and loss of enterocytes, ending with spontaneous healing of the mucosa [28, 29]. Since accurate evaluation of mucositis by intestinal biopsies is problematic in patients, mucositis is primarily diagnosed by more subjective symptoms in clinic [30]. With chemotherapy, 40-100% of patients report symptoms of mucositis, depending on the chemotherapeutic agent that is used and the given dose per cycle [30]. In children with acute myeloid leukemia, who receive multiple high doses of different chemotherapeutic agents, mucositis was found to be present in 55% of chemotherapy cycles [38]. There is a lack of objective, noninvasive markers for mucositis [30, 38], albeit recently, we and others suggested plasma citrulline level to be a good marker [3, 21, 22, 38]. Citrulline is a nonprotein amino acid, made by enterocytes. Since
plasma citrulline represents functional enterocyte mass, reduced citrulline levels during mucositis represent reduced enterocyte mass [6].

Patients with mucositis suffer from anorexia, diarrhea and stomach pain, often leading to weight loss and malnutrition [16]. These complications of mucositis are associated with an increased use of injectable analgesics, nutritional problems and longer hospitalizations [30]. Moreover, since mucositis and its associated complications lead to a dose reduction of chemotherapy, mucositis compromises overall survival in cancer patients [9].

Mucositis is histologically characterized by villus atrophy, enterocyte damage and infiltration of inflammatory cells [30-32]. Although these histological changes suggest loss of epithelial function, the digestive and absorptive capacity of enterocytes during mucositis is still not known. A number of studies showed that protein and mRNA expression of enzymes and transporters involved in nutrient absorption are decreased during mucositis, indicating maldigestion and malabsorption [8, 31, 40]. However, only a few functional digestion and absorption studies during mucositis have been performed [14]. Up to now there is still no rational feeding strategy for mucositis patients. Directed nutritional support might actually improve the nutritional state, accelerate recuperation and increase survival of mucositis patients [2, 17, 24, 27].

We chose to determine nutrient digestion and absorption in a methotrexate (MTX) induced mucositis rat model. Our ultimate objective is to design a more rational feeding strategy for mucositis patients. We focus on carbohydrate digestion and absorption because of its major role in dietary energy supply, and started with lactose. Lactose is an important carbohydrate in Western pediatric diets and formulas [26]. It is a disaccharide that has to be digested by the glycohydrolazing enzyme lactase into the monosaccharides glucose and galactose before absorption of these monosaccharides takes place [33]. Absorption of glucose and galactose occurs by active and passive transport across the epithelial border by Sodium-dependant Glucose Transporter 1 (SGLT1) and Glucose Transporter 2 (GLUT2) respectively [33]. Both the enzyme lactase and the transporters SGLT1 and GLUT 2 are normally present in the brush border of enterocytes.

In this study, we aim to determine lactose digestion and absorption of its derivative glucose in our mucositis rat model, by using stable isotope labeled [1-13C]lactose and [U-13C]glucose. We hypothesize that both digestion and absorption of these carbohydrates is decreased during mucositis.
Chapter 2

MATERIALS AND METHODS

Rats and housing
Male Wistar outbred rats (4 wk old, 95-105 g) were obtained from Harlan (Horst, the Netherlands). Rats were individually housed in plexiglass cages (42.5 x 26.6 x 18.5 cm) on a layer of wood shavings under controlled temperature (21 ± 1 °C) with a relative humidity of 55 ± 10% and a 12:12-h light-dark cycle (lights on 7 A.M.-7 P.M.). Water and purified diet (AIN-93G, Harlan Laboratories, Madison, WI, USA) were available ad libitum unless otherwise stated. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, the Netherlands.

Materials
Methotrexate was obtained from Pharmachemie Holding B.V. (Haarlem, the Netherlands). [1-13C]lactose (kindly donated by Dr. R.J. Vonk) and [U-13C]glucose of 99% isotopic purity were purchased from Isotec (Miamisburg, OH, USA).

Experimental procedures
The mucositis rat model. We developed an MTX-induced mucositis rat model to determine nutrient digestion and absorption during mucositis. To find the optimal dosage of MTX and time interval to study digestion and absorption during mucositis, we did some pilot experiments. Pilot experiments were done with different dosages of MTX (30, 45, 60, 90, 120 and 150 mg/kg) via a single intravenous injection in the tail vein under general anesthesia (day 0), based upon other mucositis rat and mouse models [8, 39, 40]. Clinical findings were recorded daily and rats were sacrificed at several days post injection (days 2, 4, 6 and 10) to study small intestinal damage. Based on results from our Pilot studies (see ‘Results’), digestion and absorption experiments were performed with MTX 60 mg/kg, 4 days after injection. Jejunal histology was used as a representative for small intestinal damage.

The lactose digestion and glucose absorption test. Two weeks after arrival at the animal facility, rats (6 wk old, 184-215 g) were injected once intravenously with MTX (60 mg/kg, n=14) or NaCl 0.9% (controls, n=7). Intake of food and water and body weight was recorded daily at 8 A.M. Four days after injection, after an overnight fast (11 P.M. day 3–8 A.M. day 4), rats received a bolus with trace amounts of [1-13C]lactose (40 mg per rat) and [U-13C]glucose (20 mg per rat) in 600 µl PBS by oral gavage to study both lactose digestion and glucose absorption. Before and at time points 7.5, 15, 30, 45, 60, 90, 120 and 180 min after bolus administration, blood samples were obtained by blood spot technique from the tail tip to measure blood glucose levels and to quantify blood enrichment of lactose-derived [1-13C]glucose and of [U-13C]glucose [35]. Before and at the end of the test, we obtained additional blood samples to measure plasma insulin levels. Samples were centrifuged immediately (10 min at 3,000 rpm) and collected plasma was stored at -80°C until further analysis.
Euthanazation. At the end of the digestion/absorption test (3 h after bolus administration), rats were euthanized under general anesthesia by obtaining a large blood sample through cardiac puncture for determination of plasma citrulline levels. Blood samples were centrifuged immediately (10 min at 3,000 rpm) and collected plasma was stored at -80°C until further analysis. Then, the abdomen was opened via a midline incision and the small intestine was excised, flushed with ice-cold PBS and divided into three segments of similar size (4.5 cm) [duodenum (proximal small intestine), jejunum (anatomical middle of the small intestine) and ileum (1/6 part proximal from the cecum)]. Smaller parts from each intestinal segment were harvested for assessment of histology and immunohistochemical protein expression (2.5 cm), myeloperoxidase (MPO) levels (0.5 cm), glycohydrolase activity (1.0 cm) and mRNA expression (0.5 cm). Small intestinal parts for histology and protein expression were fixed in formalin (1 cm) or 2% paraformaldehyde (PFA, 1 cm) dissolved in PBS, dehydrated and embedded in paraffin according to standard procedures for (immuno)histochemistry. Extra parts were frozen in isopentane (0.5 cm) and stored at -80°C until further use. The intestinal parts for MPO levels, glycohydrolase activity and mRNA expression were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Analytical methods

Histological assessment. Hematoxylin and eosin (H&E) staining was done on 3-μm-thick sections of formalin- and PFA-fixed jejunal segments to assess histology, according to standard procedures. Morphometric analysis was carried out as described previously [20]. Villus and crypt length were measured manually in well-orientated sections (10 measurements per rat) from digitized images that were evaluated at 10x magnification (1 pixel = 0.397 μm) using a calibrated image analysis system (Qwin V3.0, Leica Microsystems Inc.). Goblet cell distribution was analyzed by Alcian-Blue staining of 3-μm-thick sections of PFA-fixed material according to standard procedures.

Mucosal MPO levels. Mucosa of frozen jejunal sections was scraped on ice to make tissue homogenates in lysis buffer (reaction tubes: Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). Homogenates were 5-50 times diluted in dilution buffer before MPO levels were quantitatively measured via a solid bound antibody against MPO as described by the manufacturer (rat MPO ELISA kit, Hycult Biotech, Uden, the Netherlands).

Plasma citrulline levels. Plasma citrulline levels were measured in 30 μl plasma at room temperature by using automated ion exchange column chromatography as described before [37, 38].

Blood glucose and plasma insulin levels. Blood glucose levels were measured with a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium). Plasma insulin
levels were measured in 25 µl plasma via a solid bound antibody against insulin as described by the manufacturer (Rat Insulin Ultrasensitive EIA, Alpcro Diagnostics, Salem, NH, USA).

[1-13C]- and [U-13C]glucose absorption. After bolus administration with trace amounts of [1-13C]lactose and [U-13C]glucose, blood samples were obtained to quantify blood enrichment of lactose-derived [1-13C]glucose and of [U-13C]glucose. The quantification of lactose-derived [1-13C]glucose and [U-13C]glucose enrichment in blood from blood spots was performed according to Van Dijk et al. [35] by gas chromatography-mass spectrometry (Agilent 5957C Series GC/MSD, Agilent Technologies, Amstelveen, the Netherlands) as has been done before [20]. The calculations for blood glucose kinetics were described recently by Van Dijk et al. [34] and Laskewitz et al. [18]. In short, a single-pool, first-order kinetic model was assumed for this test. The mole percent enrichments of mass isotopomers M1 and M6, due to administered [1-13C]lactose and [U-13C]glucose respectively, were used to calculate the first order absorption process in an one-compartment model using SAAM-II software (version 1.2.1; SAAM Institute, University of Washington, Seattle, WA, USA) [36]. Absorption of lactose-derived [1-13C]glucose and [U-13C]glucose during the experimental period was calculated as area under the curve of [1-13C]glucose and [U-13C]glucose concentration (time 0-180 min) respectively.

Mucosal glycohydrolase activity. Mucosa of frozen duodenal, jejunal and ileal sections was scraped on ice to make tissue homogenates in distilled water (reaction tubes: Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). Homogenates were 100-400 times diluted before glycohydrolase activity levels were measured of lactase, sucrase, isomaltase and maltase as described previously [7; 20]. Activity levels were normalized to protein levels that were measured by the BCA method as described by the manufacturer (BCA protein assay kit, Thermo Fischer Scientific, Inc., Rockford, IL, USA).

Immunohistochemical protein expression. Jejunal protein expression of lactase, sucrase-isomaltase (SI) and SGLT1 was detected using immunohistochemistry according to standard procedures. Lactase was visualized on frozen material (4-µm slides) using a monoclonal mouse anti-rat lactase antibody (kindly donated by Dr. A. Quaroni) [25; 40], dilution 1:500, as described previously [12]. After incubation with the first antibody (30 min), endogenous peroxidase activity was blocked and slides were incubated with the peroxidase-conjugated secondary (rabbit anti-mouse) and tertiary (goat anti-rabbit) antibodies (Dako North America, Carpinteria, CA, USA). SGLT1 was also visualized on frozen material (4-µm slides) using a commercially available polyclonal goat anti-mouse antibody (Santa Cruz Biotechnology, Inc., sc-20584, Santa Cruz, CA, USA), dilution 1:20, with a slightly adapted protocol for immunofluorescent staining. After incubation with the SGLT1 antibody (overnight),
slides were incubated with fluorescent secondary (donkey anti-goat) antibody (Invitrogen Corporation, Alexa Fluor 488, Carlsbad, CA, USA). Slides were covered with fluorescent mounting medium (Dako North America, Carpinteria, CA, USA). SI was visualized on formalin fixed material (3-μm slides) using a polyclonal rabbit anti-rat SI antibody (kindly donated by Prof. dr. K.Y. Yeh) [42], dilution 1:600, as described previously [40].

**Mucosal mRNA expression.** Mucosa of frozen duodenal, jejunal and ileal sections was scraped on ice to isolate RNA, synthesize cDNA and subsequently measure mRNA expression of glycohydrolases lactase (Lct) and SI (Si) and glucose transporters SGLT1 (Slc5a1), GLUT2 (Slc2a2) and Glucose Transporter 5 (GLUT5 or Slc2a5). mRNA expression was measured by real-time PCR as described previously [1] (PCR plates and tubes: Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). Integrity of isolated RNA was checked via gel electrophoresis and disintegrated samples were not included for PCR-analysis. PCR results were normalized to β-actin (Actb) mRNA levels. Sequences of the primers and probes are listed in the supplementary data (Table S1).

<table>
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<th>Reverse Primer</th>
<th>TaqMan® probe</th>
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<td>TCT CCG GAG TCC ATC ACA ATG</td>
<td>TGT CCC TGT ATG CCT CTG GTC GTA CCA C</td>
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<td>GTG GGA AAA TGT GTC CCA GAT ACT</td>
<td>TCC TTT GCC ATC TGC TCT CCA CGC</td>
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<td>S1 (sucrase-isomaltase)</td>
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<td>Slc5a1 (SGLT1)</td>
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**Table S1. The PCR primers and TaqMan probes.** Depicted genes are stated in symbols according to the official gene nomenclature, their corresponding commonly used names are placed in parentheses.

**Statistical analysis**

Statistical analysis was performed using the Mann-Whitney U-test (SPSS 16.0 for Windows, Chicago, IL, USA). Values represent medians and first to third quartiles (Figures) or ranges (Tables) for the indicated number of rats per group. All correlations are expressed as nonparametric Spearman correlation coefficient. P values were considered statistically significant if P<0.05. NS means ‘not significant’.
RESULTS

Pilot studies
To find the optimal dosage of MTX and time interval to study nutrient digestion and absorption during mucositis, we did some pilot experiments. At day 2, MTX-treated rats (≥60 mg/kg) showed crypt loss and atrophy while villi still appeared normal. Typical histological signs of mucositis like villus atrophy and blunting, enterocyte damage and infiltration of inflammatory cells were present in most MTX-treated rats (≥60 mg/kg) at day 4. By this time, crypts tended to be elongated, a sign of crypt regeneration. From day 6 on, villi of MTX-treated rats started to recover (results not shown). Histological signs of mucositis were basically the same in the duodenum, jejunum and ileum. Typical clinical signs of mucositis, such as a decreased food intake, weight loss and diarrhea, were present in most MTX-treated rats (≥60 mg/kg) from day 2 until day 5, after which rats started to recover (results not shown). Histological and clinical signs of mucositis differed substantially between MTX-treated rats, dependant on the dosage of MTX. When lower MTX dosages were used (30-60 mg/kg), some rats developed only mild signs of mucositis. When higher dosages were used (90-150 mg/kg), all rats developed severe signs of mucositis but mortality increased. For our experiments, we chose the MTX dosage of 60 mg/kg since this caused pronounced mucositis in most rats, without causing mortality.

The mucositis rat model during the present experiment
Histological findings. We analyzed jejunal sections by H&E staining to demonstrate that MTX-treated rats developed histological signs of mucositis (Figure 1A-B). Most MTX-treated rats showed profound villus atrophy and blunting with irregular, sometimes even vacuolized enterocytes (Figure 1B). Furthermore, there was an influx of inflammatory cells into the stroma of villi (Figure 1B, arrows). However, individual signs of mucositis varied between MTX-treated rats, with some of them only showing scattered cuboidal shaped enterocytes. Villus length of MTX-treated rats was 1.8-fold decreased (p<0.01, Figure 2A) while crypt length was 1.3-fold increased (p<0.01, Figure 2B), as compared with controls. Goblet cells were evenly distributed along the crypt-villus axis in controls (Alcian-Blue staining, Figure 1C). In contrast, Goblet cells were restricted along the villi or solely present on villus tops of MTX-treated rats (Figure 1D). Our findings indicate that MTX-treated rats developed histological signs of mucositis, varying from mild to severe.

Mucosal MPO levels. We measured MPO levels in scraped mucosa of the jejunum to quantify intestinal inflammation during mucositis (Figure 1B, arrows). MPO levels were 20.3-fold increased in MTX-treated rats, as compared with controls (p<0.01, Figure 2C), indicating significant infiltration of neutrophils in the small intestine during mucositis.
Lactose Maldigestion during Methotrexate-induced Mucositis

Plasma citrulline levels. We measured plasma citrulline levels to estimate the level of functional enterocyte mass during mucositis [6] and to see whether plasma citrulline can serve as a noninvasive marker for mucositis, as has been suggested before [3, 21, 22, 38]. Citrulline levels were 3.6-fold decreased in MTX-treated rats, as compared with controls (p<0.01, Figure 2D), corresponding with significant loss of functional, citrulline producing small intestinal enterocytes during mucositis. Plasma citrulline level correlated with the severity of mucositis as measured by villus length (rho=0.90, p<0.001, Figure S1).

Clinical findings. We recorded the intake of food and water and bodyweight daily after injection with NaCl or MTX to see if MTX-treated rats developed clinical signs of mucositis. Food intake in MTX-treated rats was decreased on all days post injection (day 0) with a maximum of 1.5-fold on both day 2 and 3, as compared with controls (p<0.01, Figure 3A). On day 3, food intake of all rats was decreased since rats were fasted before the digestion and absorption test at day 4. Water intake of MTX-treated rats was decreased from day 2 on with a maximum of 1.9-fold on day 3, as compared with controls (p<0.01, Figure

Figure 1. Histological findings in the mucositis rat model. Hematoxylin and eosin (H&E) staining showing morphology (A-B), Alcian Blue staining showing Goblet cell distribution (C-D) and immunohistochemical protein expression of lactase (E-F), sucrase isomaltase (SI, G-H) and Sodium dependant Glucose Transporter 1 (SGLT1, I-J) in the jejunum of NaCl (left panels) and methotrexate (MTX)-treated rats (right panels). Arrows (B) point out infiltration of inflammatory cells in the stroma of villi. Magnification: 20x.
Body weight was decreased in MTX-treated rats from day 1 on with a maximum on day 4, as compared with controls (p<0.05, Figure 3C). Compared to the day of injection, MTX-treated rats lost 2% of initial body weight at day 4 while, in contrast, controls gained 9% of initial body weight by this time (Figure 3C). Other clinical signs of mucositis, like a sick appearance in general and watery diarrhea were present in MTX-treated rats from day 3 on. Our findings indicate that MTX-treated rats developed clinical signs of mucositis.

Figure 2. Morphometric analysis, myeloperoxidase (MPO) and citrulline levels in the mucositis rat model. Jejunal villus (A) and crypt length (B), mucosal MPO levels (C) and plasma citrulline levels (D) in NaCl- (○, n=7) and MTX-treated rats (●, n=14). Dots represent data of individual rats, horizontal lines represent medians of groups. *P<0.01 for NaCl- versus MTX-treated rats.

Figure S1. Correlation between plasma citrulline level and villus length in the mucositis rat model. The correlation is shown in NaCl- (○, n=7) and MTX-treated rats (●, n=14). The Spearman correlation (r) and P value is indicated.
Lactose Malabsorption during Methotrexate-induced Mucositis

The lactose digestion and glucose absorption test
Blood glucose and plasma insulin levels. There was hardly a rise in blood glucose after bolus administration, both in MTX-treated rats and in controls (Figure S2). Plasma insulin levels did not differ between MTX-treated rats and controls at the start or at the end of the test (results not shown).

Lactose digestion and absorption of its derivative [1\(^{13}\)C]glucose. Blood appearance of lactose-derived [1\(^{13}\)C]glucose was determined over a 3-h period after bolus administration. In controls, [1\(^{13}\)C]glucose entered the blood glucose pool 11 minutes after bolus administration (t\(_{lag}\), Figure 4A and Table 1). Maximal [1\(^{13}\)C]glucose concentration (c\(_{max}\)) was reached at 56 min after bolus administration (t\(_{max}\)), and was 0.34 mmol/l. In MTX-treated rats, [1\(^{13}\)C]glucose appearance was significantly delayed and decreased, as compared with controls (Figure 4A and Table 1). During the experimental period, the absorption of [1\(^{13}\)C]glucose was 4.2-fold decreased in MTX-treated rats, as compared with controls (p<0.01, Table 1). Absorption correlated with villus length (rho=0.86, p<0.001, Figure 4B) and with plasma citrulline level (rho=0.81, p<0.001, Figure 4C). Our findings indicate that lactose digestion and/or absorption of its derivative glucose is severely decreased during mucositis.
[U-13C]Glucose absorption. Blood appearance of [U-13C]glucose was determined over a 3-h period after bolus administration. In controls, [U-13C]glucose entered the glucose pool 5 min after bolus administration (tLAG, Figure 4D and Table 1). Maximal [1-13C]glucose concentration (c\text{max}) was reached at 33 min after bolus administration (t\text{max}), and was 0.33 mmol/l. Interestingly, [U-13C]-glucose appearance was slightly delayed (p<0.05) but not decreased in MTX-treated rats, as compared with controls (Figure 4D and Table 1). MTX treatment did not affect the absorption of [U-13C]glucose during the experimental period (Table 1). Absorption correlated with the level of mucositis as measured by villus length (rho=0.48, p=0.027, Figure 4E) but did not correlate with plasma citrulline level (rho=0.36, NS, Figure 4F). Our findings indicate that glucose absorption is still intact during mucositis, when given in trace amounts. Therefore, decreased absorption of lactose-derived [1-13C]glucose during mucositis indicates disturbed lactose digestion instead of glucose malabsorption, since [U-13C]glucose is absorbed normally.

Table 1. Blood appearance of lactose-derived [1-13C]glucose and of [U-13C]glucose during the lactose digestion/glucose absorption test. Appearance is shown after administration of the [1-13C]lactose/[U-13C]glucose bolus in NaCl- and MTX-treated rats. Data belong to the curves that are plotted in Figure 4A and D. TLAG is the point of time where the label enters the glucose pool, t\text{max} is the point of time where the concentration of the label is maximal and c\text{max} is the concentration of the label that is reached at t\text{max}. Data indicate medians of groups, ranges are in parentheses. #P<0.05 and *P<0.01 for NaCl- versus MTX-treated rats.

Lactose-derived [1-13C]glucose appearance

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<td>31 (6-52)  *</td>
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<td>t\text{max} (min)</td>
<td>56 (54-82)</td>
<td>144 (63-175) *</td>
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<tr>
<td>c\text{max} (mmol/l)</td>
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<td>0.09 (0.02-0.46) *</td>
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[U-13C]glucose appearance

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<td>45 (30-61) #</td>
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<td>AUC (mmol/l-min)</td>
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**Figure 4.** Lactose digestion and absorption of glucose (lactose-derived [1-13C]glucose and [U-13C]glucose) during the lactose digestion/glucose absorption test. Blood appearance of lactose-derived [1-13C]glucose (A) and [U-13C]glucose (D) in NaCl- (○, n=7) and MTX-treated rats (●, n=14) after oral administration of the [1-13C]lactose/ [U-13C]glucose bolus. Dots represent medians and p25-p75 at 7.5, 15, 30, 45, 60, 90, 120 and 180 min after bolus administration. #P<0.05 and *P<0.01 for NaCl- versus MTX-treated rats. B and C: Correlation between lactose derived [1-13C]glucose absorption on the one hand and villus length (B) or plasma citrulline level (C) on the other hand, in NaCl- (○) and MTX-treated rats (●). E and F: Correlation between [U-13C]glucose absorption on the one hand and villus length (E) or plasma citrulline level (F) on the other hand, in NaCl- (○) and MTX-treated rats (●). Spearman correlations (r) and P values are indicated. AUC: area under the curve of [1-13C]glucose and [U-13C]glucose concentration (time 0-180 min, Figure 4A and D), representing [1-13C]glucose absorption and [U-13C]glucose absorption respectively.
Mucosal glycohydrolase activity
We measured mucosal glycohydrolase activity of lactase to investigate whether disturbed lactose digestion during mucositis can be explained by a decreased lactase activity. We also studied activity of glycohydrolases sucrase, isomaltase and maltase. Lactase activity was most abundant in the jejunum of controls and was 19.5-fold decreased in MTX-treated rats, as compared with controls (p<0.01, Table 2). As with lactase activity, a decreased jejunal activity of sucrase (13.9-fold, p<0.05), isomaltase (17.0-fold, p<0.01) and maltase (9.1-fold, p<0.01) was found in MTX-treated rats, as compared with controls (Table 2). Our findings indicate that the hydrolyzing activities of lactase, sucrase, isomaltase and maltase are all severely decreased during mucositis. Disturbed lactose digestion during mucositis can therefore be explained by a decreased lactase activity.

<table>
<thead>
<tr>
<th>Glycohydrolase activity (µmol/mg protein/hr)</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl (n=7)</td>
<td>MTX (n=14)</td>
<td>NaCl (n=7)</td>
</tr>
<tr>
<td>Lactase</td>
<td>0.1 (0.1-2.2)</td>
<td>0.0 (0.0-0.2) *</td>
<td>1.3 (0.9-1.6)</td>
</tr>
<tr>
<td>Sucrase</td>
<td>7 (6-9)</td>
<td>0 (0-8) *</td>
<td>8 (6-10)</td>
</tr>
<tr>
<td>Isomaltase</td>
<td>18 (15-22)</td>
<td>0 (0-20) *</td>
<td>41 (32-49)</td>
</tr>
<tr>
<td>Maltase</td>
<td>63 (51-82)</td>
<td>5 (0-73) *</td>
<td>102 (80-113)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative mRNA expression (normalized to β-actin)</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl (n=6)</td>
<td>MTX (n=13)</td>
<td>NaCl (n=7)</td>
</tr>
<tr>
<td>Lactase</td>
<td>0.0 (0.0-0.0)</td>
<td>0.0 (0.0-0.1)</td>
<td>8.5 (5.9-12.6)</td>
</tr>
<tr>
<td>Sl</td>
<td>0.8 (0.5-1.1)</td>
<td>0.8 (0.1-1.4)</td>
<td>2.2 (1.4-2.5)</td>
</tr>
<tr>
<td>SGLT1</td>
<td>1.3 (0.7-1.6)</td>
<td>1.0 (0.3-1.9)</td>
<td>2.8 (2.3-3.2)</td>
</tr>
<tr>
<td>GLUT2</td>
<td>1.8 (0.9-2.1)</td>
<td>1.1 (0.3-1.8)</td>
<td>2.4 (2.1-2.8)</td>
</tr>
<tr>
<td>GLUT5</td>
<td>1.0 (0.5-1.1)</td>
<td>0.9 (0.1-1.6)</td>
<td>2.2 (1.7-2.9)</td>
</tr>
</tbody>
</table>

Table 2. Glycohydrolase activity and relative mRNA expression of glycohydrolases and glucose transporters in the mucositis rat model. Activity and/or mRNA expression profiles of lactase, sucrase, isomaltase, maltase, SGLT1, Glucose Transporter 2 (GLUT2) and Glucose Transporter 5 (GLUT5) of NaCl- and MTX-treated rats are shown. Data indicate medians of groups, ranges are in parentheses. #P<0.05 and *P<0.01 for NaCl- versus MTX-treated rats.
Immunohistochemical protein expression
We studied jejunal immunohistochemical protein expression of lactase and SI to investigate whether a decreased activity of these glycohydrolases during mucositis can be explained by a decreased protein expression. Immunohistochemical protein expression of SGLT1 was studied to investigate whether intact glucose absorption during mucositis can be explained by an intact protein expression of this glucose transporter. Protein expression of lactase (Figure 1 E-F), SI (Figure 1 G-H) and SGLT1 (Figure 1 I-J) was normally present along the brush border of villi in control rats. In contrast, expression was merely present in the remaining villus tops of MTX-treated rats. Our findings indicate that decreased lactase, sucrase and isomaltase activity during mucositis can be explained by a decreased lactase and SI protein expression. However, intact glucose absorption during mucositis can not be explained by the protein expression of SGLT1, since this was severely decreased.

Mucosal mRNA expression
We measured mRNA expression of lactase, SI and SGLT1 to investigate whether a decreased protein expression of these glycohydrolases and glucose transporter during mucositis can be explained by a decreased mRNA expression. We also studied expression of GLUT2 and GLUT5. All mRNA expression profiles were most abundant in the jejunum of controls (Table 2). Jejunal expression of lactase and SI was decreased 39.7 and 9.4-fold respectively in MTX-treated rats, as compared with controls (p<0.01 and p<0.05 respectively). Jejunal mRNA expression of SGLT1, GLUT2 and GLUT5 was decreased 9.6-, 10.1- and 9.5-fold respectively in MTX-treated rats, as compared with controls (both p<0.01). Our findings indicate that a decreased lactase, SI and SGLT1 protein expression during mucositis can be explained by a decreased mRNA expression.

DISCUSSION
In this study, we aimed to determine lactose digestion and absorption of its derivative glucose during mucositis. We hypothesized that both digestion and absorption of these carbohydrates is decreased during mucositis. Our results show that lactose digestion is severely decreased during mucositis. Interestingly, the absorption of glucose is still intact during mucositis, at least, when supplied in trace amounts.

We used an MTX-induced mucositis rat model to determine lactose digestion and absorption of its derivative glucose. Histology and mucosal MPO level (indicating infiltration of neutrophils) were studied to show that the model really represented mucositis. MTX-treated rats showed typical histological characteristics of mucositis like blunting of villi with irregular or even vacuolized enterocytes. Goblet cells were depleted and accumulated at villus tops. Crypts of MTX-treated rats tended to be elongated, which is a sign of crypt regeneration via hyperproliferation and hyperplasia after initial crypt damage caused by MTX [39,40]. Also, we saw an influx of
inflammatory cells in villus stroma and increased mucosal MPO levels during MTX treatment. Besides typical histological characteristics of mucositis, MTX-treated rats also showed typical clinical characteristics like a decreased intake of food and water, weight loss and diarrhea. These characteristics of mucositis were also found by others [8, 19, 31, 32, 40]. Histological and clinical signs of mucositis differed substantially between MTX-treated rats. Out of 14 MTX-treated rats, 3 rats showed minimal histological and clinical signs of mucositis, normal MPO and citrulline levels (Figure 2) and a normal absorption of lactose derived glucose (Figure 4B and C). The variance in observed individual signs of mucositis could be a result of genetic variability between outbred Wistar rats [30]. Also, our mucositis model is based upon 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 used [4, 10, 13, 28, 29]. The amount of subsequent crypt loss by apoptosis, crypt atrophy and ultimately villus atrophy [39] therefore differs per MTX-injected rat.

During the experimental period, the absorption of lactose-derived [1-13C]glucose was severely decreased in MTX-treated rats, as compared with controls. In contrast, the absorption of [U-13C]glucose was still intact in MTX-treated rats. We therefore concluded that decreased absorption of lactose-derived glucose during mucositis is a result of disturbed lactose digestion instead of glucose malabsorption. The hydrolysis of the disaccharide lactose into the monosaccharides glucose and galactose by the enzyme lactase must be defective during mucositis. A decreased in vitro lactase enzyme activity, as well as a decreased immunohistochemical protein and mRNA expression of lactase in MTX-treated rats, as compared with controls, further supported this conclusion. Although others already showed a decreased lactose breath test, lactase activity and lactase protein and mRNA expression during mucositis [8, 14, 31, 40], we are the first to functionally demonstrate that lactose is indeed maldigested during mucositis. Furthermore, the fact that we could confirm lactase activity and expression profiles found in other mucositis studies, demonstrates the correct establishment of a mucositis rat model in our lab using a single injection with MTX.

Here, we prove that ‘lactose malabsorption’ during mucositis is a result of defective lactose hydrolysis instead of defective absorption of its derivative glucose. Our findings imply that lactose should be omitted from the diet of mucositis patients since it can not be used as a source of energy. Lactose maldigestion might even exaggerate diarrhea and stomach pain which often is already present during mucositis [11, 26, 41]. We also found a decreased enzyme activity of other glycohydrolases such as sucrase, isomaltase and maltase in MTX-treated rats, as compared with controls. These findings indicate 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.

Because plasma citrulline level was earlier suggested to be a good, noninvasive marker for mucositis [3, 21, 22, 38], we measured plasma citrulline levels in NaCl- and MTX-treated rats. Levels of plasma citrulline, a non-protein amino acid, were severely decreased in MTX-treated rats, as compared with controls, corresponding with loss of functional enterocyte mass [6]. In individual rats, plasma citrulline level strongly correlated with the level of mucositis as measured by villus length and with lactose digestion during mucositis. Plasma citrulline level might therefore not only be an objective, non-invasive marker for the level of mucositis but, more important, for lactose malabsorption during mucositis. It could be a better alternative for the currently used, more subjective ‘National Cancer Institute Common Toxicity Criteria’ [30, 38], as a parameter for gastrointestinal mucositis. Furthermore, plasma citrulline level could be easily used in clinic to adapt the (feeding) strategy of mucositis patients.

Because the absorption of [U-13C]glucose was still intact in MTX-treated rats, we conclude that glucose transport across the epithelial border must, at least to some extent, still be intact during mucositis. However, immunohistochemical protein and/or mRNA expression of glucose transporters SGLT1 and GLUT2 was decreased in MTX-treated rats, as compared with controls, as was found by others [8, 31, 40]. It should be noted that the given bolus contained only trace amounts of [U-13C]glucose and [1-13C]lactose. Minimal glucose absorption might therefore have been possible via residual transporters on the damaged epithelial membrane, maybe in combination with leakage through damaged tight junctions since mucositis often leads to an increased gut permeability [5, 15]. Whether glucose can be an appropriate source of dietary energy for mucositis patients should be further studied by glucose absorption studies using relevant amounts of glucose.

In conclusion, our study shows that lactose digestion is severely decreased during mucositis while glucose absorption is still intact, when supplied in trace amounts. We recommend to omit lactose from the diet of mucositis patients to prevent possible negative side effects of lactose malabsorption, like lactose intolerance. Plasma citrulline level might be a useful objective, noninvasive marker for lactose malabsorption during mucositis in clinic.

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Lactose Maldigestion during Methotrexate-induced Mucositis

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