Methotrexate induces intestinal mucositis and alters gut protein metabolism independently of reduced food intake

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GUT PROTEIN METABOLISM DURING INTESTINAL MUCOSITIS

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was performed on fixed and embedded in paraffin tissue with BrdU monoclonal antibody (dilution 0.1; M0744; DakoCytomation, Trappes, France) according to a standard immunohistochemical method using a streptavidine-biotin/horseradish peroxidase detection system (5001, DakoCytomation). BrdU detection was then scored from 0 (no labeling) to 3 (high level of labeling).

Intestinal Cytokine Concentrations and GSH Content

Intestinal cytokine concentrations were evaluated by specific ELISA assay (Quantikine; R&D Systems, Abingdon, UK) for IL-1β, IL-4, IL-6, IL-10, IFNγ, and TNF-α according to manufacturer’s instructions and as previously described (26). Concentrations were expressed as picograms per milligrams of tissue. GSH content was assessed in the jejunum by a standardized spectrophotometric assay as previously described (1, 26). GSH content was expressed as micromoles per grams of tissue.

Amino Acid Concentrations

Free amino acid concentrations were determined in plasma and in the duodenum as previously described (14).

Evaluation of Mucosal Protein and Mucin Contents

The protein content in the jejunal mucosa was assessed using the Bio-Rad dye reagent kit (Bio-Rad Laboratories, Reineck, Switzerland). For mucin quantification, jejunal samples were processed, and mucins were quantified as previously described (13). The mucin content of each sample was then correlated with the weight of the initial tissue and expressed as micrograms of mucins per grams of tissue.

Evaluation of Mucin Gene Expression by Real-Time Quantitative PCR on TaqMan

TaqMan real-time quantitative PCR was carried out in an ABI 5700 Thermocycler (Applied Biosystems, Roche, Switzerland) using total RNA extracted from whole-thickness jejunum using the TriPure isolation reagent (Roche Diagnostics, Waldbronn, Germany) for quality verification, and reverse transcribed. Primers and TaqMan probes for mucin genes were designed (Supplemental Table S1; supplemental data for this article are available online at the Am J Physiol Endocrinol Metab website) and synthesized for mucin 2, mucin 3, and mucin 4. The Taqman probes presented in the 5′-end an additional 6-carboxyfluorescein. The reactions were carried out in

Histological Parameters

For histological and immunohistochemistry assessments, jejunal samples were fixed in formalin 10% and treated as previously described (26). Briefly, sections were scored by the same pathologist blinded to the treatment allocation. Epithelial necrosis, inflammatory cells infiltration, and exocytosis were assessed using semiquantitative scores that ranged from 0 (no damage) to 3 (severe damages) for each parameter: villus atrophy, necrosis, inflammation, and exocytosis. Villus height was measured in 10 well-oriented villi from each rodent using the analysis software Leica QWin (Leica Microsystems, Ben-shipen, Germany).

Evaluation of Cell Proliferation

The rate of cell proliferation was evaluated by the incorporation of 60 mg/kg 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich, Saint-Quentin-Fallavier, France) injected intraperitoneally 1 h before euthanasia as previously described (26). Briefly, immunohistochemistry

Fig. 1. Enrichment kinetics in different pools. Enrichment (means ± SE; mol % excess) measured in intratissular free amino acid (A; ⋄) or plasma (A; □) precursor pools and in mucosal proteins (B). Rats were injected intravenously with 1,500 μmol/kg of L-[ring-2H5]phenylalanine and killed after 5, 10, 15, or 20 min (n = 3 at each time).

Daily Clinical State Monitoring

From D3 to euthanasia day, body weight and food intake were monitored at 24-h intervals.

Euthanasia and Tissue Sampling

Animals were decapitated after carbon dioxide inhalation, and blood samples were collected. Plasma samples were stored at −80°C as previously described (14). Then, the duodenum and jejunum were taken and rinsed with ice-cold PBS (140 mM NaCl, 3 mM KCl, 8 mM as previously described (14). During 1 wk, 200- to 250-g male Sprague-Dawley rats (Charles River, L’Arbresle, France) were acclimatized at 25°C with a 12-h light-dark cycle. Animals were individually housed in metabolic cages 4 days before study. Rats were given free access to water and standard chow until D3. Afterwards, MTX-treated rats had free access to a protein-rich diet. Control rats were either ad libitum or pair-fed vs. MTX rats with the same diet. Rats were subcutaneously injected during the first 3 days (D0, D1, and D2) with 2.5 mg/kg MTX (Teva Pharma, Courbevoie, France) or NaCl solution (0.9%) as a control (26). Rats were euthanized on D4 or D7 for pair-fed and MTX-treated rats and at D7 for ad libitum rats. D4 and D7 were chosen to be in the acute (D4) and the recovery (D7) phases of mucositis after preliminary experiments (data not shown).

Animals, Housing, Diet, and MTX Injections

Animal care and experimentation complied with both French regulation and European Community regulations (Official Journal of the European Community L 358, 18/12/1986), and M. Coeffier is authorized by the French Government to use an animal model (authorization No. 76-60). During 1 wk, 200- to 250-g male Sprague-Dawley rats (Charles River, L’Arbresle, France) were acclimatized at 25°C with a 12-h light-dark cycle. Animals were individually housed in metabolic cages 4 days before study. Rats were given free access to water and standard chow until D3. Afterwards, MTX-treated rats had free access to a protein-rich diet. Control rats were either ad libitum or pair-fed vs. MTX rats with the same diet. Rats were subcutaneously injected during the first 3 days (D0, D1, and D2) with 2.5 mg/kg MTX (Teva Pharma, Courbevoie, France) or NaCl solution (0.9%) as a control (26). Rats were euthanized on D4 or D7 for pair-fed and MTX-treated rats and at D7 for ad libitum rats. D4 and D7 were chosen to be in the acute (D4) and the recovery (D7) phases of mucositis after preliminary experiments (data not shown).

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triplicate. Data were analyzed using the GeneAmp 5700 SDS software (Applied Biosystems). Relative gene expression was calculated after normalization to $H_{2}$-microglobulin, the primers and TaqMan probe of which were purchased from Applied Biosystems.

**Protein Metabolism Study**

**Protein synthesis.** In vivo protein synthesis was assessed by the infusion of a large dose of $L\text{-}[\text{ring-}{^2}\text{H}_5]\text{phenylalanine (1,500 }\mu\text{mol/kg; 90}\%\text{ molar percent excess; Mass Trace, Woburn, MA)}$ via a lateral tail vein as previously described (16). Animals were killed 10 min after the tracer administration. Tissues were immediately excised, and the mucosa of the jejunum was rapidly separated and frozen in liquid nitrogen. Under these conditions, plasma-free phenylalanine enrichment decreased slightly and linearly, as has been previously verified in rats killed at various time (Fig. 1). The enrichment of $[^2\text{H}_5]$phenylalanine was determined in the mucosal intracellular free amino acid pools and in the tissue proteins by gas-chromatography-mass spectrometry (MSD 5975; Agilent Technologies, Palo Alto, CA), using tert-butyldimethylsilyl derivatives as previously reported (4, 8). Appropriate standard curves were run simultaneously for determination of the enrichments. Protein synthesis rate was expressed as fractional synthesis rate (FSR) calculated as follows: $FSR (\%/\text{day}) = \left(\frac{E_t - E_o}{E_p}\right) \times \frac{1}{t} \times 24 \times 100$, where $E_t$ is the enrichment in tissue protein at time $t$ in %; $E_o$ is the natural abundance of the labeled amino acid in intestinal mucosal protein in %; $E_p$ is the enrichment of intracellular free amino acid pool at time $t$; and “$t$” is the duration of the tracer infusion in hours (s).

**Proteolysis.** The evaluation of proteolytic pathway activities was performed by measuring fluorogenic substrate degradation, as previously described (25, 26). Briefly, lysosomal cathepsin D activity was quantified with InnoZyme Cathepsin D Immunocapture Activity Assay Kit Fluorogenic (Calbiochem, San Diego, CA). The evaluation of calpain (1 and 2) and proteasome chymotrypsin-like activity was performed by spectrofluorimetry on microtiter plate fluorometer Mithras LB.
940 (Berthold Technologies, Bad Wildbad, Germany) using the fluorogenic proteasome substrate Suc-LLVY-MCA (PSII; Calbiochem) in the presence or absence of specific inhibitors.

The evaluation of gene expressions for proteolytic pathway components was realized by real-time quantitative PCR on LightCycler as previously described (26).

Calculation Methods and Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA) and consisted of a two-way ANOVA with Bonferroni post hoc tests when appropriate. For daily monitored parameters, repeated measures two-way ANOVA was performed. For all, \( P < 0.05 \) was considered significant.

RESULTS

Food Intake and Body Weight

After MTX treatment, food intake decreased until D5 (−60% at D2, −96% at D5, both \( P < 0.0001 \) vs. D0) and then increased (−30% at D7, \( P < 0.0001 \) vs. D5). No difference in food intake was observed between pair-fed and MTX groups (data not shown).

We compared body weight evolution of ad libitum, pair-fed, and MTX rats during 11 days from D3 to D7 (Fig. 2). Before MTX or saline treatment, body weight increased in the three groups at the same rate (5%). After injection of rats with 2.5 mg/kg of MTX or saline, body weight increased over time in ad libitum rats but progressively decreased from D2 until D6 (about −15%) in a similar manner in pair-fed and MTX-treated rats. Thus body weight was significantly reduced in pair-fed and MTX-treated rats compared with ad libitum controls.

Histological Damages and Cellular Proliferation in the Jejunum

Intestinal morphometry (Fig. 3) was similar in the pair-fed group at D4 and D7 compared with ad libitum controls. The MTX-treated group presented a marked decrease of villus height at D4 (Fig. 3, A and B) and an increase of inflammation score (Fig. 3C) compared with ad libitum and pair-fed groups. In MTX-treated rats, the necrosis score was 1.7 ± 0.2 at D4 and 0.7 ± 0.3 at D7, whereas any necrosis event was observed in control ad libitum and pair-fed rats. At D7, villus height was restored to that of ad libitum and pair-fed rats and inflammation score began a decrease. The cellular proliferation rate did not significantly differ between pair-fed and MTX-treated rats but was increased at D7 compared with D4 (Table 1).

Gut Protective Factors: Mucins and GSH

The mucin content was markedly impaired by MTX treatment compared with pair-fed group (Table 1). At D4, in MTX-treated rat mucosa, the MUC2 gene expression was significantly reduced (\( P < 0.05 \)), while thereafter it significantly increased (\( P < 0.01 \)) at D7 (Fig. 4). In contrast, MUC3 and MUC4 gene expressions were significantly increased (\( P < 0.05 \)) at both D4 and D7 (Fig. 4). GSH content was similar in pair-fed and MTX-treated rats and increased at D7 in both groups (Table 1).

Inflammatory Response

TNF-\( \alpha \) concentration in jejunal mucosa was higher in MTX-treated rats than in ad libitum and pair-fed rats (\( P < 0.05 \); Fig. 5A). In contrast, the IL-1β concentration was increased in pair-fed and MTX-treated rats compared with ad libitum controls but no difference was observed between pair-fed control rats and MTX-treated rats (Fig. 5B). IL-4, IL-10, and IFN-γ remained undetectable in the majority of jejunal samples (data not shown).

Protein Metabolism

Plasma and duodenal amino acid concentrations are displayed in Tables 2 and 3, respectively. The food restriction imposed to pair-fed rats was associated with modifications of the concentrations of various amino acids at D4 and D7. More importantly, at D4, plasma concentrations of citrulline, isoleucine, and threonine were lower in MTX-treated rats than in control ad libitum and pair-fed rats. At D7, only citrulline concentration remained low. By contrast, plasma concentra-
Selected plasma amino acid concentrations in control and MTX-treated rats at D4 and D7

Table 2. Selected plasma amino acid concentrations in control and MTX-treated rats at D4 and D7

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Ad Libitum</th>
<th>Pair-Fed</th>
<th>MTX-Treated</th>
<th>Treatment</th>
<th>Day</th>
<th>Interaction</th>
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<tr>
<td></td>
<td>D4</td>
<td>D7</td>
<td>D4</td>
<td>D7</td>
<td></td>
<td></td>
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<tr>
<td>Ala</td>
<td>645±26</td>
<td>562±17</td>
<td>729±24‡</td>
<td>615±21</td>
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<td>89±21*</td>
<td>84±14*</td>
<td>78±33*</td>
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</tr>
<tr>
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<td>95±51</td>
<td>14±3*</td>
<td>39±35*‡</td>
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<td>226±89</td>
<td>140±22*</td>
<td>207±38</td>
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<td>475±12*</td>
<td>474±15*</td>
<td>353±12*†</td>
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<tr>
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<td>105±29*</td>
<td>93±10*</td>
<td>115±21*‡</td>
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<td>411±14*</td>
<td>313±8*†</td>
<td>348±18*†</td>
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<td>Thr</td>
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<td>373±10</td>
<td>400±11</td>
<td>297±10*†</td>
<td>358±21‡</td>
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<tr>
<td>Val</td>
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<td>217±13*</td>
<td>229±20*</td>
<td>226±20*</td>
<td>276±30</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. Amino acid concentrations are given in μmol/l. *P < 0.05 vs. control ad libitum rats; †P < 0.05 vs. control pair-fed rats; and ‡P < 0.05 vs. D4.

At D4, cathepsin D activity, a component of the lysosomal proteolytic pathway, was significantly increased both in pair-fed and MTX-treated rats at D4 and D7 compared with ad libitum controls (P < 0.05; Fig. 7A). Moreover, cathepsin D activity was significantly increased in MTX-treated rats compared with pair-fed rats both at D4 and D7 (P < 0.05; Fig. 7A), even if cathepsin D activity decreased significantly between D4 and D7 in MTX-treated rats. Cathepsin D mRNA level was also increased in MTX-treated rats compared with pair-fed rats (Supplemental Table S2).

Regarding the Ca"+"-activated pathway, calpain activities were significantly increased in MTX-treated rats at D4 compared with pair-fed rats but not compared with ad libitum rats and then were decreased at D7 (Fig. 7B). Calpain 1 mRNA was affected both by treatment and day, being higher in MTX-treated rats compared with control pair-fed rats and increasing between D4 and D7 in both groups (Supplemental Table S2). Calpain 2 mRNA level was significantly increased by MTX treatment but without any day effect (Supplemental Table S2).

At D4 and D7, the proteasome chymotrypsin-like activity was significantly decreased in pair-fed and MTX-treated rats compared with ad libitum rats (P < 0.001; Fig. 7C). However,
this activity did not differ significantly between MTX-treated and pair-fed rats at D4 (Fig. 7C). Between D4 and D7, proteasome activity increased significantly in the pair-fed group but not in MTX-treated rats. Consequently, proteasome activity was lower in MTX-treated rats than in pair-fed rats at D7. Ubiquitin mRNA expression, a key component of proteasomal degradation, increased after MTX treatment (Supplemental Table S2).

**DISCUSSION**

Mucositis, a severe side effect of chemotherapy, may cause reduction or interruption of cancer treatment. Thus a better understanding of its pathophysiological mechanisms is required to develop adapted therapeutic strategies. We (26) previously reported alterations of the proteolytic pathways during chemotherapy-induced enterocolitis in rats (26), but whether these alterations were due to chemotherapeutic drugs or to the decrease of food intake that could be related to gastrointestinal pains or to direct or indirect central effects of drugs remains unknown. Indeed, in this precedent study, the control group was fed ad libitum. Therefore, we investigated in the present study the specific effects of MTX on the intestine independently of the reduction of body weight in rats as previously described (17).

In the present study, the marked reduction of food intake resulted in a rapid (−10% at D4) and profound (−20% at D6) decrease of body weight in rats as previously described (17). Surprisingly, we did not observe a further decrease of body weight in MTX-treated rats compared with pair-fed rats, indicating that body mass loss observed after MTX treatment would originate from food intake reduction per se.
In contrast, MTX-treated rats presented major histological mucosa damages in the jejunum compared with ad libitum and pair-fed rats. In particular, MTX-treated rats showed necrosis processes that were not present in pair-fed rats. Moreover, a strong reduction of villus height was observed in MTX-treated rats. At the same time, citrulline concentration was dramatically low in the duodenum and plasma of MTX-treated rats. This was especially the case on D4, since citrulline values of MTX-treated animals represent only 24 and 18% of control pair-fed values, respectively, in the duodenum and plasma. Interestingly, intestinal mucosa atrophy was correlated with reduced plasma citrulline concentration (Fig. 8). In MTX-treated rats, the low concentration of this nonprotein amino acid, synthesized by enterocytes, could reflect reduction of the enterocyte mass, as previously described in different diseases affecting the gut (11, 12), and could suggest a reduction of absorptive function. Our results demonstrate that MTX treatment induced a mucosal atrophy correlated with the citrulline decrease, independently of the anorexic effect of the drug.

To explain these histological differences, we compared protein metabolism, as well as the mucosal status in two key players of host defense mechanisms, namely mucins and GSH in the jejunum of MTX-treated vs. pair-fed rats. Mucosal damages could be related to alterations of protein metabolism, and we (26) previously reported that MTX treatment was associated with enhanced lysosomal proteolysis and decreased proteasome activity when compared with ad libitum rats (26). However, mRNA expressions of proteolytic pathway components, such as ubiquitin, and C2, C8, C9 subunits (proteasome) or cathepsin D (lysosome) and m-calpain (Ca²⁺-activated proteolytic pathway) were significantly increased by 48 h of starvation in the whole jejunum (19, 36) but not in the jejunal mucosa (35). In the present study, we show that during the acute phase of enterocolitis (D4) cathepsin D-mediated proteolysis, which was considered as a marker of lysosomal activity, was enhanced specifically by MTX treatment as previously described (26). We also observed that calpain activities were increased but only compared with pair-fed rats. These parameters were completely or partially restored at D7. In contrast, proteasome activity remained low at D4 and D7 in pair-fed and MTX-treated rats. In addition, calpain activities were correlated with the mRNA level of calpain 2 (r = 0.3; P < 0.05; data not shown), suggesting that calpain activities were mainly related to calpain 2. Taken together, these results suggest that the main proteolytic pathway that was activated in the jejunum after MTX treatment was the cathepsin D system whatever the rats used as control, namely pair-fed or ad libitum animals in the present study, or ad libitum animals in our previous work (26). In inflammatory bowel diseases, cathepsin D is enhanced in macrophages of gut mucosa (18).

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animal model of inflammatory bowel diseases, cathepsin D inhibition by drugs limits intestinal lesions (31). Thus we propose that cathepsin D might be targeted during MTX treatment to limit intestinal damage.

Proteasome modifications deserve discussion. In our previous study (26), we observed that proteasome activity was decreased in MTX-treated rats compared with ad libitum rats during both the acute and the recovery phases. Moreover, in the present work, a similar decrease of proteasome activity in pair-fed and MTX-treated rats compared with ad libitum rats at D4 was shown. At D7, these activities increased in pair-fed rats but not in MTX-treated rats. These data indicate that nutritional starvation, which is allowed by the decrease of food intake until D5, reduces proteasome activity as previously suggested (35). In addition, the increase of food intake from D5 to D7 is associated with higher proteasome activity in pair-fed but not in MTX-treated rats. Hence, we can hypothesize that either MTX directly inhibits proteasome or there is an activation of survival or economic processes in MTX-treated animals (39, 41).

In the present study, protein synthesis was reduced in jejunal mucosa compared with ad libitum and pair-fed rats during the acute phase of enterocolitis and was restored at D7. To evaluate protein synthesis rate, we used a previously reported method (16) that may influence phenylalanine and tyrosine levels. Thus we did not take account of phenylalanine and tyrosine levels. Altogether, these data suggest that a decrease of protein synthesis and an increase of lysosomal and Ca\(^{2+}\)-activated pathway activities are involved in the more marked damages observed in MTX-treated rats.

In addition to this global approach of protein metabolism, we focused our interest on specific proteins and particularly on intestinal mucins, the major component of the mucus layer playing a key role in the nonimmune gut barrier function (40). The mucus layer and mucin production are known to be qualitatively and quantitatively altered in many situations of intestinal stress, including inflammatory events (10), in the case of malnutrition (37), or in specific amino acid deprivation (14). Nevertheless, transient nutritional starvation similar to that observed in pair-fed rats in the present study has not been associated with modification of composition of mucins in goblet cells (23). In the present study, the reduced mucosal mucin content observed at D4 in the jejunum of MTX-treated rats compared with pair-fed rats indicates a deleterious effect of the chemotherapy treatment by itself. Together with the impaired gene expression of the main secreted intestinal mucin MUC2, this likely alters the protection and therefore increases the vulnerability of the jejunal epithelium. Further investigations are required to evaluate the specific role of membrane-bound mucins (MUC3 and 4) that behave differently than MUC2. At D7, defense mechanisms took place, as shown by an increased gene expression of both secreted (MUC2) and membrane-bound (MUC3 and 4) mucins. However, the lack of response at the protein level may reflect a posttranslational limitation of mucin synthesis. As shown by a low threonine plasma level in MTX compared with control rats, a reduced availability in threonine is likely occurring in MTX-treated rats, as previously suggested in other stress situations (13, 15).

The intestinal inflammatory response appeared higher in MTX-treated rats than in pair-fed controls, since we observed an increase in the histological inflammation score. It has been suggested that proinflammatory cytokines, e.g., TNF-α and IL-1β, may be involved in the amplification phase of intestinal mucositis (28) but that inflammation may be the functional consequence of the weakened barrier function, i.e., weakened epithelial integrity (atrophy and altered protein metabolism) and altered mucus protection. However, TNF-α concentration was the only proinflammatory cytokine affected in MTX-treated compared with pair-fed rats in the present study. This could be related to the timing of mucosa sampling, since the proinflammatory peak may have been observed early in time after MTX treatment as reported in irinotecan-treated rats (27). In addition, malnutrition is able to induce by itself cytokine expression in intestine (29) and to enhance the production of IL-1β but not of TNF-α by monocytes (7). Our results confirm this precedent work since we observed an increased intestinal IL-1β level in the pair-fed groups at D4 and D7, but no further modifications in the MTX groups. Thus IL-1β was overexpressed after the food restriction and not further stimulated by MTX itself. These data suggest that only TNF-α is involved in the pathophysiology of MTX-induced intestinal mucositis independently of food intake reduction.

All these lesions could have metabolic implications not only at the level of the gut but also at the systemic level as shown by the disturbances of amino acid profile in the duodenum and plasma. For instance, we observed a decrease both in the duodenum and plasma for taurine and citrulline, suggesting, respectively, an increased utilization for antioxidant response or an altered gut production. For glutamine, which is increased in plasma, this potentially suggests an increased liberation from the muscle. These data underline the need to better adapt nutritional interventions during chemotherapy as objectives to correct metabolic alterations in the gut but also distant from gut.

In conclusion, we show that MTX treatment alters intestinal mucosa and protein metabolism in a specific manner with a decrease of protein synthesis, in particular of mucin production, and an increase of proteolysis mediated by lysosomal pathway. Nutritional starvation that is associated with the treatment due to the decrease of food intake could enhance these modifications, but numbers of the MTX-induced disturbances were observed independently of the reduction of food intake. These alterations could be targeted by a specific nutritional intervention during chemotherapeutic treatment.

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