

The morphological changes of the colonic goblet cells and mucin profile in oncohematological patients under Epirubicin-based chemotherapy

CORALIA ADINA COTORACI¹⁾, ALCIONA SASU¹⁾, ALEXANDRU FICA MIRCEA ONEL¹⁾, DANA IOVĂNESCU²⁾, EFTIMIE MIUȚESCU²⁾, SAMI GHARBIA³⁾, ALINA LILIANA CICEU³⁾, HILDEGARD HERMAN³⁾, ANCA OANA HERMENEAN^{3,4)}

¹⁾Department of Hematology, Faculty of Medicine, Vasile Goldiș Western University of Arad, Romania

²⁾Department of Gastroenterology, Faculty of Medicine, Vasile Goldiș Western University of Arad, Romania

³⁾Aurel Ardelean Institute of Life Sciences, Vasile Goldiș Western University of Arad, Romania

⁴⁾Department of Histology, Faculty of Medicine, Vasile Goldiș Western University of Arad, Romania

Abstract

Changes in the lining of the small intestine following chemotherapy have been extensively studied, although also occurs in the large intestine. The aim of this study was to assess the consequences of Epirubicin-based therapy on goblet cells (GCs) and mucus production on colonic mucosa, immediately and after short-time of chemotherapy administration to oncohematological patients, by clinical and histopathological analysis. We assessed the mucus production, composition, and distribution by Alcian Blue (pH 2.5)–Periodic Acid–Schiff (PAS) staining, alongside with the immunoexpression of mucin (MUC)2, MUC4 and inflammatory markers in a series of oncohematological patients, immediately and after short-time of Epirubicin-based chemotherapy cumulative therapy cessation. We showed that GCs number decrease slightly at 48 hours, while mucous secretion became mixed (with a few neutral) after three weeks. Overall, the secretion was increased immediately after the Epirubicin administration, due to the activation of inflammatory pathways, assessed by increased immunostaining of tumor necrosis factor-alpha (TNF- α) at 48 hours. The MUC2 and MUC4 showed a decreased immunoexpression at 48 hours after the Epirubicin administration compared to controls and partially restored three weeks after the cessation. Overall, it is highly plausible that all these key players revolve around the chemotherapy-induced mucositis in oncohematological patients and highlights the morphofunctional particularities of the GCs, which further modulates the clinical outcome of the patient.

Keywords: oncohematological patients, Epirubicin, mucositis, colon, goblet cells, mucins.

Introduction

Goblet cells (GCs) secrete acidic glycoproteins, named mucins, which form a gel-like protective barrier over the mucosa against pathogenic bacterial proliferation and mucosal penetration [1], as well providing attachment sites for commensal bacteria [2]. Mucins also protect mucosa from digestion by microflora [3] and act as a shield against translocation of bacteria by increasing mucus acidity and viscosity [1]. Moreover, mucins protect the intestinal mucosa from chemical and mechanical stress [4] and promote intestinal transmucosal transport. Moreover, the mucous layer is rich in electrolytes, water, and secreted immunoglobulins [3].

In the colon, the intestinal mucus forms a thick layer (200 μ m in humans and 50 μ m in mice), consisting of an adherent part and an outer part rich in nutrients for the resident commensal bacteria; it contains mainly glycoprotein mucin 2 (MUC)2 produced by GCs in the epithelial lining and released into the lumen of the colon [5].

GCs discharges secretion of different mucins from surface epithelium and provide a mechanism to assure the protection by acidic secretion to the presence of bacteria or toxic agents in the lumen [2].

Changes in the lining of the small intestine following chemotherapy have been extensively studied, although also occurs in the large intestine. Thus, it has been noticed harmful effects occurring in the colon after treatment with Irinotecan [6].

During and after chemotherapy, the intestinal mucus produced by colonic GCs exhibit a more diverse staining pattern, with increased mucous production. Moreover, the number of GCs decreases, and severe architectural changes occurs [7]. In a previous study, we demonstrated the Epirubicin-induced mucosal barrier injury of the gastrointestinal tract in mice [8], but up to date, there are any pathoclinical observations of an intestinal changes, following a standard antineoplastic Epirubicin-based protocol applied to oncohematological patients.

Aim

The aim of this study was to assess the consequences of Epirubicin-based therapy on GCs and mucus production on colonic mucosa, immediately and after short-time of chemotherapy administration, by clinical and histopathological methods. We also evaluated the effects of this chemotherapy on GC composition and distribution, alongside with mucin accumulation.

☒ Patients, Materials and Methods

Oncohematological patients – clinical evaluation and colonic biopsies

Thirty oncohematological patients in the records of the Clinic of Hematology, Emergency County Hospital of Arad, Romania, having 182 applications of standard antineoplastic Epirubicin-based protocol, were included. The ethical approval of the Research Ethics Commission of the Emergency County Hospital of Arad was received. All data was collected between June 2015 and September 2016. Nine of the oncohematological patients were biopsied after cumulating 6–8 cycles of chemotherapy, 48 hours, and three weeks, after the last Epirubicin administration. The control was represented by healthy volunteers.

Alcian Blue (pH 2.5)–Periodic Acid–Schiff (PAS) staining

The proximal and distal sigmoid colonic biopsies taken at 20 cm and 40 cm from the anus were flushed with chilled sterile, distilled water, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by dehydration in gradients of ethanol concentrations, then clarification in toluene, embedding in paraffin, and sliced into 5 μm -thick paraffin sections.

Sections were stained in Alcian Blue (pH 2.5) for 30 minutes, then in sodium tetraborate solution for 10 minutes and oxidized in Periodic Acid solution. After washing, the sections were immersed in Schiff reagent, according to Hotchkiss–McManus, and leave to act 20 minutes and two minutes in potassium metabisulfite solution. The nuclei were stained with Hematoxylin. Slides were dehydrated through ascending alcohols, cleared, mounted, and examined to Olympus BX43 microscope. The acidic mucins were stained in blue turquoise with Alcian Blue (pH 2.5), while the neutral mucins were colored magenta red with Schiff reagent. Mixtures of the two mucins will appear from purple to dark blue, due to the positive reactions with both Alcian Blue and Schiff reagent.

To determine the effect of Epirubicin on intestinal mucus secretion, GCs were counted in the colonic crypts, as previously described [8].

Immunohistochemistry

Colonic sections of 5 μm thick have been deparaffined and rehydrated in a gradual series of alcohols. After PBS washing, slides were subjected to heat-mediated antigen retrieval in citrate buffer (pH 6.5). The endogenous peroxidase activity was blocked, and slides were incubated overnight, at 4°C, with the primary antibodies [MUC2, Santa Cruz Biotechnology, sc-15334, 1:100 dilution; MUC4, Abcam, ab60720, 3 $\mu\text{g}/\text{mL}$; tumor necrosis factor- α (TNF- α), ab270264, 1:100 dilution] on the experimental sections. Immunoexpressions were seen employing a Novocastra kit (Leica Biosystem, Germany), according to the manufacturer's instructions.

Electron microscopy

For electron microscopy examination, colonic biopsies were prefixed in 2.7% glutaraldehyde solution in 0.1 M

PBS, washed in PBS and post-fixed in 2% osmic acid solution, dehydrated in acetone and embedded in the epoxy resin. Double counterstaining was done with uranyl acetate solution and lead citrate and ultrathin sections were analyzed with FEI Tecnai 12 Biotwin transmission electron microscope (TEM).

Statistical data analysis

One-way analysis of variance (ANOVA) (Stata 13 software, StataCorp, USA) was applied for statistical analysis of the data. A value of $p < 0.05$ was considered to be statistically significant.

☒ Results

Clinical evaluation of the early symptoms of mucositis in oncohematological patients treated with Epirubicin

Symptoms as nausea were recorded in 112 (61%) of chemotherapy applications, in 15 patients, grade 2–3 [according to *Common Terminology Criteria for Adverse Events* (CTCAE)]. The frequency of nausea was higher during the first cycle of therapy, and Adriamycin–Bleomycin–Vinblastine–Dacarbazine (ABVD) therapy induced nausea at each application. Each patient had at least one episode of nausea during chemotherapy.

Post-chemotherapy diarrhea was registered after 10% of the applications of chemotherapy, grade 1–2 patients who were biopsied during this experiment had nausea ($n=2$, number of chemotherapy administrations: 12), without vomiting or diarrhea.

Effect of Epirubicin-based chemotherapy on GC composition and distribution

At 48 hours following chemotherapy treatment, most of the mucins were acidic, and the GCs were dilated (Figure 1), compared to the control one (Figure 2). Three weeks following chemotherapy administration, GCs remain dilated, and the mucous secretion increased, whereas the mucins were mainly mixed, with a few neutral mucins (Figure 3). These morphological characteristics of the GCs were confirmed by electron microscopy (Figure 4).

Effect of Epirubicin-based chemotherapy on mucin discharge

To determine the effect of Epirubicin on mucus secretion, GCs were counted in colonic crypts on Alcian Blue (pH 2.5)–PAS slides. Epirubicin-based chemotherapy caused marked decreases in the number of mucus cells. Total GCs in the colon of untreated patients numbered 40.6 ± 0.54 cells per crypt. A slight decrease in total GCs appeared early after therapy (48 hours) compared to control and increased significantly to week three post-therapy (Table 1).

Table 1 – Colonic GC density in colonic crypts from control and oncohematological patients 48 hours and three weeks following Epi-based chemotherapy ($n=10$)

Patients	Control	Epi 48 hours	Epi three weeks
Colonic mucosa	40.4 \pm 2.07	38 \pm 1.22	39.6 \pm 1.14

Epi: Epirubicin; GC: Goblet cell. Counts expressed as mean \pm standard deviation (SD).

Effect of Epirubicin-based chemotherapy on the immunoeexpression of mucins

The mucins represent the main protein secretion of GCs. Therefore, we analyzed the immunohistochemical (IHC) expression of gel-forming MUC2 and membrane-bound MUC4. Both of MUC2 and MUC4 immunostaining appeared reduced in both of the sigmoid segments from

of the oncohematological patients 48 hours following Epirubicin-based chemotherapy, compared to control (Figures 5, 6 and 9). After three weeks of chemotherapy cessation, MUC2 immunoeexpression was restored, whereas MUC4 immunoeexpression remained below the control (Figures 7, 8 and 10).

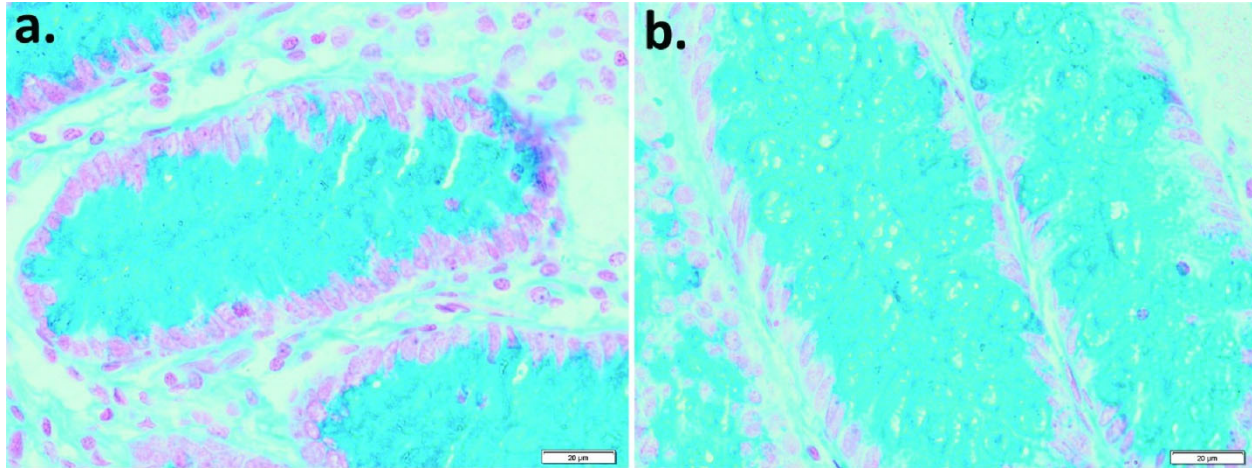


Figure 1 – Alcian Blue–PAS staining in the proximal (a) and distal (b) sigmoid colon of the oncohematological patients 48 hours following Epirubicin-based chemotherapy. Scale bar: 20 μm . PAS: Periodic Acid–Schiff.

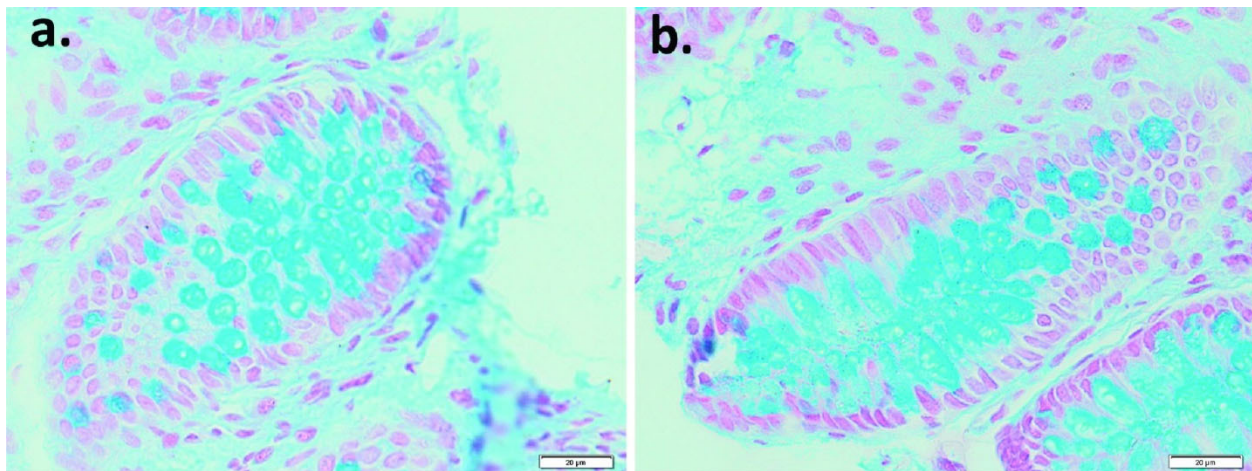


Figure 2 – Alcian Blue–PAS staining in the proximal (a) and distal (b) sigmoid colon of the controls. Scale bar: 20 μm . PAS: Periodic Acid–Schiff.

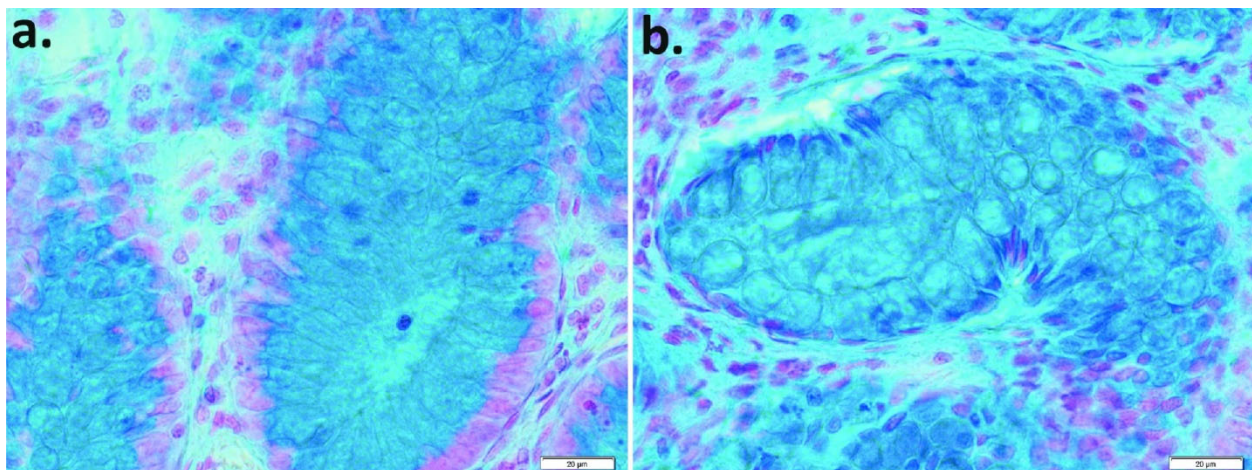


Figure 3 – Alcian Blue–PAS staining in proximal (a) and distal (b) sigmoid colon of the oncohematological patients three weeks following Epirubicin-based chemotherapy. Scale bar: 20 μm . PAS: Periodic Acid–Schiff.

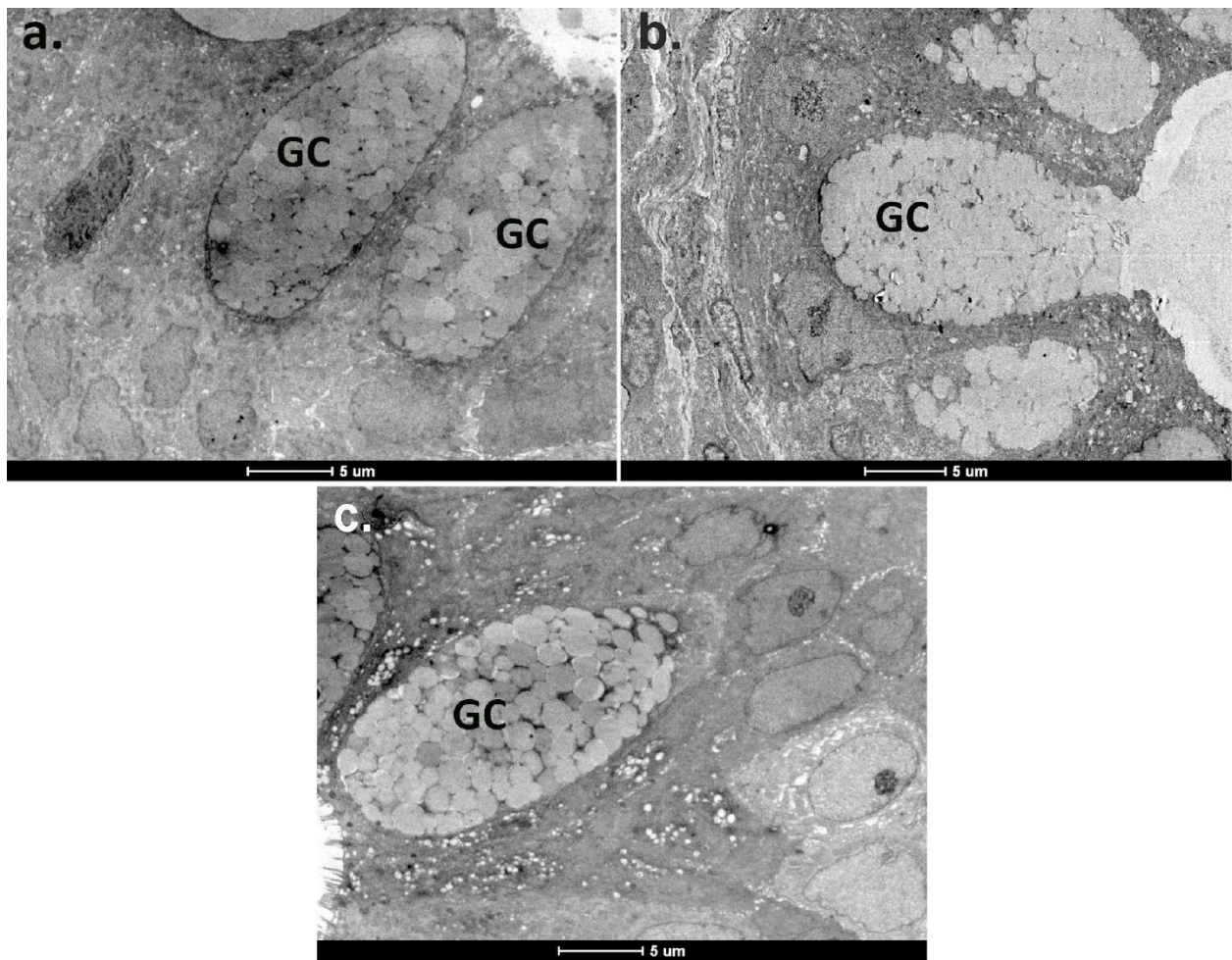


Figure 4 – Ultrastructural morphology in the GCs in the colonic mucosa of the control (a), oncohematological patients 48 hours following Epirubicin-based chemotherapy (b), and three weeks after the last administered dose (c). Scale bar: 5 µm. GC: Goblet cell.

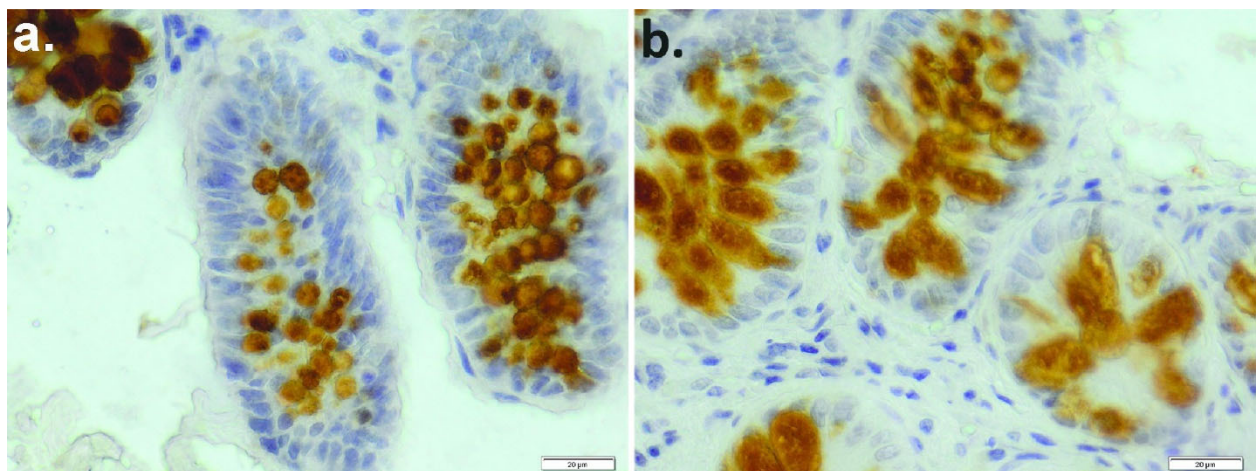


Figure 5 – MUC2 immunostaining in the proximal (a) and distal (b) sigmoid colon of the controls. Scale bar: 20 µm. MUC2: Mucin 2.

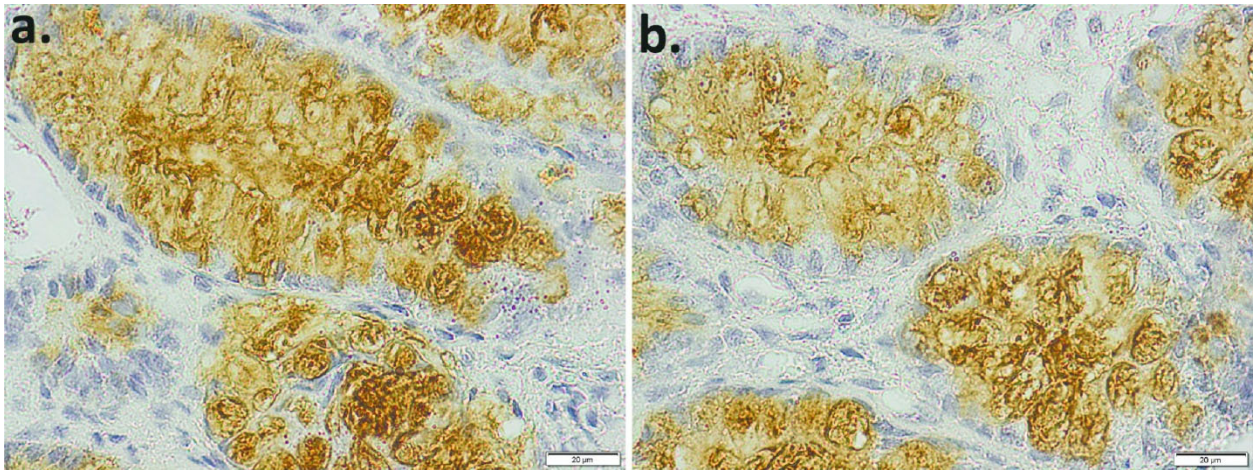


Figure 6 – *MUC2 immunostaining in the proximal (a) and distal (b) sigmoid colon of the oncohematological patients 48 hours following Epirubicin-based chemotherapy. Scale bar: 20 μm. MUC2: Mucin 2.*

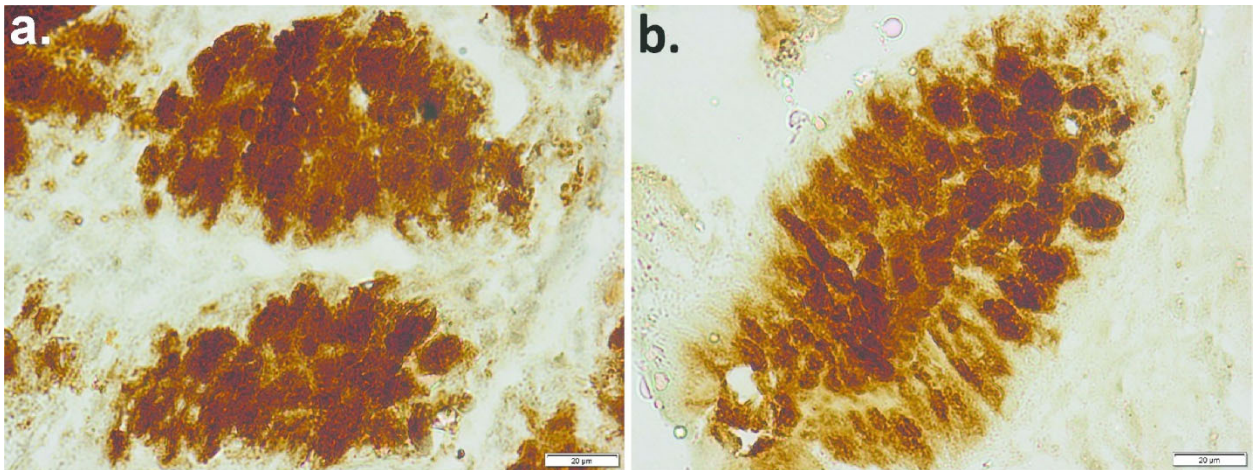


Figure 7 – *MUC2 immunostaining in the proximal (a) and distal (b) sigmoid colon of the oncohematological patients three weeks following Epirubicin-based chemotherapy. Scale bar: 20 μm. MUC2: Mucin 2.*

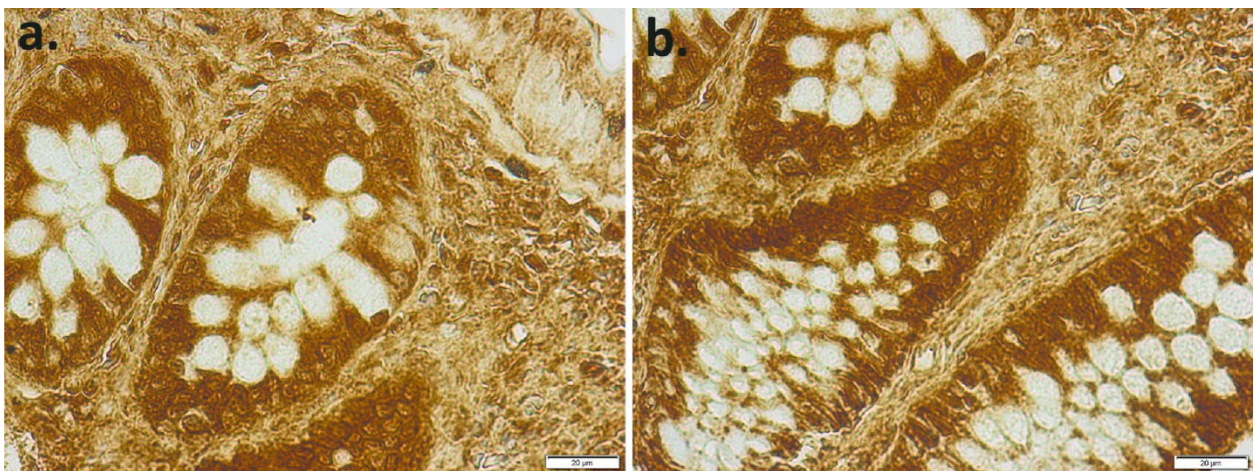


Figure 8 – *MUC4 immunostaining in the proximal (a) and distal (b) sigmoid colon of the controls. Scale bar: 20 μm. MUC4: Mucin 4.*

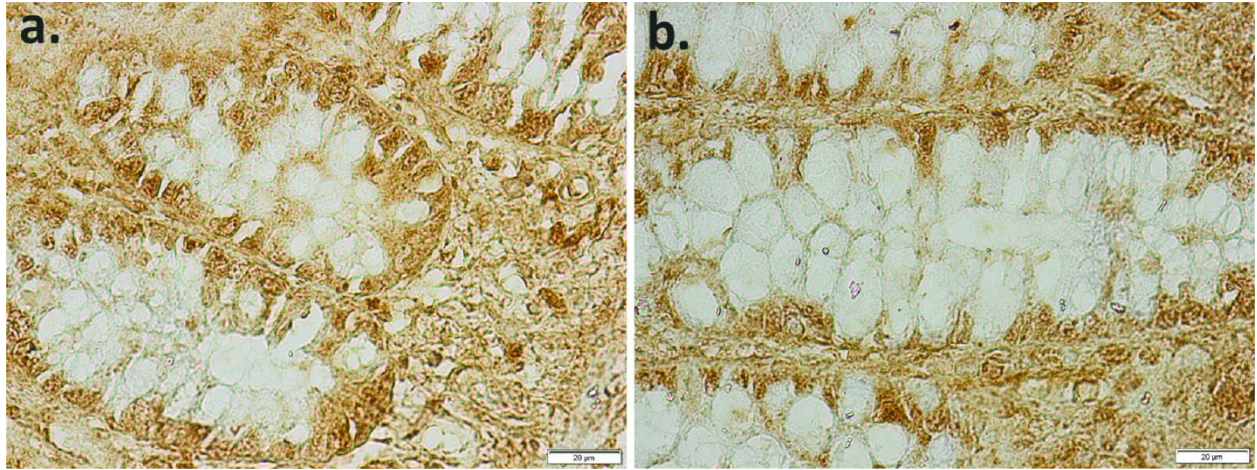


Figure 9 – MUC4 immunostaining in the proximal (a) and distal (b) sigmoid colon of the oncohematological patients 48 hours following Epirubicin-based chemotherapy. Scale bar: 20 µm. MUC4: Mucin 4.

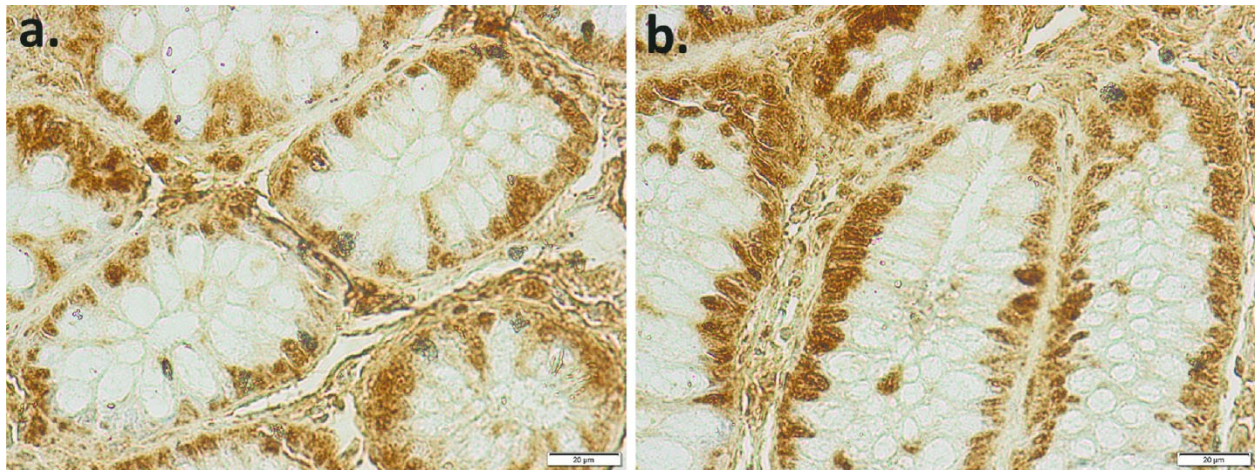


Figure 10 – MUC4 immunostaining in the proximal (a) and distal (b) sigmoid colon of the oncohematological patients three weeks following Epirubicin-based chemotherapy. Scale bar: 20 µm. MUC4: Mucin 4.

Effect of Epirubicin-based chemotherapy on the TNF- α immunoexpression

Since mucus production by the GCs is highlighted by the inflammation, IHC expression of TNF- α pro-inflammatory cytokine was analyzed. TNF- α immuno-

staining was marked 48 hours following Epirubicin-based chemotherapy, compared to control (Figures 11 and 12). After three weeks of chemotherapy cessation, TNF- α immunoexpression was very much reduced (Figure 13).

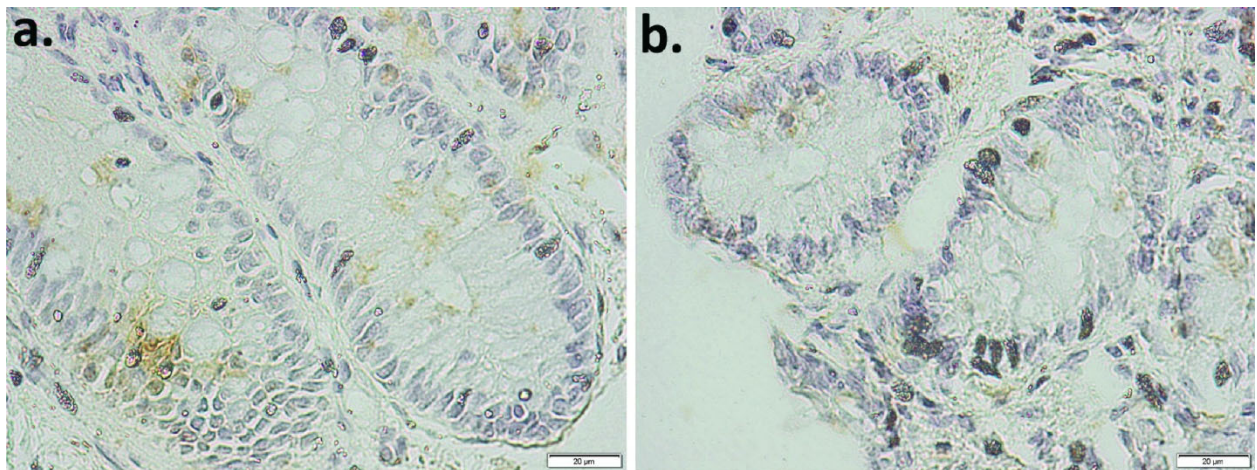


Figure 11 – TNF- α immunostaining in the proximal (a) and distal (b) sigmoid colon of the controls. Scale bar: 20 µm. TNF- α : Tumor necrosis factor-alpha.

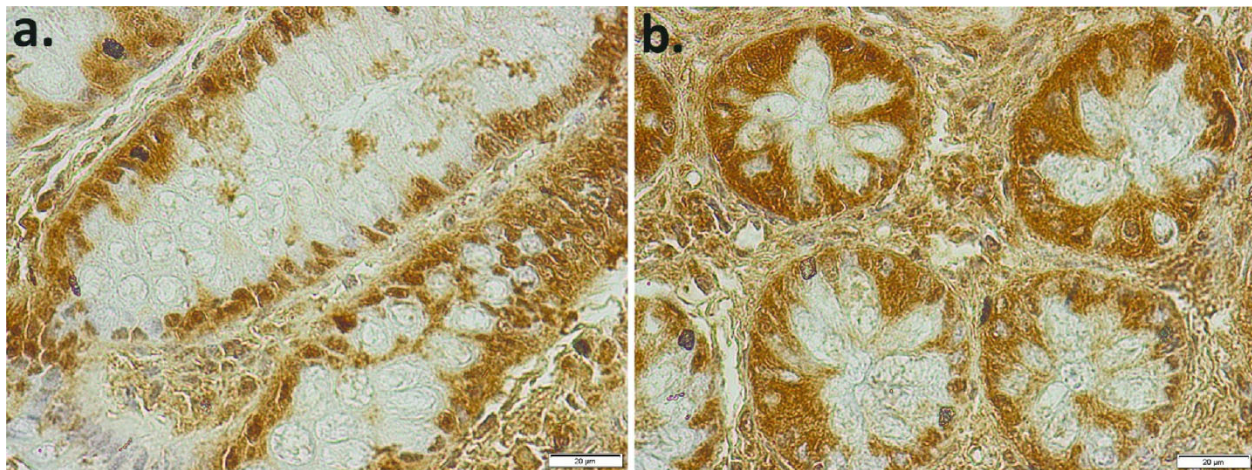


Figure 12 – TNF- α immunostaining in the proximal (a) and distal (b) sigmoid colon of the oncohematological patients 48 hours following Epirubicin-based chemotherapy. Scale bar: 20 μ m. TNF- α : Tumor necrosis factor-alpha.

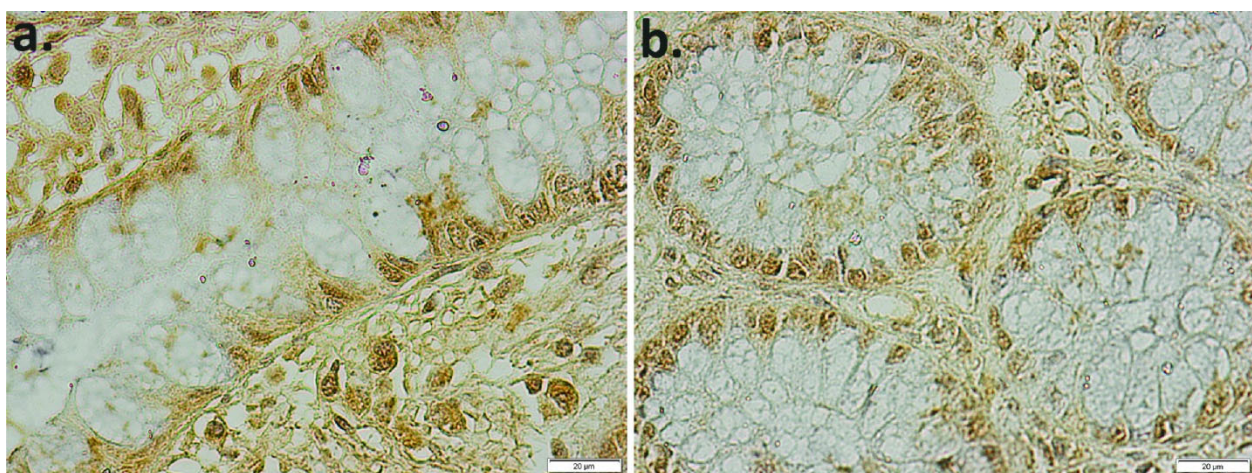


Figure 13 – TNF- α immunostaining in the proximal (a) and distal (b) sigmoid colon of the oncohematological patients three weeks following Epirubicin-based chemotherapy. Scale bar: 20 μ m. TNF- α : Tumor necrosis factor-alpha.

☒ Discussions

GCs are exocrine cells that secrete mucins, and their numbers increase in the large intestine, from the cecum to the rectum [3]. After chemotherapy, colonic GCs exhibited various staining pattern and increased mucus secretion [7]. Moreover, GCs number decrease, as we recently reported on Epirubicin-induced mucositis in mice gastrointestinal tract [8]. Stringer *et al.* [9] showed significant changes in GC numbers both in the small and large intestines of rats treated with Irinotecan.

Intestinal mucins are glycoproteins consisting in oligosaccharide chains attached by covalent bonds to proteins.

Mucin synthesis involves the activity of glycosyl transferases, assuring the addition of monosaccharides [10]. The secretory mucins of the small intestine contain hexosamines and sialic acid. By sulfating of specific saccharides, mucins become acidic [11] and appear to be less degradable by bacterial glycosidases and proteases [12]. Acidic mucins have been shown to protect against the transluminal translocation of bacteria [12]. In our study, we noticed that composition of mucins slightly changed from acid to mixed and few neutral, which makes the mucosa more vulnerable to pathogens.

MUC2 is the main secretion of GCs in the colonic mucosa and small intestine, while MUC4 is expressed in normal stomach and colon [13]. MUC2 and MUC4 immunoexpressions decreased in the colonic mucosa 48 hours after the administration of Epirubicin-based treatment and almost restored after three weeks of chemotherapy cessation. This indicates that Epirubicin causes a net increase in mucous production, despite a slightly decrease in GCs number, whereas the immunoexpression of MUC2 and MUC4 in the large intestine became injured following chemotherapy and restored almost to the level of control in three weeks after the last doses administered. Irinotecan has been shown to induce GC depletion and MUC2 immunoexpression to be decreased during villous atrophy in the small intestine [14]. Meanwhile, we observed that the increased mucous secretion is associated with highly expressed TNF- α at 48 hours. Previous data have been noticed that inflammatory cytokines as interleukins (ILs) and TNF- α stimulate the rapid release of mucin from cells [12]. Other study revealed the relationship between intestinal mucositis induced by chemotherapy and the increased level of cytokines, as IL-6, TNF- α [15, 16, 17]. Previous results have suggested a relationship between increased mucus secretion and altered mucin immunoexpression and may contribute to

chemotherapy-induced diarrhea [18]. Clinical studies noticed grade 3/4 diarrhea to 32% of treated patients with 5-Fluorouracil (5-FU) bolus, 16–22% with Irinotecan and 4% with Docetaxel/Paclitaxel. Overall, is considered that approximately 10% patients with advanced cancer will be affected consequently a chemotherapy treatment [10].

☒ Conclusions

In this study, we have assessed the mucus production, composition, and distribution by Alcian Blue (pH 2.5)–PAS staining, alongside with the immunoeexpression of MUC2, MUC4 and inflammatory markers in a series of oncohematological patients, immediately and after short-time of Epirubicin-based chemotherapy cumulative therapy cessation. We showed that GCs number decrease slightly at 48 hours, while mucous secretion became mixed (with a few neutral) after three weeks. Overall, the secretion was increased immediately after the Epirubicin administration, due to the activation of inflammatory pathways, assessed by increased TNF- α immunostaining at 48 hours. MUC2 and MUC4 showed a decreased immunoeexpression at 48 hours after the Epirubicin administration compared to controls and partially restored three weeks after the cessation. Overall, it is highly plausible that all these key players revolve around the chemotherapy-induced mucositis in oncohematological patients and highlights the morphofunctional particularities of the GCs, which further modulates the clinical outcome of the patient.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Coralia Adina Cotoraci, Alciona Sasu, and Eftimie Miușescu have equal contributions to the study.

References

- [1] Thorpe D, Stringer A, Butler R. Chemotherapy-induced mucositis: the role of mucin secretion and regulation, and the enteric nervous system. *NeuroToxicology*, 2013, 38:101–105. <https://doi.org/10.1016/j.neuro.2013.06.007> PMID: 23827812
- [2] Robbe C, Capon C, Coddeville B, Michalski JC. Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. *Biochem J*, 2004, 384(Pt 2): 307–316. <https://doi.org/10.1042/BJ20040605> PMID: 15361072 PMID: PMC1134114
- [3] Specian RD, Oliver MG. Functional biology of intestinal goblet cells. *Am J Physiol*, 1991, 260(2 Pt 1):C183–C193. <https://doi.org/10.1152/ajpcell.1991.260.2.C183> PMID: 1996606
- [4] Smirnov A, Perez R, Amit-Romach E, Sklan D, Uni Z. Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. *J Nutr*, 2005, 135(2):187–192. <https://doi.org/10.1093/jn/135.2.187> PMID: 15671211
- [5] Zhang K, Horneff MW, Dupont A. The intestinal epithelium as guardian of gut barrier integrity. *Cell Microbiol*, 2015, 17(11): 1561–1569. <https://doi.org/10.1111/cmi.12501> PMID: 26294173
- [6] Keefe DM. Intestinal mucositis: mechanisms and management. *Curr Opin Oncol*, 2007, 19(4):323–327. <https://doi.org/10.1097/CCO.0b013e3281214412> PMID: 17545794
- [7] Gibson RJ, Bowen JM, Inglis MRB, Cummins AG, Keefe DMK. Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. *J Gastroenterol Hepatol*, 2003, 18(9):1095–1100. <https://doi.org/10.1046/j.1440-1746.2003.03136.x> PMID: 12911669
- [8] Sasu A, Herman H, Mariasiu T, Rosu M, Balta C, Anghel N, Miutescu E, Cotoraci C, Hermenean A. Protective effects of Silymarin on Epirubicin-induced mucosal barrier injury of the gastrointestinal tract. *Drug Chem Toxicol*, 2015, 38(4):442–451. <https://doi.org/10.3109/01480545.2014.992072> PMID: 25609004
- [9] Stringer AM, Gibson RJ, Bowen JM, Logan RM, Ashton K, Yeoh ASJ, Al-Dasooqi N, Keefe DMK. Irinotecan-induced mucositis manifesting as diarrhoea corresponds with an amended intestinal flora and mucin profile. *Int J Exp Pathol*, 2009, 90(5):489–499. <https://doi.org/10.1111/j.1365-2613.2009.00671.x> PMID: 19765103 PMID: PMC2768147
- [10] Stringer AM, Gibson RJ, Bowen JM, Logan RM, Yeoh ASJ, Keefe DMK. Chemotherapy-induced mucositis: the role of gastrointestinal microflora and mucins in the luminal environment. *J Support Oncol*, 2007, 5(6):259–267. PMID: 17624050
- [11] Jass JR, Walsh MD. Altered mucin expression in the gastrointestinal tract: a review. *J Cell Mol Med*, 2001, 5(3):327–351. <https://doi.org/10.1111/j.1582-4934.2001.tb00169.x> PMID: 12067494 PMID: PMC6517815
- [12] Deplancke B, Gaskins HR. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am J Clin Nutr*, 2001, 73(6):1131S–1141S. <https://doi.org/10.1093/ajcn/73.6.1131S> PMID: 1139319
- [13] Choudhury A, Moniaux N, Winpenny JP, Hollingsworth MA, Aubert JP, Batra SK. Human MUC4 mucin cDNA and its variants in pancreatic carcinoma. *J Biochem*, 2000, 128(2): 233–243. <https://doi.org/10.1093/oxfordjournals.jbchem.a022746> PMID: 10920259
- [14] Verburg M, Renes IB, Meijer HP, Taminiu JA, Büller HA, Einerhand AW, Dekker J. Selective sparing of goblet cells and Paneth cells in the intestine of Methotrexate-treated rats. *Am J Physiol Gastrointest Liver Physiol*, 2000, 279(5):G1037–G1047. <https://doi.org/10.1152/ajpgi.2000.279.5.G1037> PMID: 11053002
- [15] Araújo RS, de Barros ALB. Intestinal mucositis induced by chemotherapy: an overview. *J Mol Pharm Org Process Res*, 2015, 3(3):e123. <https://doi.org/10.4172/2329-9053.1000e123>
- [16] Logan RM, Stringer AM, Bowen JM, Yeoh AS, Gibson RJ, Sonis ST, Keefe DM. The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: pathobiology, animal models and cytotoxic drugs. *Cancer Treat Rev*, 2007, 33(5):448–460. <https://doi.org/10.1016/j.ctrv.2007.03.001> PMID: 17507164
- [17] Beutheu S, Ouelaa W, Guérin C, Belmonte L, Aziz M, Tonnoune N, Bôle-Feysot C, Galas L, Déchelotte P, Coëffier M. Glutamine supplementation, but not combined glutamine and arginine supplementation, improves gut barrier function during chemotherapy-induced intestinal mucositis in rats. *Clin Nutr*, 2014, 33(4):694–701. <https://doi.org/10.1016/j.clnu.2013.09.003> PMID: 24095638
- [18] Stein A, Voigt W, Jordan K. Chemotherapy-induced diarrhea: pathophysiology, frequency and guideline-based management. *Ther Adv Med Oncol*, 2010, 2(1):51–63. <https://doi.org/10.1177/1758834009355164> PMID: 21789126 PMID: PMC3126005

Corresponding author

Coralia Adina Cotoraci, Professor, MD, PhD, Department of Hematology, Faculty of Medicine, Vasile Goldiș Western University of Arad, 94–96 Revoluției Avenue, 310025 Arad, Romania; Phone +40257–280 260, e-mail: ccotoraci@yahoo.com