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# Increased Severity of Ulcerative Colitis in the Terminal Phase of the Metabolic Syndrome

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Ulcerative colitis is chronic immune-mediated disorder that affects primarily colonic mucosa. The metabolic syndrome has increasing global prevalence with a significant impact on biology of chronic diseases, such as ulcerative colitis. Today it is known that the metabolic syndrome attenuates severity of ulcerative colitis. Still, there is no evidence that different stages of metabolic syndrome alter the course of the ulcerative colitis. The aim of this study was to dissect out how progression of the metabolic syndrome impacted the biology of ulcerative colitis and severity of clinical presentation. Seventy-two patients (41 men and 31 women, 22-81 years old) were enrolled in this observational cross-sectional study. Concentrations of pro- and anti-inflammatory cytokines in serum and feces samples were measured and phenotype of colon infiltrating cells was analyzed. Patients in the terminal phase of the metabolic syndrome have clinically and pathohistologically more severe form of ulcerative colitis, which is followed by decreased concentrations of systemic galectin-1, increased values of systemic pro-inflammatory mediators and increased influx of lymphocytes in affected colon tissue. Our data suggest that reduced concentrations of galectin-1 and predomination of the pro-inflammatory mediators in patients with terminal stage of the metabolic syndrome enhance local chronic inflammatory response and subsequent tissue damage, and together point on important role of galectin-1 in immune response in ulcerative colitis patients with the metabolic syndrome.

**Keywords:** disease severity; immune response; metabolic syndrome progression; ulcerative colitis
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## Introduction

Ulcerative colitis (UC) is chronic, immune-mediated disorder that affects the gastrointestinal mucosa. The pathological process begins in the intestinal lamina propria of the rectum and spread further into the colon. The etiology of UC involves interaction between environmental factors and genetic predisposition (Thompson-Chagoyan et al. 2005). Abnormal immune response to intestinal flora seems to play the crucial role in pathogenesis of UC (DuPont and DuPont 2011; Huang and Chen 2016). UC is associated with many other diseases such as psoriasis, hypothyroidism, lupus and the metabolic syndrome (MetS) (Hemminki et al. 2010; Yorulmaz et al. 2011). The most common co-morbidity is MetS (Nagahori et al. 2010; Maconi et al. 2014).

MetS has global prevalence with a significant impact on human health and development of chronic diseases (Rao et al. 2014). MetS strongly affects the immune system of

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the host. There were registered cases of unsuccessful vaccination and complications of infections in patients suffering from MetS, indicating on suppressive effect of MetS on the immune response (Milner and Beck 2012; Paragh et al. 2014; Andersen et al. 2016). Recent studies confirmed significant impact of MetS on the immune response as well as on many disorders, making its co-morbidity with UC particularly interesting (Nagahori et al. 2010; Yorulmaz et al. 2011; Maconi et al. 2014). UC outcome in patients with MetS is something worth paying attention to (Dragasevic et al. 2020).

Recently, we revealed that patients with MetS have milder form of UC (Jovanovic et al. 2019). It appears that MetS attenuates colon inflammation that is diagnosed as clinically and endoscopically milder disease. However, there is still no evidence to clarify whether and how MetS progression affects severity of UC. The aim of our study was to dissect out how progression of MetS affected the biology of UC and severity of clinical presentation. With this study, we came to the evidence that patients in terminal stage of MetS have more severe form of UC and also have systemic predomination of pro-inflammatory cytokines over galectin-1 and subsequent intense accumulation of natural killer (NK) and $CD8^+$ T cells in lamina propria of affected colon tissue.

## Materials and Methods

*Compliance with ethics guidelines*

The study was performed at the Center for Gastroenterology, Clinical Center of Kragujevac and the Center for Molecular Medicine and Stem Cell Research at the Faculty of Medical Sciences, University of Kragujevac, Serbia. The Ethics Committees of these institutions approved the study. Furthermore, the Principle of Good Clinical Practice and the Declaration of Helsinki were adhered to at all times. Written informed consent was obtained from every patient for the analysis of blood and tissues. All patients were constantly supervised by medical staff at the Clinical Center Kragujevac.

*Population*

In this observational and cross-sectional study, we have included UC patients with MetS. Seventy-two patients, comprising 41 men and 31 women aged 21-81 years, were enrolled. All of them had UC, histologically confirmed at the beginning, as well as MetS. Demographic and clinical data of every participant in the study were imported into the database using the SPSS Statistical Analysis Software. Excluding criteria for patients were: previously diagnosed colorectal cancer, Crohn's disease or UC previously treated with antibiotics, aminosalicylates, corticosteroids, immunosuppressants, statins and biological therapy. Physical examination, routine laboratory tests, and diagnostic imaging, such as chest X-ray, ultrasound and computed tomography scan of abdominal and endoscopy, were performed for every patient.

*Metabolic syndrome staging*

In line with Adult Treatment Panel (ATP) III criteria for MetS diagnosis, the minimal requirement for patients was to have 3 of the 5 following disorders, in order to be diagnosed with MetS: dysglycemia (fasting plasma glucose > 5.5 mM and/or 2 h-post load plasma glucose > 7.8 mM or actively treated dysglycemia), arterial blood pressure (arterial tension > 130/85 mmHg or actively treated), central type of obesity and low high-density lipoprotein (HDL) cholesterol and high triglycerides values (Huang 2009). All of the patients included in this study met the criteria entirely. Patients with MetS were further divided into 4 groups, based on plasma glucose levels and plasma insulin levels during oral glucose tolerance testing (OGTT). These groups correspond with the developmental stages of MetS: group I) normal tolerance of glucose (fasting plasma glucose lower than 5.5 mM and plasma glucose lower than 7.8 mM at 120 min of OGTT) and normoinsulinemia; group II) normal tolerance of glucose and hyperinsulinemia; group III) pathological tolerance of glucose and hyperinsulinemia; and group IV) pathological tolerance of glucose and normo/hypoinsulinemia. Stage IV of MetS characterizes impaired glucose tolerance and normo- or hypoinsulinemia. Longstanding insulin resistance leads to diminished insulin production from the pancreas, resulting in hypoinsulinemia and elevated blood glucose concentrations, features of type 2 diabetes also (Nathan et al. 2004).

*Evaluation of UC severity*

UC severity was diagnosed and assessed using histological and clinical scores in all individual cases (Truelove and Witts 1955; Geboes et al. 2000; Rutgeerts et al. 2005; Pineton de Chambrun et al. 2010; Magro et al. 2017). In order to provide consistent evaluation of the mucosa, the same endoscopist (NZ) committed every endoscopy. The Mayo endoscopic sub-score, with scores between 0 and 3, consisting of erosions/ulcerations, mucosal erythema, vascular pattern visibility and provoked/spontaneous bleeding, was used to determine the endoscopic lesions severity (Rutgeerts et al. 2005; Magro et al. 2017).

The Truelove and Witts clinical activity index and the Mayo clinical index were used for the evaluation of the clinical score (Truelove and Witts 1955; Gomollon et al. 2013; Walsh et al. 2014). The Truelove and Witts severity index can be valorized as mild, moderate and severe based on frequency of defection, rectal bleeding, pyrexia, tachycardia, anemia and erythrocyte sedimentation rate (Truelove and Witts 1955). Mayo clinical index was calculated taking into account frequency of defection, rectal bleeding, endoscopic finding and general assessment by physician and is expressed numerically from 0 to 3 (Gomollon et al. 2013; Walsh et al. 2014). The Geboes grading was used for the evaluation of the histological score (Geboes et al. 2000). Histology scoring was performed on biopsies taken from the most inflamed area of the colon, 10-40 cm from the anal verge. Two pathologists blindly inspected the histological

sections independently of one another by examining the architectural changes, crypt destruction, mucosal membranes erosion, eosinophilic infiltration, neutrophilic infiltration and chronic inflammatory infiltration. In line with the Montreal classification of the UC lesions localization, such as E1 (proctitis), E2 (left-sided colitis) or E3 (pancolitis), we have assorted the UC patients (Satsangi et al. 2006). Evaluation of noticeable extraintestinal manifestations included fatty liver, primary sclerosing cholangitis, cholelithiasis, dermatological manifestations (pyoderma gangrenosum or erythema nodosum), and changes in bone, joints, hematopoiesis, the reproductive system and eyes.

*Measurement of cytokines levels in serum and feces*

As described in our previous study, the blood and feces were collected from UC patients with MetS at 8:00 a.m. (Jovanovic et al. 2019). Sera and liquid feces fraction were separated and stored at −80°C before use. The commercially available ELISA tests were used following the instructions of the manufacturer (R&D Systems; Minneapolis, MN, USA) to measure the concentrations of tumor necrosis factor alpha (TNF-$\alpha$), interleukin-6 (IL-6), IL-17, C-X-C Motif Chemokine Ligand 8 (CXCL8), Galectin-1 (Gal-1) and Galectin-3 (Gal-3) in serum and feces samples.

*Analysis of colon infiltrating cells by flow cytometry*

As described previously, immune cells of the colon were isolated from all patients included in the study (Rogler et al. 1998; Acovic et al. 2018; Jovanovic et al. 2019). After the biopsy, tissue samples were washed and incubated for 10 minutes in medium with 1 mM EDTA at 37°C. In order to remove intestinal epithelial cells, gentle shaking was also performed. After that, specimens were incubated in 2 ml of Dulbecco's Modified Eagle Medium (DMEM) (Lonza; Basel, Swissland) along with 1 mg/ml type I collagenase (Sigma-Aldrich; St. Louis, MO, USA), 0.1 mg/ml DNAse (Sigma-Aldrich) and 1 mg/ml hyaluronidase (Sigma-Aldrich) without fetal bovine serum (FBS) at 37°C for 20 to 30 minutes. The cells were centrifuged with a gradient of Ficoll density at 690 g for 20 minutes. After removing of the interphase, the suspensions of cells were washed two times using FACS medium.

Flow cytometry was performed with $1 \times 10^6$ cells per sample incubated with antibodies against human CD3 (a pan-marker of T cells), CD4 (a marker of Th cells), CD8 (a marker of cytotoxic T cells), CD19 (B cells marker) and CD56 (NK cell marker) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin Chlorophyll A Protein (PerCP) and allophycocyanin (APC), respectively (BD Pharmingen; San Diego, CA, USA). Intracellular staining was performed after the treatment of cells with phorbol myristate acetate (PMA) and ionomycin for 4 hours at 37°C in the presence of 1 $\mu$g/ml of Golgi plug. Furthermore, for IL-10 and forkhead box P3 (Foxp3, transcription factor specific for regulatory T cells), we used the fixation/permeabilization buffer kit (BD Biosciences; San Jose, CA, USA). We have performed the flow cytometry using FACSCalibur (BD Biosciences; San Jose, CA, USA), and data analyses using FlowJo (Tree Star).

*Statistical analysis*

Statistical Analysis Software IBM SPSS (version 23.0) was used for performing all data analyses. The significance tests used for suitable purposes were Mann–Whitney U test, one-way ANOVA or Kruskal–Wallis test with appropriate post-hoc test (Tukey's honestly significant difference (HSD) post-hoc test, or a series of Mann–Whitney U tests). Data were shown as mean ± standard error of mean, and the significant difference was $p < 0.05$.

## Results

*Clinical feature in patients with ulcerative colitis and the metabolic syndrome*

All of recruited UC patients met the criteria for MetS, according to ATP III criteria for the diagnosis of MetS (Huang 2009). Among them, 41 (56.94%) were male. The mean age was 55.5 years. UC patients with MetS were divided into 4 groups, based on plasma glucose level and plasma immunoreactive insulin level during oral glucose tolerance testing (OGTT), that corresponded with the developmental stages of MetS: I) normal glucose tolerance and normoinsulinemia; II) normal glucose tolerance and hyperinsulinemia; III) pathological glucose tolerance and hyperinsulinemia; IV) pathological glucose tolerance and normo/hypoinsulinemia. Fig. 1 shows the movement of anthropological and metabolic parameters during the developmental stages of the MetS (plasma glucose level and plasma immunoreactive insulin level during OGTT, HOMA-RI and HOMA-beta). Patients with MetS were at the upper limit of normal nutrition (data not shown). Mean plasma glucose concentrations reached their highest values in 60 min of OGTT, with the lowest values recorded in the group I (Fig. 1A). Still, plasma glucose levels were significantly lower in the group I than in the group III ($p < 0.001$) and group IV ($p < 0.001$), and there was significant difference between the groups III and IV ($p = 0.037$). This trend continued until the end of oral glucose load test (Fig. 1A). Almost the same trend of plasma immunoreactive insulin level dynamic and the ratio between insulin resistance parameters and beta cell function were detected during the OGTT test as with plasma glucose level (Fig. 1B, C). Clinical and pathohistological parameters of UC, parameters of systemic and local immune response, cellular composition and functional phenotype of the infiltrating leukocytes in lamina propria were monitored in groups as defined herein.

Baseline characteristics, laboratory findings and clinical features of all patients are included in Table 1. There were 22 patients with noticeable extraintestinal manifestations. Still, there was not any difference in proportion of patients with extraintestinal manifestations, according to MetS stage (Table 1). There was no significant difference

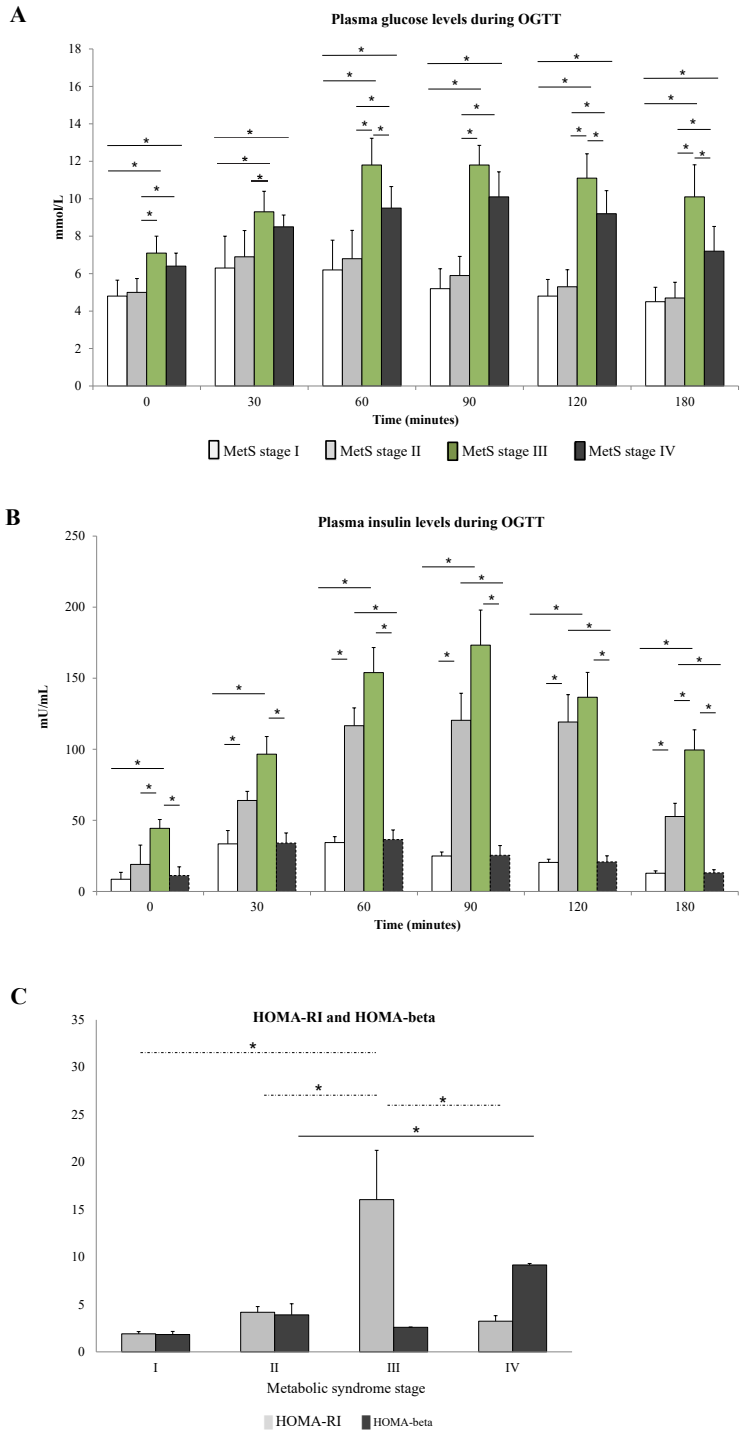


Fig. 1. Dynamic of anthropological and metabolic parameters during the progressive stages of the metabolic syndrome (MetS).

Ulcerative colitis (UC) patients with MetS were divided into 4 groups which correspond to the developmental stages of this syndrome: normal glucose tolerance and normoinsulinemia (I), normal glucose tolerance and hyperinsulinemia (II), pathological glucose tolerance and hyperinsulinemia (III) and pathological glucose tolerance and normo/hypoinsulinemia (IV). All UC MetS patients were submitted to oral glucose tolerance test and blood samples were collected for glucose and immunoreactive insulin measurements. HOMA-IR was calculated according to the formula: fasting insulin ($\mu$U/L) x fasting glucose (mmol/L)/22.5, while HOMA-beta was calculated using the following formula: 20 × fasting insulin ($\mu$U/ml)/(fasting glucose (mmol/L) − 3.5). The graphs display plasma glucose levels during OGTT (A) and plasma immunoreactive insulin levels during OGTT (B), as well as HOMA-RI and HOMA-beta indexes (C) in UC patients in all progressive stages of MetS, respectively. Results are expressed as mean ± SD. Statistical significance was tested by one-way ANOVA test with post-hoc Tukey's HSD (Honestly Significant Difference) Test.

between previously defined groups with respect to platelet count, hemoglobin, cholesterol, high density lipoprotein (HDL)-cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), urea, albumin and globulin of patients (Table 1).

*Patients in the terminal stage of the metabolic syndrome have severe form of ulcerative colitis*

Patients with UC in the stage IV of MetS have significantly higher Mayo endoscopic score ($p = 0.017$) and Truelove and Witts clinical score ($p = 0.049$) (data not shown). Twenty five percent of patients in stage IV of MetS was recorded for Mayo ES 3 as well as Truelove and Witts clinical score 3, while none of patients in the first stage of MetS was recorded for higher Mayo ES and Truelove and Witts clinical score (Fig. 2A, B), indicating that majority of the patients in stage IV of MetS have severe UC. The Mayo clinical score was also higher in patients in stage IV of MetS, but this difference did not reach statistical significance (Fig. 2C). Endoscopic findings are presented in Fig. 2D. Edematous mucosa, erythema, loss of vascular markings, and mucosal friability were present in UC patients in first developmental stages of MetS, while erosions, ulcers, frank friability and spontaneous bleeding were common in patients in stage IV of MetS (Fig. 2D).

Endoscopic findings and clinical outcome are in line with pathohistological characteristics (Fig. 3). The pathohistological parameters of UC were analyzed in all developmental stages of MetS. Patients in stage IV of the MetS have significantly more pronounced chronic inflammatory infiltrates ($p = 0.049$) (data not shown). Thirty three percent of patients in IV stage of MetS was recorded for stage 3 of chronic inflammatory infiltrates, while 10 percent of patients in stage I of MetS was recorded for stage 3 of chronic inflammatory infiltrates (Fig. 3A). The values of other pathohistological parameters of the disease severity, such as eosinophilic infiltration (Fig. 3B), neutrophil infiltration (Fig. 3C), crypt destruction (Fig. 3D), erosion of the mucous membranes (Fig. 3E), and architectural changes (Fig. 3F) in 4th stage MetS patients were also higher, but these differences did not reach statistical significance. Representative pathohistological characteristics are shown in Fig. 3G.

*Progression of the metabolic syndrome alters the systemic and local immune response in patients with ulcerative colitis*

The patients in stage IV of MetS had significantly higher systemic values of pro-inflammatory cytokine IL-6 ($p = 0.049$) and chemokine CXCL8 ($p = 0.01$), while lower

Table 1. Clinical and laboratory findings in ulcerative colitis (UC) patients.

| Characteristics | Reference range | All [n = 72] | Metabolic syndrome stages | | | | *P* value |
|---|---|---|---|---|---|---|---|
| | | | I [n = 25] | II [n = 21] | III [n = 14] | IV [n = 12] | |
| Age [years], Median [range] | | 55.5 [22-81] | 53 [23-81] | 48 [22-75] | 61.5 [42-81] | 59 [32-70] | n. s. |
| Sex: Male [percentage %] | | 41 [56.94] | 15 [60.00] | 9 [42.85] | 8 [57.14] | 9 [75.00] | n. s. |
| Female [percentage %] | | 31 [43.05] | 10 [40.00] | 12 [57.15] | 6 [42.86] | 3 [25.00] | n. s. |
| Localization [PT/LC/PC][#] | | 11/43/18 | 4/13/8 | 3/12/6 | 2/9/3 | 2/9/1 | n. s.⁂ |
| Extraintestinal manifestations [+/-] | | 22/50 | 7/18 | 9/12 | 3/11 | 3/9 | n. s.⁂ |
| HbA1c [%] | < 6.0 | 6.21 ± 2.67 | 5.04 ± 0.74 | 5.19 ± 0.68 | 9.57 ± 3.41 | 6.50 ± 3.26 | **0.001** |
| WBC [$10^9$/l] | 4-10 | 7.68 ± 0.48 | 7.83 ± 0.96 | 8.11 ± 0.76 | 6.45 ± 0.89 | 8.08 ± 1.35 | n. s. |
| Platelet [$10^9$/l] | 140-440 | 371.57 ± 11.46 | 373.32 ± 20.63 | 375.86 ± 19.21 | 376.50 ± 23.38 | 354.67 ± 34.59 | n. s. |
| Hb [g/l] | 120-140 | 125.7 ± 1.97 | 127.64 ± 3.36 | 125.95 ± 3.97 | 125.14 ± 5.23 | 128.50 ± 5.32 | n. s. |
| Cholesterol [mmol/l] | 3.1-5.2 | 5.19 ± 0.19 | 4.66 ± 0.29 | 5.52 ± 0.34 | 5.74 ± 0.52 | 5.05 ± 0.49 | n. s. |
| Triglycerides [mmol/l] | 0.1-1.7 | 1.80 ± 0.11 | 1.80 ± 0.19 | 1.68 ± 0.19 | 2.18 ± 0.30 | 1.55 ± 0.28 | n. s. |
| HDL [mmol/l] | 1.1-2.5 | 1.49 ± 0 .07 | 1.26 ± 0.11 | 1.53 ± 0.13 | 1.43 ± 0.14 | 1.38 ± 0.16 | n. s. |
| LDL [mmol/l] | 0.1-3.5 | 2.89 ± 0.18 | 2.57 ± 0.26 | 3.27 ± 0.35 | 3.32 ± 0.46 | 2.36 ± 0.50 | n. s. |
| AST [U/l] | 0-40 | 32.31 ± 2.05 | 27.80 ± 2.65 | 37.10 ± 5.21 | 36.14 ± 4.43 | 28.83 ± 2.64 | n. s. |
| ALT [U/l] | 0-40 | 35.53 ± 5.24 | 28.12 ± 2.72 | 50.14 ± 17.15 | 31.42 ± 3.72 | 30.17 ± 5.04 | n. s. |
| GGT [U/l] | < 60 | 39.53 ± 7.82 | 30.64 ± 2.97 | 61.81 ± 25.52 | 24.71 ± 4.51 | 36.50 ± 10.68 | n. s. |
| Urea [mmol/l] | 3.0-8.0 | 5.47 ± 0.32 | 5.21 ± 0.49 | 5.39 ± 0.70 | 6.44 ± 0.74 | 5.01 ± 0.68 | n. s. |
| Albumin [g/l] | 35-52 | 41.19 ± 0.39 | 40.12 ± 0.77 | 40.43 ± 0.63 | 42.29 ± 0.45 | 43.50 ± 0.88 | n. s. |
| Globulin [g/l] | 26-46 | 27.35 ± 0.42 | 27.08 ± 0.98 | 27.76 ± 0.54 | 27.43 ± 0.61 | 27.08 ± 0.97 | n. s. |

[#]PT, proctitis; LC, left-sided colitis; PC, pancolitis.

HbA1c, hemoglobin A1c; WBC, white blood cells; Hb, hemoglobin; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase.

Values are expressed as mean ± SEM. The one-way ANOVA test was applied.

⁂ The Kruskal–Wallis test was applied. n. s., no statistically significant difference ($p > 0.05$).

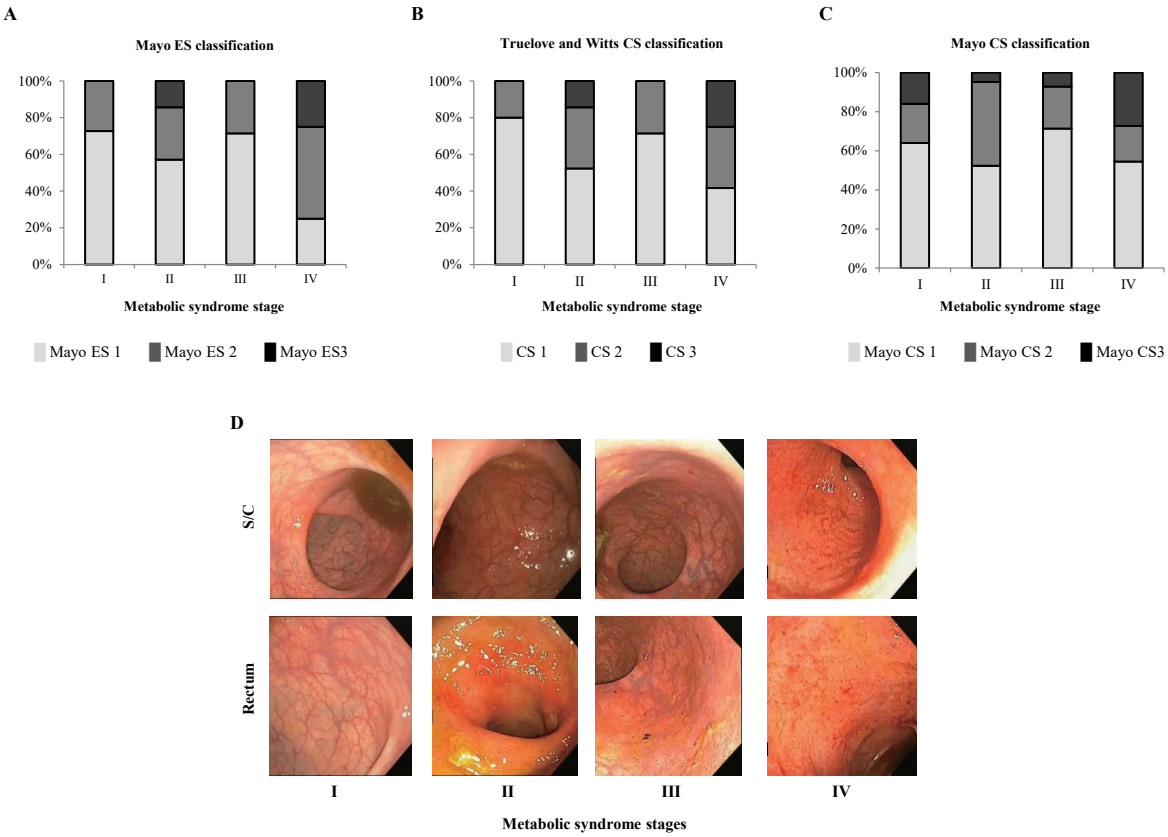


Fig. 2. Clinical characteristics of ulcerative colitis in progressive stages of the metabolic syndrome (MetS). Ulcerative colitis (UC) patients with MetS were divided into 4 groups which correspond to the developmental stages of syndrome (I-IV). Mayo endoscopic subscore (A), Truelove and Witts clinical score (B) and Mayo clinical score (C) of UC patients in all four progressive stages of MetS were presented. Representative images of Sigma/Colon (S/C) and Rectum were presented (D).

Gal-1 ($p = 0.04$) compared to those in early stages of MetS (Table 2). Interestingly, there wasn't significant difference in TNF-$\alpha$, IL-17 as well as in Gal-3 concentrations between defined groups (Table 2). Further, serum concentration of Gal-1 negatively correlated with stage of MetS ($r = -0.236$, $p = 0.026$), plasma glucose level ($r = -0.362$, $p = 0.004$) and plasma immunoreactive insulin level ($r = -0.283$, $p = 0.018$). In order to determinate the relationship between immunomodulatory galectins and proinflammatory mediators in all stages of MetS we analyzed ratio of systemic values of Gal-1 and Gal-3 with values of TNF-$\alpha$, IL-6 and IL-17. Analysis revealed that patients with UC in the stage IV of MetS had significantly decreased Gal-1/TNF-$\alpha$ ($p = 0.04$), Gal-1/IL-6 ($p = 0.011$) and Gal-1/IL-17 ($p = 0.03$), compared to the subjects in the stage I of MetS (Table 2). We did not find difference in Gal-3/TNF-$\alpha$, Gal-3/IL-6 and Gal-3/IL-17 ratios between same groups (Table 2).

When it comes to fecal values of cytokines, the same pattern of cytokine values were observed. The patients in the stage IV of MetS had higher values of pro-inflammatory cytokines IL-6, TNF-$\alpha$ and IL-17, while lower Gal-1, but these differences did not reach statistical significance (Table 3). Analysis of ratios of Gal-1 and pro-inflammatory mediators revealed that patients with UC in the stage IV of MetS had significantly decreased Gal-1/TNF-$\alpha$ ($p = 0.043$), Gal-1/IL-6 ($p = 0.032$) and Gal-1/IL-17 ($p = 0.036$), compared to the subjects in the stage I of MetS (Table 3).

FACS analysis revealed no significant differences in the percentage of $CD3^+CD56^+$ NKT cells, $CD4^+$ T cells and $CD19^+$ B cells among defined groups (data not shown). A significantly higher percentage of infiltrating $CD56^+$ NK cells ($p = 0.03$) and $CD8^+$T cells ($p = 0.016$) were recorded in affected tissue of patients in stage IV of MetS (Fig. 4). Further analysis revealed strong positive correlation between accumulation of $CD56^+$ NK cells and parameters of disease severity Mayo ES ($r = 0.949$, $p = 0.015$), and Mayo CS ($r = 0.866$, $p = 0.048$). We did not notice difference in Foxp3 and Gal-3 expression nor in cytokine production in all analyzed leukocytes between defined groups (data not shown).

## Discussion

Previous studies indicate significant association between the UC and MetS (Maconi et al. 2014). These diseases often occur jointly and undoubtedly affect one another. Recently, we have published results showing that

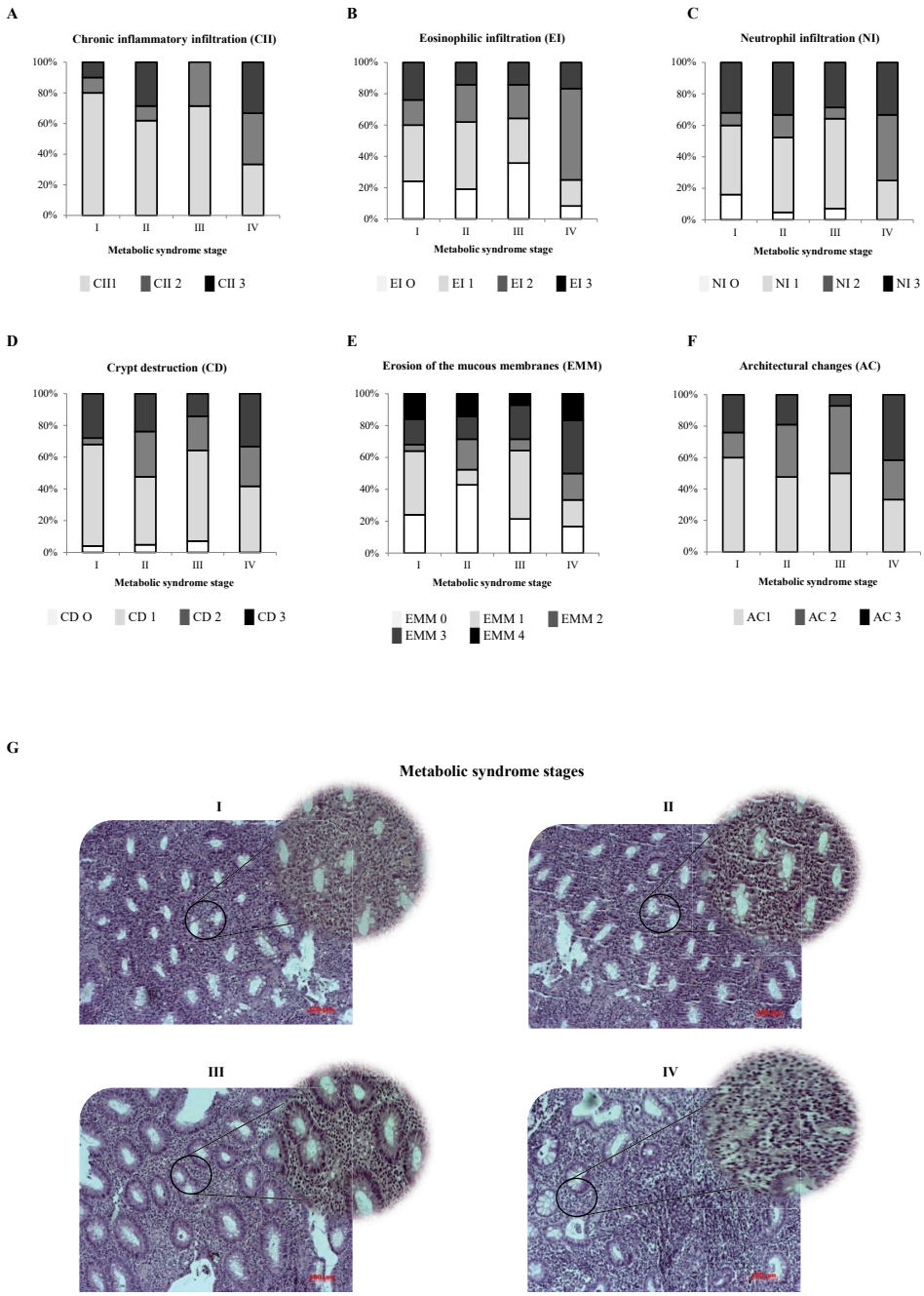


Fig. 3. Pathohistological parameters of ulcerative colitis (UC) in progressive stages of the metabolic syndrome (MetS). Histological score was analyzed for chronic inflammatory infiltration (A), eosinophilic infiltration (B), neutrophilic infiltration (C), crypt destruction (D), erosion of the mucous membranes (E) and architectural changes (F), in all four stages of MetS (I-IV). UC patients in phase IV of MetS had more severe chronic inflammatory infiltration in the injured colon. Representative H&E images were presented, illustrating intensive inflammatory infiltration (arrows) (G).

MetS affects the course of the UC by alleviating the clinical presentation and severity of the disease (Jovanovic et al. 2019). Milder form of UC in patients with MetS was accompanied by higher level of Gal-3 and interleukin-10 and less pronounced lymphocytes infiltration in affected tissue (Jovanovic et al. 2019). Still, MetS is complex disease that goes through multiple stages and it is not known how individual stages affect the course of the UC. To our knowledge, this is the first investigation of the influence of stages of MetS on biology of UC. We recruited *de novo* histologically confirmed UC patients with MetS and without previous treatment with antibiotics, aminosalicylates for at least two months, corticosteroids, statins, immunosuppressive agents as well as any kind of biological therapy previously. All patients were divided into 4 groups that corresponded with the progressive stages of MetS (Fig. 1).

Table 2. Systemic cytokine profile of ulcerative colitis (UC) patients with progressive stages of the metabolic syndrome (MetS).

| Cytokines (pg/ml) | Metabolic syndrome stages | | | | *P* value (I vs. IV) |
|---|---|---|---|---|---|
| | I Mean ± SD | II Mean ± SD | III Mean ± SD | IV Mean ± SD | |
| IL-6 | 542.7 ± 12.5 | 542.8 ± 13.2 | 594.5 ± 56.1 | 706.7 ± 65.5 | p = 0.049 |
| CXCL8 | 59.4 ± 22.3 | 93.9 ± 22.3 | 126.5 ± 62.3 | 297.7 ± 95.9 | p = 0.010 |
| TNF-*α* | 393.1 ± 15.5 | 388.6 ± 19.4 | 481.7 ± 74.7 | 419.5 ± 57.4 | n. s. |
| IL-17 | 581.0 ± 16.5 | 561.2 ± 17.4 | 588.9 ± 33.4 | 580.2 ± 33.5 | n. s. |
| Gal-1 | 1697 ± 245 | 997 ± 66 | 1003 ± 123 | 810 ± 36 | p = 0.038 |
| Gal-3 | 1130 ± 153 | 998 ± 168 | 594 ± 148 | 797 ± 106 | n. s. |
| Cytokines ratio | Ratio ± SD | Ratio ± SD | Ratio ± SD | Ratio ± SD | |
| Gal-1/TNF-*α* | 4.06 ± 1.01 | 2.63 ± 0.35 | 2.44 ± 0.14 | 2.08 ± 0.03 | p = 0.040 |
| Gal-1/IL-6 | 2.80 ± 0.67 | 1.93 ± 0.18 | 1.65 ± 0.15 | 1.44 ± 0.08 | p = 0.011 |
| Gal-1/IL-17 | 2.75 ± 0.59 | 1.81 ± 0.18 | 1.87 ± 0.28 | 1.39 ± 0.07 | p = 0.030 |
| Gal-3/TNF-*α* | 3.09 ± 0.67 | 3.43 ± 0.63 | 2.01 ± 0.51 | 2.32 ± 0.03 | n. s. |
| Gal-3/IL-6 | 2.37 ± 0.37 | 2.61 ± 0.42 | 2.10 ± 0.72 | 1.73 ± 0.41 | n. s. |
| Gal-3/IL-17 | 2.08 ± 0.42 | 2.34 ± 0.41 | 1.49 ± 0.31 | 1.60 ± 0.11 | n. s. |

Serum concentrations of IL-6, CXCL8, Gal-1, TNF-*α*, IL-17 and Gal-3 were determined by ELISA. Ratios of Gal-1/TNF-*α*, Gal-1/IL-6, Gal-1/IL-17, Gal-3/TNF-*α*, Gal-3/IL-6, and Gal-3/IL-17 were evaluated for each patient, separately. The Mann–Whitney U test was applied to evaluate statistically significant differences. The results are expressed as the mean ± SD or Ratio ± SD.
n.s., no statistically significant difference ($p > 0.05$).
IL-6, interleukin 6; CXCL-8, C-X-C motif chemokine ligand 8; TNF-*α*, tumor necrosis factor *α*; IL-17, interleukin 17; Gal-1, galectin 1; Gal-3, galectin 3.

Interestingly, BMI is similar for all groups (data not shown), and we believe it is due to comorbidity, UC. The conducted study is cross-sectional with only one time point evaluation.

Analyzing the clinical and pathohistological characteristics of the disease revealed that patients with UC in the stage IV of the MetS have significantly higher Mayo endoscopic and clinical scores as well as more pronounced chronic inflammatory infiltrates in affected colon tissue (Figs. 2 and 3). Thus, although UC patients with MetS have clinically and endoscopically milder disease in comparison to UC patients without MetS, as previously published (Jovanovic et al. 2019), those in stage IV have more severe disease compared to those in early stages of MetS.

Since various components of the immune system are involved in the pathogenesis of UC, in continuation of the study we analyzed the local and systemic parameters of the immune response in order to dissect out the causes of more severe disease in subjects with stage IV of MetS. The analysis revealed that patients with stage IV of MetS had significantly higher systemic values of pro-inflammatory cytokine IL-6 and chemokine CXCL8 (Table 2). It is well established role of IL-6 in facilitating innate and acquired immune response (Takac et al. 2014). Increased systemic values of IL-6 are detected in patients with inflammatory bowel diseases (Hyams et al. 1993) and positively correlate with the intensity of the inflammatory reaction (Reinisch et al. 1999). Moreover, Mazzucchelli et al. (1994) showed that the expression of CXCL8/IL-8 correlated with the severity of inflammation in gastrointestinal tract. During active form of UC, expression of CXCL8/IL-8 is increased in different immune cells, such as neutrophils, macrophages and intestinal epithelial cells (Mazzucchelli et al. 1994; Grimm et al. 1996). Additionally, it was noted that the CXCR1 expression and the CXCL8/IL-8 receptor were increased on macrophages and lymphocytes in the colon of patients with active UC (Williams et al. 2000).

There was no significant difference in Gal-3 concentration between defined groups, while Gal-1 was significantly lower in stage IV, compared to early stages of MetS (Table 2). Earlier studies have shown elevated systemic levels of Gal-1 in metabolic disorders (Liu et al. 2009; Fryk et al. 2016; Acar et al. 2017). Significant increment of Gal-1 concentration in sera of patients with UC and mild inflammation of the colon tissue compared with patients with severe inflammatory process suggest protective role of Gal-1 for intestinal epithelium (Simovic Markovic et al. 2016). In line with these findings, we detected lower Gal-1 concentration in UC patients with stage IV of MetS. Previous studies have shown that Gal-1 directs the acquired immune response toward type 2, while it inhibits the production of IFN-*γ*, TNF-*α*, IL-2, and IL-12 (Allione et al. 1998; Baum et al. 2003; Santucci et al. 2003). Some studies suggest that Gal-1 can inhibit effector T lymphocytes and consequently suppress potent immune response (van den Brule et al. 2001; van der Leij et al. 2004; Camby et al. 2006; Simovic Markovic et al. 2016). The anti-inflammatory properties of Gal-1 have been confirmed in several

Table 3. Fecal cytokine profile of ulcerative colitis (UC) patients with progressive stages of the metabolic syndrome (MetS).

| Cytokines (pg/g) | Metabolic syndrome stages | | | | *P* value (I vs. IV) |
|---|---|---|---|---|---|
| | I Mean ± SD | II Mean ± SD | III Mean ± SD | IV Mean ± SD | |
| IL-6 | 274.4 ± 42.9 | 284.2 ± 47.2 | 270.5 ± 65.5 | 383.4 ± 94.6 | n. s. |
| CXCL8 | 1057 ± 295 | 963 ± 211 | 863 ± 444 | 1029 ± 268 | n. s. |
| Gal-1 | 141.9 ± 35.4 | 158.5 ± 30.0 | 135.7 ± 42.8 | 98.1 ± 17.9 | n. s. |
| TNF-*α* | 398.6 ± 71.4 | 385.4 ± 63.7 | 392.9 ± 78.5 | 563.8 ± 95.2 | n. s. |
| IL-17 | 499.5 ± 91.2 | 492.8 ± 90.8 | 445.9 ± 85.8 | 625.8 ± 98.3 | n. s. |
| Cytokines ratio | Ratio ± SD | Ratio ± SD | Ratio ± SD | Ratio ± SD | |
| Gal-1/TNF-*α* | 0.63 ± 0.17 | 0.59 ± 0.13 | 0.38 ± 0.20 | 0.18 ± 0.06 | p = 0.043 |
| Gal-1/IL-6 | 0.75 ± 0.21 | 0.91 ± 0.22 | 0.53 ± 0.26 | 0.23 ± 0.06 | p = 0.032 |
| Gal-1/IL-17 | 0.49 ± 0.13 | 0.53 ± 0.12 | 0.32 ± 0.15 | 0.14 ± 0.04 | p = 0.036 |

Fecal concentrations of IL-6, CXCL8, Gal-1, TNF-*α* and IL-17 were determined by ELISA. Ratios of Gal-1/TNF-*α*, Gal-1/IL-6, and Gal-1/IL-17 were evaluated for each patient, separately. The Mann–Whitney U test was applied to evaluate statistically significant differences. The results are expressed as the mean ± SD or Ratio ± SD.
n. s., no statistically significant difference ($p > 0.05$).
IL-6, interleukin 6; CXCL-8, C-X-C motif chemokine ligand 8; Gal-1, galectin 1; TNF-*α*, tumor necrosis factor *α*; IL-17, interleukin 17.

models of chronic inflammatory diseases and autoimmune diseases, including experimental autoimmune encephalomyelitis, arthritis, uveitis, hepatitis, and diabetes (Offner et al. 1990; Rabinovich et al. 1999; Santucci et al. 2000; Perone et al. 2006; Toscano et al. 2006). Systemic Gal-1 also negatively correlated with stage of MetS, and plasma glucose level. Moreover, significantly higher ratios of Gal-1/TNF-*α*, Gal-1/IL-6 and Gal-1/IL-17 in patients with early stadium of MetS in comparison to subjects in stage IV indicate on predominance of Gal-1 over proinflammatory cytokines in early stages of MetS, in which UC is mild (Table 2). Predomination of Gal-1 over proinflammatory cytokines was confirmed in feces in early stages of MetS (Table 3). These findings indicate that with the progression of MetS, pro-inflammatory agents are prevalent over Gal-1, which is accompanied by more intense tissue damage and more severe disease.

In continuation of the study, we analyzed cellular makeup of infiltrates in mucous membrane of affected tissue, derived from UC patients. Despite no difference in the percentage of $CD3^+CD56^+$ NKT cells, $CD4^+$ helper T cells, $CD19^+$ B cells and $CD4^+Foxp3^+$ regulatory T cells (data not shown), we detected increased accumulation of $CD8^+$ T cells and $CD56^+$ NK cells in UC patients in stage IV of MetS (Fig. 4). $CD8^+$ T cells and natural killer (NK) cells usually infiltrate mucous membrane of UC patients and present one of main effectors in immune mediated tissue damage (Ueyama et al. 1998). Moreover, accumulation of $CD56^+$ NK cells is in positive correlation with more progressive UC (higher Mayo ES and Mayo CS, respectively). This suggests an enhanced local immune response resulting in abundant tissue damage and severe disease. Increased systemic IL-6 values in MetS subjects in stage IV is accompanied by increased influx of effector lymphocytes, which is consistent with the earlier finding of positive correlation of this cytokine and the intensity of the inflammatory reaction (Reinisch et al. 1999).

Interestingly, there was no difference in Gal-3 expression in any of analyzed infiltrating lymphocytes (data not shown). We previously established increment of Gal-3, locally and systemically, in UC patients with MetS and that Gal-3 and IL-10 play important roles in attenuating inflammation and subsequent clinical outcome of UC (Jovanovic et al. 2019). However, expression and production of Gal-3 did not alter significantly through progressive stages of MetS.

Although MetS, in general, have attenuating effect on UC biology, its effect varies depending on progressive stage. Patients in the stage IV of MetS have clinically and pathohistologically severe UC. There is evident predomination of mechanisms that attenuate local and systemic inflammatory response and subsequent tissue damage during the initial stages of MetS, whereas in the stage IV of MetS these mechanisms alleviate, which is accompanied by more pronounced inflammatory response and greater tissue damage. The enhanced inflammation in the stage IV of MetS can be, at least, due to decreased immunomodulatory influence of Gal-1 (Fig. 5). Our findings revealed the nature of influence of MetS progressive stages on UC severity and points on Gal-1 as an important regulator of immune response interplay in UC patients with MetS.

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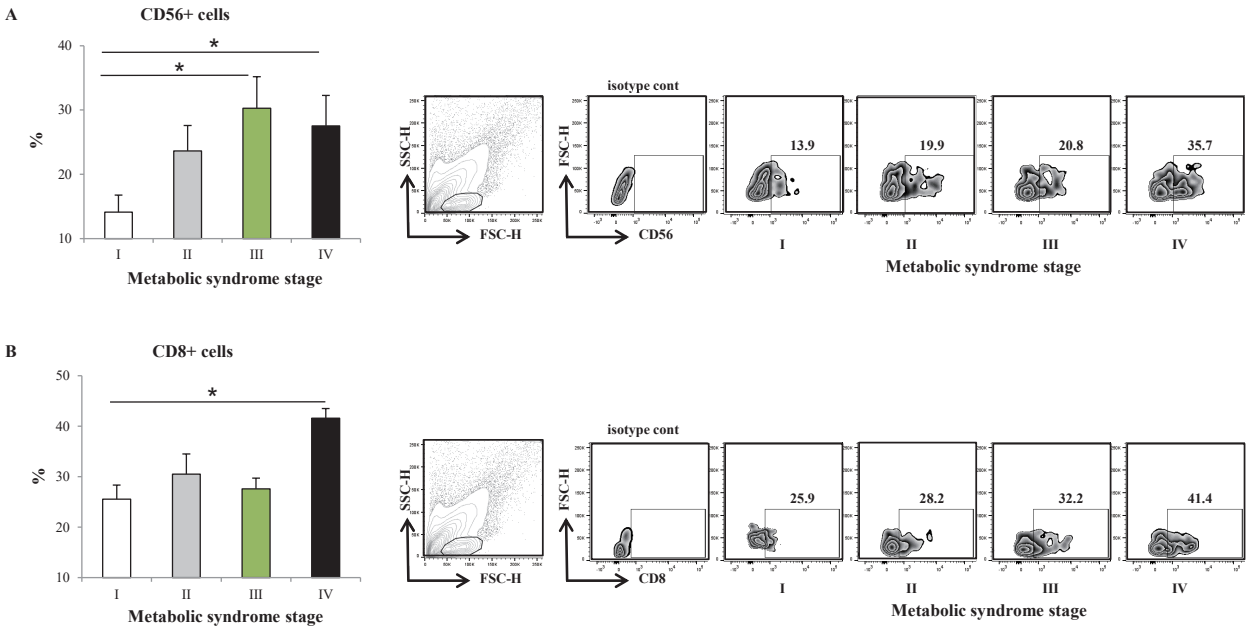


Fig. 4. Cellular make up of colon-infiltrating lymphocytes.
Overall frequencies and representative FACS plots of NK cells and CD8$^+$ T cells infiltrating the lamina propria detected in UC patients in all progressive stages of MetS are presented. The Kruskal–Wallis test (with post-hoc Mann–Whitney U-test) was applied to evaluate statistically significant differences.

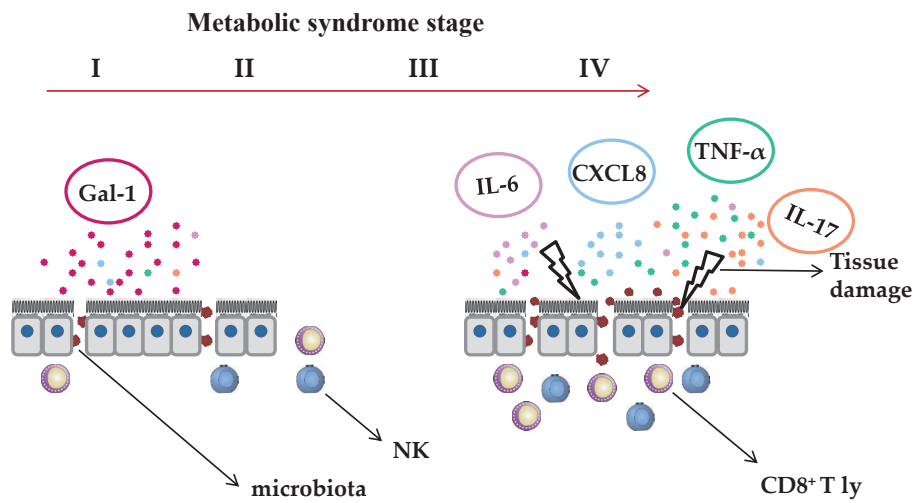


Fig. 5. Overview of systemic and local immune events in ulcerative colitis (UC) patients during the metabolic syndrome (MetS) progression.
During MetS progression, hypo-insulinemia and decreased systemic Gal-1 are accompanied by increased production of pro-inflammatory mediators IL-6, CXCL8, TNF-$\alpha$ and IL-17 and by more intense inflammatory reaction in affected colon tissue. All this facilitates local tissue damage and clinically severe UC. Gal-1, galectin 1; IL-6, interleukin 6; CXCL-8, C-X-C motif chemokine ligand 8; TNF-$\alpha$, tumor necrosis factor $\alpha$; IL-17, interleukin 17; NK, natural killer cells; CD8$^+$ T Ly, CD8$^+$ T lymphocytes.

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## Conflict of Interest

The authors declare no conflict of interest.

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