Medical University -Varna
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THE ROLE OF NECROPTOSIS IN INFLAMMATORY BOWEL DISEASE

ABSTRACT

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The dissertation contains 166 standard pages and is illustrated with 16 tables, 90 figures and 2 photos. The literature reference includes 283 literary sources, of which 9 in Cyrillic and 274 in Latin.

The dissertation was discussed and directed to the defense of the Department Council of the Department of "Propaedeutics of Internal Medicine" at the Medical University "Prof. Dr. Paraskev Stoyanov ”- Varna on April 27, 2022

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The official defense of the dissertation will take place on 22.06.2022 by…. hours at the Faculty of Medicine - Varna at an open meeting of the Scientific Jury.

The materials on the defense are available in the Scientific Department of MU - Varna and are published on the website of the Medical University - Varna.

Note: In the abstract the numbers of the tables and figures correspond to the numbers in the dissertation.

CONTENTS

ABBREVIATIONS USED

ACD	Accidental cell death
Bcl-2	B-cell lymphoma-2
CARD 15	Caspase recruitment domain family, member 15(formerly NOD2)
CD	Crohn's Disease
CDAI	Crohn's Disease Activity Index
cIAPs	Cellular inhibitor of apoptosis proteins
CYLD	Cylindromatosis
DAI	Disease Activity Index
DAMPs	Danger associated molecular patterns
DD	Death Domain
DISC	Death-inducing signaling complex
DRs	Death receptors (DR 1-6)
DRLs	Death receptor ligands
FADD	Fas associated Death Domain
FAS	First apoptosis signal receptor
FASL	FAS ligand
FLICE	FADD-like IL-1β-converting enzyme
cFLIP	cellular FLICE-like Inhibitory Protein
GALT	Gut - associated lymphoid tissue
HMGB1	High mobility group box 1
HSP70	Heat shock protein 70
IBD	Inflammatory Bowel Disease
IECs	Intestinal epithelial cells
IKK 1,2	IkB киназа 1 и 2
IL	Интерлевкин
JNK	c-Jun N-terminal kinases
LUBAC	Linear ubiquitination assembly complex
MAPK	Mitogen-activated protein kinase
MLKL	Mixed lineage kinase domain-like protein
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor κB, ядрен фактор κB
NOD	Nucleotide-binding oligomerization domain
NLRs	NOD-like receptors
PAMPs	Pathogen-associated molecular pattern
PCD	Programmed Cell Death
PRRs	Pattern recognition receptors
RAGE	Receptor for Advanced Glycation Endproducts
RHIM	RIP homotypic interaction motif
RIPK1	Receptor interacting protein kinase 1
RIPK3	Receptor interacting protein kinase 3
RIG	Retinoic acid-inducible gene
RLR	RIG-like receptors (Retinoic acid-inducible gene I (RIG-I)-like receptors)
ROS	Reactive oxygen species

CRP	C-reactive protein
TAB 1/2	TAK1-binding protein 1/2
TAK1	TGF - β activated kinase
TGF- β	Transforming grouth factor β
TNF-α	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
TRADD	TNFR1 – associated death domain protein
TRAFs	TNFR – associated factor
TRAIL	Tumor necrosis factor – related apoptosis inducing ligand
TRAILR1	TNF- related apoptosis inducing ligand receptor 1
TRIF	TIR-domain-containing adapter-inducing interferon-β
UC	Ulcerative colitis

INTRODUCTION

Inflammatory bowel disease (IBD), with two main representatives, Crohn's disease (CD) and ulcerative colitis (UC), is a group of idiopathic diseases characterized by chronic recurrent inflammation of the gastrointestinal tract. These are diseases that mainly affect young people and have a lifelong evolution. They alternate between periods of remission and relapse, with phases of activity varying in duration and often leading to local and systemic complications. This impairs the quality of life of patients, requires long-term treatment and is associated with significant socio-economic costs.

Despite intensive efforts over the years to understand the etiology and pathogenesis of IBD, they still remain unclear. This limits therapeutic options to the extent that symptoms are treated, remission is maintained, and relapses are avoided, but not to definitively cure patients.

The accumulated evidence in recent years reveals that impaired intestinal barrier function, as a primary defect, is a key factor in the development of IBD.

In order to ensure the structural integrity and stability of the barrier, it is necessary that the cell death of intestinal epithelial cells be strictly regulated.

Recent experimental studies have identified a new type of cell death in the intestinal epithelium called necroptosis, which leads to inflammation, with characteristics similar to inflammatory bowel disease. This has suggested its involvement in the pathogenesis of these diseases.

Necroptosis is a regulated form of cell death that occurs when apoptosis is inhibited, but extracellular apoptotic stimulation continues. It has morphological characteristics similar to passive necrosis, but similar to apoptosis is strictly regulated by the intracellular protein platform.

Necroptosis is thought to have evolved as a "backup" of apoptosis in cases where pathogens have developed mechanisms to inhibit the apoptotic machine.

As lytic cell death, it results in the rapid destruction of the cell membrane and the release into the extracellular environment of immunogenic cytosolic content, which activates the immune system and causes an inflammatory response. Thus, necroptosis directly causes inflammation and is perceived as an inflammatory pattern of cell death. Necroptosis in the intestinal epithelium may also indirectly induce inflammation by disrupting barrier integrity, allowing the invasion of commensal and pathogenic bacteria and further stimulating the immune system and exacerbating inflammation in the intestinal mucosa. Therefore, necroptosis

plays a role in initiating, exacerbating and creating a vicious circle of chronic inflammation, which is at the root of the pathogenesis of IBD.

The RIPK3 protein has been identified as a key molecule required for the necroptosis pathway, and its expression correlates with the sensitivity of cells to undergo necroptosis.

Therefore, inhibition of key molecules involved in the necroptotic pathway may provide new opportunities for the treatment of these diseases by not only reducing or suppressing the symptoms of intestinal inflammation, but also hindering the underlying molecular processes.

There are few data in the literature on the role of necroptosis in IBD in humans. In view of this, there is a need to study the expression of RIPK3 in IBD patients as a first step in a personalized approach to treatment, follow-up and staging.

PURPOSE, TASKS AND HYPOTHESIS

2.1. Purpose

The aim of this study was to investigate the presence of necroptosis in patients with inflammatory bowel disease (Crohn's disease and ulcerative colitis), determining the level of expression of the marker for necroptosis - RIPK3 in the intestinal mucosa and its relationship with clinical and pathological indicators in patients with inflammatory bowel disease.

2.2. Tasks

1. To study the level of RIPK3 expression in endoscopically altered areas (intestinal resect) in CD patients and compare with clinical, laboratory, endoscopic and histological indices of activity.
2. To study the level of RIPK3 expression in endoscopically altered areas in UC patients and compare with clinical, laboratory, endoscopic and histological indices of activity.
3. To compare the levels of RIPK3 expression in patients with inflammatory bowel disease and healthy controls.
4. To compare the levels of RIPK3 expression in CD and UC patients.
5. To establish the potential of RIPK3 levels as a prognostic marker for progression and development of severe disease in IBD patients.

2.3. Hypothesis

1) It is assumed that there is a significant difference in the expression of RIPK3 in IBD patients and can be used as a marker to distinguish CD from UC and as a prognostic marker for the development of severe disease and progression.
2) It is assumed that the expression of RIPK3 does not differ significantly in CD and UC and cannot be used as a prognostic marker.

MATERIAL AND METHODS

3.1. Patients

The study included 170 patients over 18 years of age diagnosed with IBD (of which 85 patients with Crohn's disease and 85 with ulcerative colitis), as well as 30 healthy controls passed through the structures of the University Hospital "St. Marina“ - Varna for the period from 2011 - м.06.2020.

The study is retrospective, as the participants included in the study are patients diagnosed with CD and UC according to the criteria of the ESSO. Complete medical records as well as biopsy material are available for each patient. The medical documentation of each patient was reviewed in detail and anamnestic, clinical, laboratory, imaging, endoscopic and morphological data were collected and used in the analyzes..

The patients included in the control group have no history of disease and do not take any medication.

All clinical, laboratory, imaging, endoscopic (fibrogastroduodenoscopy, ileocolonoscopy) and morphological examinations, as well as surgical interventions with resections of segments of the gastrointestinal tract (incisional biopsies), which were used for immunohistochemical analysis were performed on the territory of the University Hospital "St. Marina" – Varna.

The study received a positive assessment from Research Ethics Committee at MU-Varna with a protocol with №96 / 24.09.2020.

The study selected patients who met pre-defined inclusion criteria.

3.1.1. Inclusion criteria

- Patients hospitalized in the University Hospital "St. Marina "- Varna for the period from 2011 to 06.2020 diagnosed with Crohn's disease according to ECCO criteria.
- Patients hospitalized in the University Hospital "St. Marina "- Varna for the period from 2011. until the month of 06.2020 diagnosed with ulcerative colitis according to ECCO criteria.

- Patients with available complete medical documentation (data on the patient's disease - anamnestic, clinical, laboratory, imaging, endoscopic and histological), proving the diagnosis and determining the activity of the disease.
- Patients over 18 years of age, allo- and autopsychically oriented.

3.1.2. Excluding criteria:

- Patients under <18 years of age, allo- and autopsychically disoriented.
- Patients who do not meet the inclusion criteria.

3.1.3. Control group

3.1.3.1. Inclusion criteria

- Patients hospitalized in the University Hospital "St. Marina "- Varna for the period from 2011. to 06.2020, diagnosed with functional gastrointestinal disorders, in which there are no clinical, laboratory, imaging, endoscopic and histological data on gastrointestinal diseases, as well as no data on inflammatory, oncological and severe concomitant diseases.
- Persons over 18 years of age.

3.1.3.2. Excluding criteria

- Persons under 18 years of age.
- Patients who do not meet the inclusion criteria.

3.2. Methods

3.2.1. Clinical trials

Patients were selected according to their medical records diagnosed with Crohn's disease and ulcerative colitis according to ECCO Criteria.

3.2.1.1. Clinical trials in patients with CD

In the group of patients with CD information was collected and analyzed in detail about:

- Gender
- age
- number of hospitalizations
- statute of limitations for the disease
- age at debut

- clinical symptoms
- localization (L)
- disease behavior (B)
- activity - CDAI
- presence of extraintestinal manifestations (EIM)
- presence of complications
- presence of operations
- conducted treatment
- presence of comorbidities

3.2.1.1.1. Clinical classification of CD

The Montreal Classification was used to determine the location and course of patients with CD (Silverberg MS et al., 2005). (Table 1)

Table 1. Montreal classification for CD

	Монреалска класификация на БК (2005)
Age of diagnosis (A)	A1: < 16 years
	A2: < 17 - 40 years
	A3: > 40 years
Location (L)	L1 – ileal
	L2 – colonic
	L3 – ileocolonic
	L1 -3 + L4 - upper GI
Disease behavior (B)	B1 – inflammatory (non-stricturing, non-penetrating)
	B2 - stricturing
	B3 – penetrating
	B 1-3 + p – perianal disease

3.2.1.1.2. Determination of clinical activity

The most commonly used in clinical trials index for the assessment of disease activity in CD (CDAI), also known as the Best index, which consists of eight clinical parameters (Table 2). According to the number of points assessed by CDAI, the disease is divided into inactive (remission) - CDAI <150, mild - CDAI = 150 to 219, moderate - CDAI = 220 to 450 and severe with CDAI values> 450 (Best WR et al. , 1976).

Table 2. ***Crohn's Disease Activity Index – CDAI***

Clinical or laboratory variable	***Weighting factor***	***Points***
Number of liquid or soft stools each day for 7 days	*x 2*	
Abdominal pain (graded from 0 to 3 based on severety) each day for 7 days	*x 5*	
General well-being : - *generally well = 0* - *slightly under par = 1* - *poor= 2* - *very poor = 3* - *terrible = 4*	*x 7*	
Number of complications	*x 20*	
Use of opiates for diarrhea: - *yes = 0* - *no= 1*	*x 30*	
Abdominal mass: - *none = 0* - *questionable = 2* - *definite = 5*	*x 10*	
Deviation of normal hematocrit from 47 % in men and 42 % in women	*x 6*	
Percentage deviation from standart weight	*x 1*	
Total CDAI		

3.2.1.2. Clinical trials in patients with UC

In the group of patients with UC information was collected and analyzed in detail about:

- Gender
- age
- number of hospitalizations
- statute of limitations for the disease
- age at debut
- clinical symptoms
- extent (E)
- Severety (S)
- Index of activity – Mayo scor
- presence of extraintestinal manifestations (EIM)
- presence of complications
- presence of operations
- conducted treatment
- presence of comorbidities

3.2.1.2.1. Clinical trials of UC

The Montreal classification was used to determine the extent and clinical UD activity (Magro F et al., 2017; Silverberg MS et al., 2005). (Table 3 and Table 4)

Table 3. Montreal classification of extent of UC

Extent	***Anatomy***
E1 – Ulcerative proctitis	Rectum
E2 – Left sided UC	Involvement limited to a portion of the colorectum distal to the splenic flexure
E3 – Extensive UC	Involvement extends proximal to the splenic flexure

Table 4. Montreal classification of severity of UC

Severety	***Definition***
S0 – Clinical remission	Asymptomatic
S1 – Mild UC	Passage of 4 or fewer stools/day (with or without blood) Absence of any systemic ilness Normal inflammatory markers
S2 – Moderate UC	Passage of more than 4 stools dayly Minimal signs of systemic toxicity
S3 – Severe UC	Passage of at least 6 bloody stools dayli + Pulse rate > 90/min; T> 35,5C; Hb <105; CRP>30

To compare the results, we used the Montreal Classification (2005) of CD for age at onset (A) and divided patients with UC with the same age groups: A1: <16 years, A2: <17 - 40 years, A3:> 40 years.

Index of Activity

The most commonly used in clinical practice Mayo index (score) (Magro F et al., 2017) was used to assess the severity of UC (Table 5). According to the sum of points the remission are accepted: ≤2; for mild colitis: 3-5; moderate colitis: 6-10; severe UC: 11-12.

Table 5. Mayo-scoring system for assessment of UC activity (DAI = disease activity index)

Mayo scor	**0**	**1**	**2**	**3**
Stool frequency	Normal No of stools for this patient	1-2 stools more than normal	3-4 stools more than normal	>5 stools more than normal
Rectal bleeding	No blood seen	Streaks of blood with stool less than half the time	Obvious blood with stool most of the time	Blood alone passed
Endoscopis finding	Normal or inactive disease	Erithema Decreased vascular pattern Mild friability	Marked erythema Absent of vascular pattern Friability	Spontaneous bleeding Ulceration

			Erosions	
Physicion's global assessment	Normal	Mild disease	Moderate disease	Severe disease

3.2.2. Laboratory tests

The patients collected information about the laboratory tests performed during the hospitalization - ESR, peripheral blood smear, C reactive protein (CRP), albumin, serum iron, ferritin, FCP, as well as virological and microbiological tests.

3.2.3. Imaging studies

In the patients included in the study, abdominal ultrasound and, if necessary, additional imaging studies (CT, CT-enterography, MRI / MR-enterography, X-ray contrast examination of the GI tract) were performed to assess the extent of the changes.

3.2.4. Endoscopic examinations

3.2.4.1. Endoscopic examinations in CD

Endoscopy plays an essential role in the diagnosis, determination of therapeutic behavior, prognosis and follow-up of patients with IBD. Unlike UC, where the signs of endoscopic activity are clearly defined, and in fibrocolonoscopy the changes are easily accessible, in CD the changes are scattered and varied, and changes in the course of the small intestine can not always be visualized directly. Due to the complexity of using most endoscopic indices in CD, the reflection of endoscopic changes in everyday work is usually descriptive. In order to fulfill the set goals and objectives in the present work and to systematize the descriptive endoscopic protocols, we evaluated the endoscopic activity in the area from which the biopsy was taken for immunohistochemical examination using the recently validated simplified endoscopic point system for mucosal assessment in CD (SEMA-CD , Simplified endoscopic mucosal assessment for Crohn's disease) (Adler J et al., 2021) (Table 6). 0 is considered to be the absence of mucosal changes in the ileum or colon; 1 - several aphthous lesions, the remaining mucosa is normal; 2 - scattered aphthous lesions or small ulcers, no large ulcers (ie ulcers> 2 cm); 3 - scattered large ulcers or multiple scattered small ulcers or multiple stenoses that allow the endoscope to pass; 4 - multiple scattered large ulcers, stenosis that can not be passed or the presence of visible fistula. Thus, we quantified the changes in the affected

segments of the gastrointestinal tract and compared the degree of endoscopic activity in the affected segment and the degree of expression of RIPK3.

The Rutgeerts score was administered to patients with BC after surgery (Rutgeerts P et al., 1990) (Table 7).

Table 6 Simplified endoscopic mucosal assessment for Crohn's disease (SEMA-CD).

Points	Ileum/Colon	Описание
0	**Endoscopic remission**	Normal
1	**Minimal disease**	No more than a few aphthous ulcers. Otherwise normal.
2	**Mild disease**	Scattered aphthous ulcers or small ulcers. No large ulcers (>2 sm)
3	**Moderate disease**	Scattered large ulcers or widespread small ulcers. Multiple passable stenosis.
4	**Severe disease**	Widespread large ulcers. Nonpassable stricture or visible fistula.

Табл. 7. Rutgeerts score in CD after resection

i0	No lessions (post-surgery remission)
i1	< 5 aphthous ulcers = post-surgery remission
i2	> 5 aphthous ulcers with normal intervening mucosa (substantial post-surgery recurrence)
i3	Diffuse aphthous ileitis with diffusely inflamed mucosa (advanced post-surgery recurrence)
i4	Diffuse inflammation with large ulcers, nodules and/ or narrowing (advanced post-surgery recurrence)

Rutgeerts score ≥ i2 – endoscopic recurrence and activity.

3.2.3.2. Endoscopic examinations in UC

Endoscopic Mayo score (EMS) was used to determine endoscopic activity in patients with UC (Magro F et al., 2017). (Table 8)

Table 8. Endoscopic Mayo score (EMS)

	0 points	1 point	2 points	3 points
Endoscopic Mayo Score (EMS)	Normal or inactive disease	Erithema Decreased vascular pattern Mild friability	Marked erythema Absent of vascular pattern Friability Erosions	Spontaneous bleeding Ulceration

3.2.5. Histological examinations

Histological materials from ready paraffin blocks from the histology of the Clinic of General and Clinical Pathology of the University Hospital "St. Marina" - Varna, diagnosed with Crohn's disease, ulcerative colitis and irritated colon from endoscopic and incisional biopsies.

The biopsy materials received at the Clinic of General and Clinical Pathology of the University Hospital "St. Marina" - Varna were fixed for a minimum of 24 hours in 10% neutral formalin, then included in paraffin with a melting point of 52-54ºC for the preparation of paraffin blocks. Histological sections 4 μm thick, mounted on slides and stained with hematoxylin and eosin, were used for morphological diagnosis.

The biopsies were re-evaluated and the histological changes in the areas with inflammatory activity were analyzed in detail.

The main characteristic of histological activity in IBD is determined by the presence of active inflammation of the intestinal mucosa, expressed by the presence of neutrophilic granulocytes in the epithelium and / or lumen of intestinal crypts, and the presence of erosions and / or ulceration of the lamina propria. The absence of active inflammation corresponds to histological remission.

For the purposes of this study, the histological parameters for inflammatory activity in IBD were determined - the percentage of glands with cryptitis, crypt abscesses, erosions and ulcerations. For cryptitis - the presence of neutrophilic granulocytes in the epithelium of glandular crypts is considered, for crypt abscesses - the presence of neutrophilic granulocytes in the lumen of the crypts, for erosions - massive exfoliation of the mucosal epithelium to the level of muscularis mucosae with underlying non-inflammatory reaction for ulcerations - cases in which the mucosal defect passes beyond the muscularis mucosae of the intestinal wall.

The simplified score system of the British Society of Gastroenterology (IBD biopsy pathology guidelines) was used to assess active inflammation in biopsy specimens in patients with IBD. Inflammation was assessed on a 4-point scale - inactive disease, mild, moderate and severe activity (Feakins RM, 2013). (Table 9)

Table 9. Simplified score system of the British Society of Gastroenterology

Degrees	***Criteria***
Inactive disease	No intraepithelial neutrophils, erosions or ulcerations
Mild degree of activity	Cryptitis in <25% of crypts or crypt abscesses up to 10% or both
Moderate degree of activity	Cryptitis in> 25% of crypts or crypt abscesses in> 10% of crypts or rare small foci of surface erosion or a combination of these
Severe degree of activity	Ulcerations or multiple foci of erosion

3.2.6. Immunohistochemical analysis

3.2.6.1. Antibodies, staining reagents and working concentrations used

An indirect immunoperoxidase method was used for immunohistochemical analysis using the mini KIT high Ph DAKO K8024. The antibody, staining reagents and operating concentrations used are presented in table. 10. and table. 11.

The antibody Anti-RIPK3, catalog No. ab56164, was used. The antibody is manufactured by ABCAM's RabMab technology.

Negative controls: In negative controls, instead of the primary antibody, sections of the paraffin blocks used are incubated with normal non-immune serum.

Liver tissues stained with Anti-RIPK3 were used for positive controls.

Table 10. Reagents used

Antibody	**Dilution**	**Positive control**	**Marker for**	**Manufacturer company**
Anti-RIPK3(ab56164) Rabbit polyclonal to RIP3	1:100	Черен дроб	Некроптоза	ABCAM's RabMab technolog

Table 11. Staining system and other reagents

HRP- DAB System	Original coloring system	Dako
Mayer’s hematoxilin	Counterstaining	Dako

3.2.6.2. Preparation of biopsy materials for immunohistochemical examination

From the biopsy materials, fixed in neutral formalin and included in paraffin blocks, 4 μm thick sections are prepared, which are mounted on silanized glasses.

Dewaxing was performed in descending order of alcohol concentrations as follows: Ethanol 100% 3 minutes, Ethanol 90% 3 minutes, Ethanol 80% 3 minutes, Ethanol 70% 3 minutes, Xylol 3 x 10 minutes. The sections are then washed under running water and placed in distilled water.

Antigen detection was performed with a pre-heated to 65 ° C working solution En Vision FLEX Target Retrieval Solution in a PT Link container. The sections were incubated for 20 minutes at 97 ° C and pH = 9. After cooling, the samples were washed at room temperature with FLEX Wash Buffer (20x) for 1-5 minutes.

3.2.6.3. Immunohistochemical protocol

The sections are stained by FLEX protocol, using a humid chamber for all steps.

Incubate with peroxidase blocking solution (3% H 2 O 2) for 5 minutes at room temperature to block endogenous peroxidase activity. Rinse with wash buffer for 5 minutes.

Incubation with Primary Anti-RIPK3 antibody (ab62344) diluted for 20 minutes at room temperature.

Wash with wash buffer for 2 x 5 minutes at room temperature.

Incubation with labeled HRP polymer for 20 minutes at room temperature.

Wash with wash buffer for 3 x 5 minutes at room temperature.

Incubation of sections with chromogen DAB peroxidase solution for 2 x 5 minutes under continuous microscopy.

Wash with buffer for 2 minutes.

Rinse with distilled water for 2 minutes.

Countercolor with Mayer's hematoxylin for 5 min.

Rinse the samples with distilled water for 5 minutes.

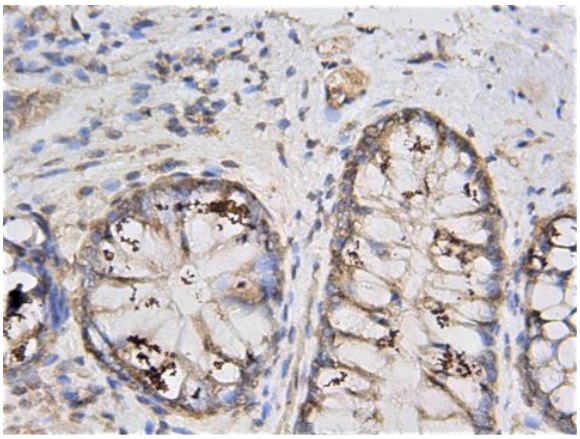
Dehydration in reverse order ethanol 70%, ethanol 80%, ethanol 90%, ethanol 100% with the same duration as the dewaxing.

Placement in a mounting environment.

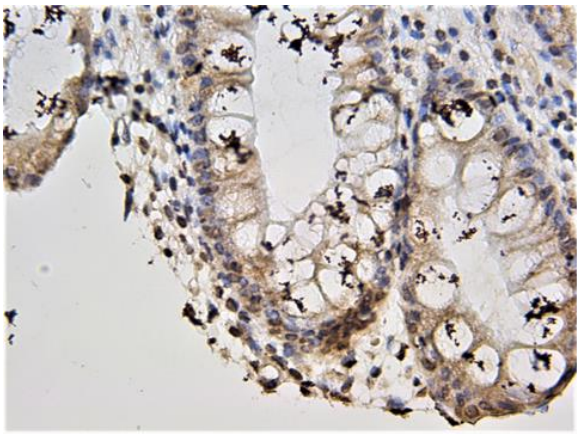
3.2.6.4. Determination of the expression level of RIP3 and interpretation of the results.

Immunohistochemical expression of RIP3 was assessed using the H-score (histological score) on tissue samples. The intensity of nuclear expression was determined for each cell as follows (picture 1 and picture 2):

- (0) - lack of nuclear expression, nuclei are colorless
- (1+) - weak nuclear expression - nuclei are light yellow to light brown (slightly colored)
- (2+) - moderate nuclear expression - nuclei are light brown (moderately colored)
- (3+) - intense nuclear expression - the nuclei are dark brown (pronounced color)



Picture 1. Colorectal mucosa without inflammatory activity, from the control group, with low expression



Picture 2. Colitis with high RIP3 expression: different degrees of nuclear antibody expression in different epithelial cells are clearly visible, H-score 218; RIP3 x 400

The percentage of positive cells for each intensity was determined, and finally the H-score was calculated using the following formula:

1x (% cells with 1+) + 2x (% cells with 2+) + 3x (% cells with 3+) = H-score (rate of RIP3 expression)

Thus calculated, the range of H-score varies from 0 to 300.

3.2.7. Statistical methods

The statistical software package - IBM SPSS for Windows, v.20.0 was used in data processing.

An acceptable level of significance $p < 0.05$ with a 95% confidence interval is assumed for all analyzes performed.

- Analysis of variance (ANOVA) to assess whether the influence of a factor is statistically significant or not.
- Variation analysis to study the quantitative characteristics of indicators.
- Event risk assessment analysis (OR, HR, RR).
- Correlation analysis to assess the relationship between the studied indicators. The estimation of the strength of the dependence between the variables is based on the results

of Pearson's coefficient (r) and Spearman's (p) coefficient, with Spearman's coefficient calculating the correlation based on monotonic relationships and Pearson's based on linear relationships.

The degree of association between the variables is defined as:

- $0<r(p)<0.3$ – weak correlation
- $0.3<r(p)<0.5$ – moderate correlation
- $0.5<r(p)<0.7$ – significant correlation
- $0.7<r(p)<0.9$ – high correlation
- $0.9<r(p)<1$ – very high correlation

- Regression analysis to assess the possible functional relationships between the studied indicators. Investigation of causal relationships.
- ROC curve analysis to determine the cut-off value, to distinguish between low and high expression of the tested antibodies.
- Prognostic analysis - Positive predictive value (PPV) to determine the prognostic value for the occurrence of an event according to the specific sample.

$$\mathbf{PPV} = \frac{\textbf{The number of truly positive answers}}{\textbf{The number of truly positive answers + The number of false positives}}$$

- Prognostic analysis - Negative predictive value (NPV) to determine the prognostic value for the occurrence of a negative event according to the specific sample

$$\mathbf{NPV} = \frac{\textbf{The number of truly negative answers}}{\textbf{The number of truly negative answers + The number of false negative responses}}$$

- Comparative analysis (assessment of hypotheses) - $\chi 2$, t-test Student's to compare quantitative and qualitative indicators and study the difference between them.
- Graphic and tabular method of displaying the obtained results.

RESULTS

4.1. Characteristics of the studied groups and determination of the cut-off values of RIPK3

The expression of RIPK3 was studied in 200 individuals, of whom 170 were with IBD and 30 were healthy controls, and the distribution is shown in Fig. 10.

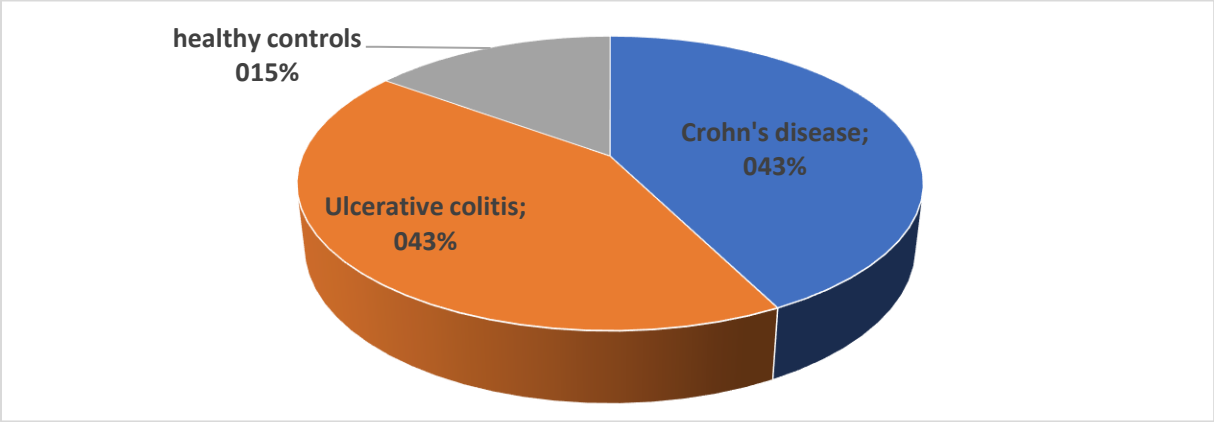


Fig. 10. Distribution of patients according to the studied groups

On the table. 12 presents the characteristics of the studied patients and the control group.

Of the IBD persons 50.6% (86) are men and 49.4% (84) - women with a mean age at the time of observation 41.67 years ± 14. years, with a minimum age of 18 years and a maximum of 83 years.

There was a significant difference in the age of the subjects ($p < 0.001$), with CD patients being the youngest.

A significant difference was also found in terms of gender ($p = 0.015$), with women predominating in CD patients and healthy controls, while men predominated in UC patients.

Regarding the localization in the patients with BC 27 (31.8%) patients have ileal localization (L1), 15 (17.6%) - with colonic localization (L2) and 36 (42.4%) - with ileocolonic localization (L3) (Table 12). Seven (8.3%) of patients with CD also have upper GI involvement.

The highest percentage are patients with pancolitis (E3) - 46 / 54.1%, followed by left colitis (E2) - 38 / 44.7% and only one patient has proctitis (E1).

Table 12. General characteristics of the examined persons

Parameters		**Crohn's disease (n=85)**	**Ulcerative colitis (n=85)**	**Healthy controls (n=30)**
Age, years	**mean±SD (range)**	**40.8±14.0 (18.0-74.0)**	**42.6±15.9 (18.0-83.0)**	**53.3±13.1 (28.0-81.0)**
Gender	**Men**	**40/47.1%**	**46/54.1%**	**7/23.3%**
	Women	**45/52.9%**	**39/45.9%**	**23/76.7%**
Localization CD/UC*	**L1/E1**	27/31.8%	1/1.2%	-
	L2/E2	15/17.6%	38/44.7%	-
	L3/E3	36/42.4%	46/54.1%	-
	L1+L4	2/2.4%	-	-
	L2+L4	1/1.2%	-	-
	L3+L4	4/4.7%	-	-
Disease behavior *	**B1**	41/48.2%	-	-
	B2	27/31.8%	-	-
	B3	4/4.7%	-	-
	B2+B3	13/15.3%	-	-
Years from diagnosis	**mean±SD (range)**	4.2±4.1 (0-20)	4.6±5.2 (0-20)	-
CDAI	**Remission**	3/3.5%	-	-
	Mild activity	29/34.1%	-	-
	Moderate avtivity	48/56.5%	-	-
	Severe activity	5/5.9%	-	-
S (severity)*	**Remission**	-	-	-
	Mild activity	-	7/8.2%	-
	Moderate avtivity	-	26/30.6%	-
	Severe activity	-	52/61.2%	-
Mayo score (DAI)	**Remission**	-	-	-
	Mild activity	-	9/10.6%	-
	Moderate avtivity	-	59/69.4%	-
	Severe activity	-	17/20.0%	-
Endoscopic Mayo score	**Mild activity**	-	12/14.2 %	-
	Moderate avtivity	-	28/32.9%	-
	Severe activity	-	45/52.9%	-
Extraintestinal manifestations		54/63.5%	51/60.0%	-
Thromboembolic complications		5/5.9%	1/1.2%	-
Intestinal complications		**64/75.3%**	**19/22.4%**	-
Accompanying diseases		**61/71.8%**	**30/35.3%**	-
Operation		**26/30.6%**	**3/3.5%**	-
Treatment	**5-ASA**	65/87.8%	64/87.7%	-
	Corticosteroids	30/40.5%	22/30.1%	-
	Immunomodulators	**24/32.4%**	**11/15.1%**	-
	Biological treatment	**24/28.2%**	**8/9.4%**	-

It is presented in bold p<0.05 * Montreal classification

It was found that in CD patients intestinal complications were significantly higher than in UC patients (respectively 75.3% for CD to 22.4% for UC; p <0.001). It can be said that in the present study intestinal complications strongly correlated with BC (r = 0.530; p <0.001).

A significant difference was also found with regard to accompanying diseases (p <0.001), with a higher incidence of this group of diseases in CD patients. There was a moderate relationship between the presence of accompanying diseases and CD (r = 0.366; p <0.001).

Regarding the performed operations, it was found that in CD patients there are 10 times more surgical interventions than in UC patients (respectively 30.6% to 3.5%; p <0.001). A moderate relationship between surgery and CD was also found (r = 0.360; p <0.001).

In CD patients, the relative share of persons treated with immunomodulators is twice as high as in UC patients (p = 0.011). There was a weak relationship between immunomodulatory treatment and CD (r = 0.204; p = 0.013).

The results of the analysis show that biological therapy was used more often in CD patients (p = 0.006), with low dependence (r = 0.245; p = 0.001).

Patients with debute were 26 (15.3%), of which 12 (46.2%) had CD and 14 (53.8%) had UC. Of the patients with CD with debut, 41.7% were women and 58.3% were men, and the male: female distribution was 50:50. There was no difference in the average age at the onset of the disease, as in CD patients it was 36.75 years ± 19.21 years, and in UC patients it was 38.0 years ± 14.26 years.

On the table. 13 presents the characteristics of patients according to laboratory parameters. There was a significant difference between the levels of FCP in CD patients and UC patients (p = 0.042), with the former being significantly lower. For the other indicators, no significant difference was observed between the two groups of patients.

Table 13. General characteristics of the examined patients - laboratory parameters

Parameters		**Crohn's disease (n=85)**	**Ulcerative colitis (n=85)**
Erythrocyte sedimentation rate (ESR)	mean±SD (range)	62.65±33.69 (3.0-120.0)	51.08±31.37 (6.0-120.0)
	Not increased	27/31.8%	28/32.9%
	Increased	58/68.2%	57/67.1%
CRP	mean±SD (range)	45.79±52.53 (0.78-232.0)	35.72±52.45 (0.11-270.0)
	Not increased	19/22.4%	22/25.9%
	Increased	66/77.6%	63/74.1%
Leukocytes	mean±SD (range)	9.55±4.04 (4.0-26.90)	9.38±3.42 (3.40-20.20)
	Not increased	55/64.7%	55/64.7%
	Increased	30/35.3%	30/35.3%

Thrombocytes	mean±SD (range)	369.0±130.4 (48.0-917.0)	375.9±127.7 (137.0-791.0)
	Normal	68/80.0%	64/75.3%
	Decreased	1/1.2%	1/1.2%
	Increased	16/18.8%	20/23.5%
Albumin	mean±SD (range)	39.69±5.74 (24.0-53.0)	38.32±6.73 (22.0-53.0)
	Normal	76/89.4%	71/83.5%
	Decreased	9/10.6%	14/16.5%
Ferritin	mean±SD (range)	75.62±105.59 (0-432.0)	125.39±195.72 (6.70-668.0)
	Normal	7/36.8%	10/50.0%
	Decreased	11/57.9%	8/40.0%
	Increased	1/5.3%	2/10.0%
Hemoglobin	mean±SD (range)	125.31±20.50 (53.0-165.0)	124.04±18.32 (70.0-166.0)
	Normal	51/60.0%	48/56.5%
	Decreased	33/38.8%	37/43.5%
Fe	mean±SD (range)	9.29±6.94 (0.60-34.0)	7.67±4.68 (1.0-24.0)
	Normal	29/34.1%	21/24.7%
	Decreased	56/65.9%	64/75.3%
FCP	**mean±SD (range)**	**703.19±597.29 (39.9-1747.0)**	**1636.38±1393.63 (359.0-5910.0)**

It is presented in bold p<0.05.

Due to the lack of validated RIPK3 reference values in IBD patients, the cut off values of the marker were calculated using ROC curve analysis. On the table. 14 presents the cut-off values of RIPK3 expression for distinguishing patients with IBD from healthy controls, as well as for distinguishing CD and UC patients. This table also presents the sensitivity and specificity of RIPK3, as well as the positive predictive value (PPV) and the negative predictive value (NPV).

Table 14. RIPK3 cut-off for distinguishing IBD from robust controls

	Cut-off	**AUC 95%CI**	**P value**	**Sensitivity / Specificity**	**PPV**	**NPV**
Distinguish healthy controls from IBD patients	163.5	0.718 (0.653-0.784)	<0.001	70.0%/70.0%	92.9%	29.2%
Distinguish healthy controls from CD patients	174.5	0.418 (0.335-0.502)	0.048	45.9%/46.1%	100 %	39.5%
Distinguish healthy controls from UC patients	179.5	0.696 (0.621-0.770)	<0.001	68.2%/70.0%	100 %	52.6%
Distinguish patients with CD	185.5	0.642 (0.558-0.726)	0.001	61.2%/62.4%	61.9%	61.6%

from UC patients						

When determining the cut-off value of nuclear expression of RIPK3 to distinguish healthy controls from patients, cut-off -163.5 was found (AUC = 0.718 (0.653-0.784); p <0.001) with a sensitivity and specificity of 70%. (Fig. 11)

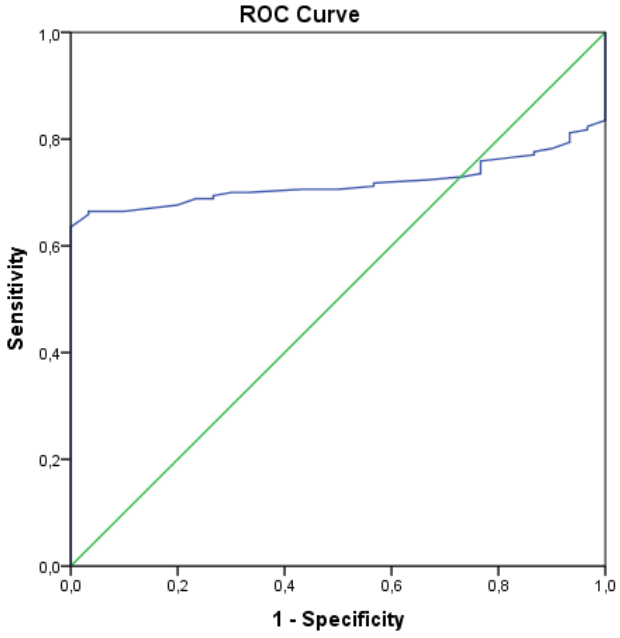


Fig. 11. ROC curve analysis to determine the cut-off value of RIPK3 to distinguish healthy controls from IBD patients

When determining the cut-off value of nuclear expression of RIPK3 to distinguish patients with UC from patients with CD, cut-off of -185.5 was found (AUC = 0.642 (0.558-0.726); p = 0.001) with a sensitivity of 61.2% and a specificity of 62.4%. (Fig. 12)

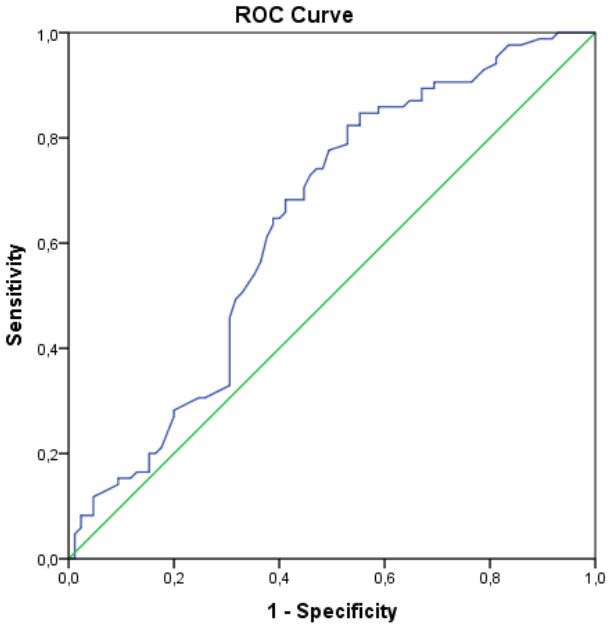


Fig. 12. ROC curve analysis to determine the cut-off value of RIPK3 to distinguish UC patients from CD patients

When examining the relationship between RIPK3 expression levels and the presence of IBD, it was found that high expression correlated with the IBD presence relative to healthy controls ($r = 0.398$; $p < 0.001$).

There was a significant difference in nuclear expression in patients with CD, UC and control subjects, with the lowest expression observed in healthy controls (158.9) and the highest in patients with UC (189.4) (Fig. 13).

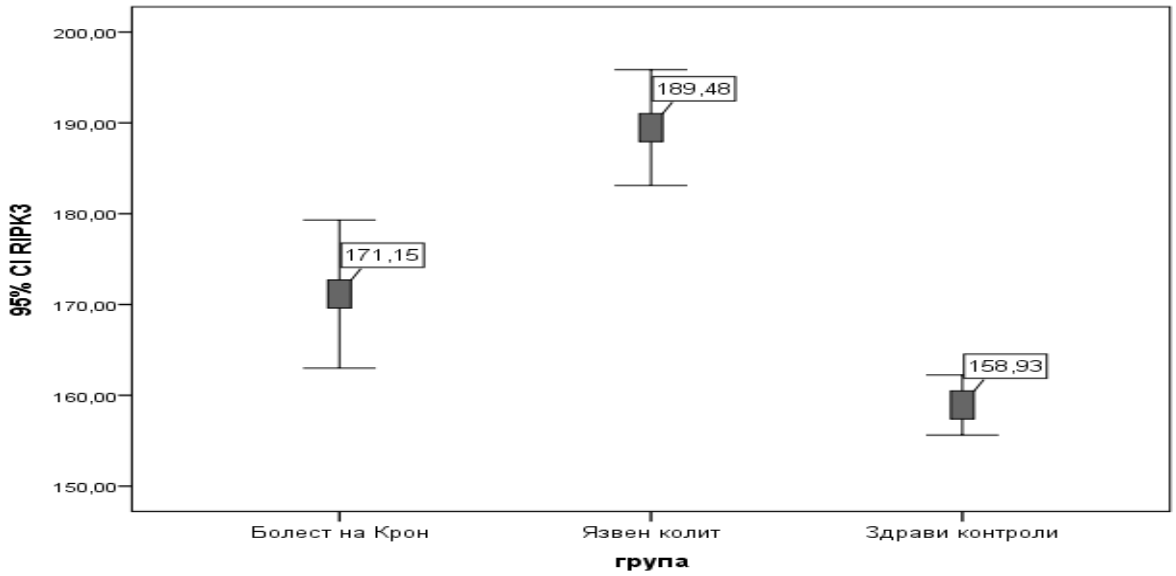


Fig. 13. Mean values of nuclear expression of RIPK3

An additional analysis of RIPK3 expression in IBD patients and healthy controls found that high marker expression resulted in a 4.14-fold higher risk of high IBD activity (RR = 4.15 (2.01-8.57); p <0.001).

In the assessment of the distinction between CD patients and UC patients according to the expression of RIPK3, it was found that high expression is associated with more than 2 times more likely IBD patients to have UC (RR = 2.61 (1.41-4.85)); <0.05).

4.2. Study of the level of RIPK3 expression in endoscopic, altered areas or intestinal resect in CD patients and comparison with clinical, laboratory, endoscopic and histological indices of activity.

In the analysis of the relationship between RIPK3 expression and the age of patients with CD and the age of the disease, no relationship was found between the indicators. There was no significant difference in the expression of RIPK3 according to age at the onset of CD, with patients with onset in the lowest age group (A1 <16 g) showing higher levels of marker expression than others (Fig. 14).

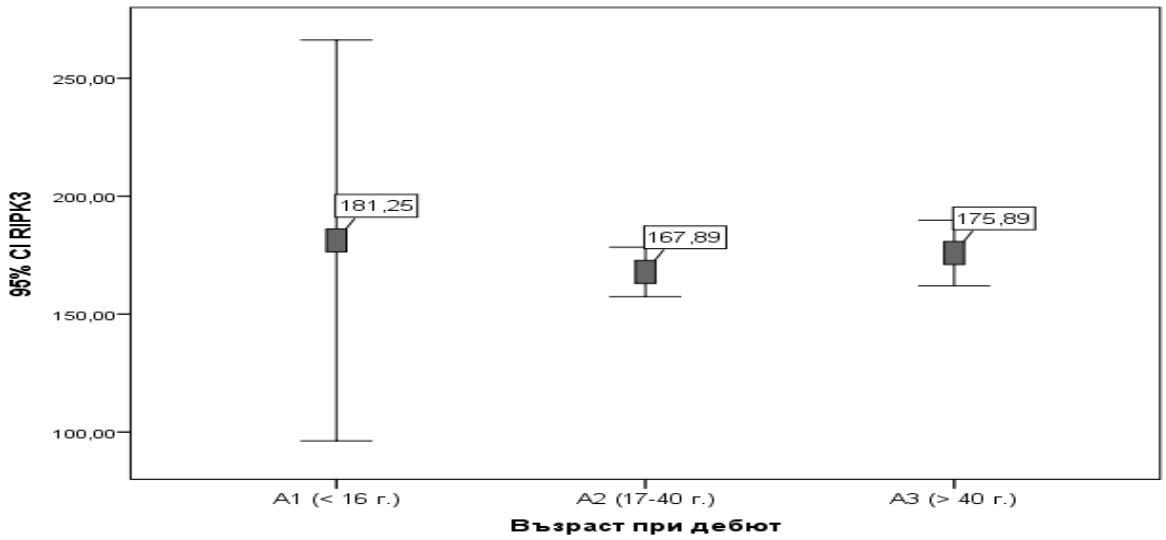


Fig. 14. Mean values of RIPK3 according to the age group of CD patients at onset

No relationship was found with regard to gender, although in women the expression was slightly lower (Fig. 15).

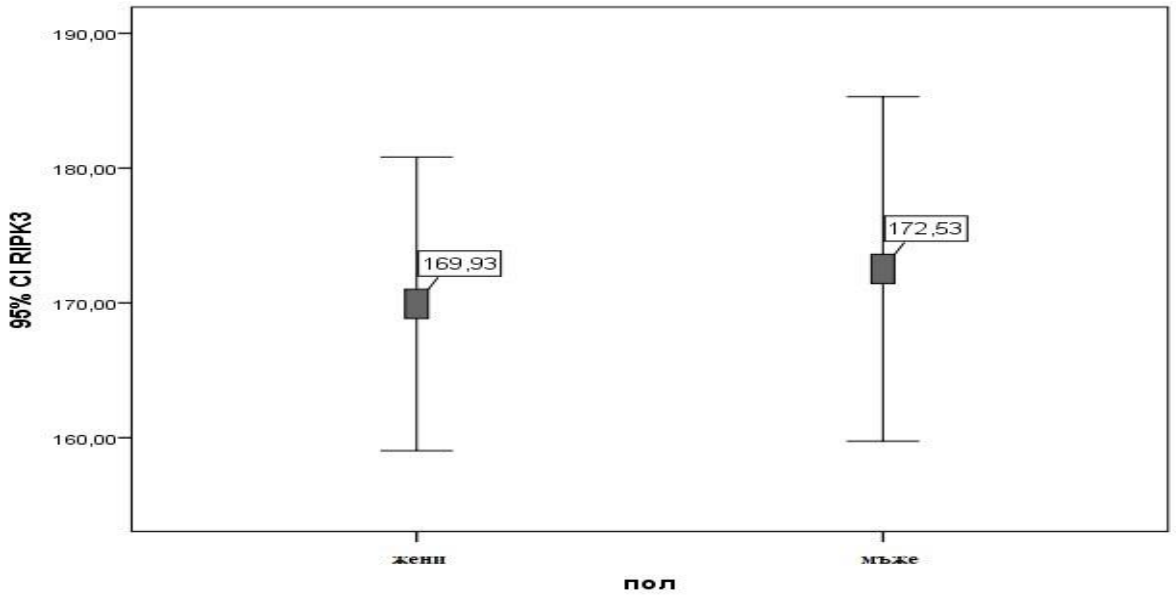


Fig. 15. Mean values of RIPK3 according to gender

Of the patients studied, only 12 (14.1%) had the onset of CD, and in these patients a slightly lower expression of RIPK3 was observed (Fig. 16).

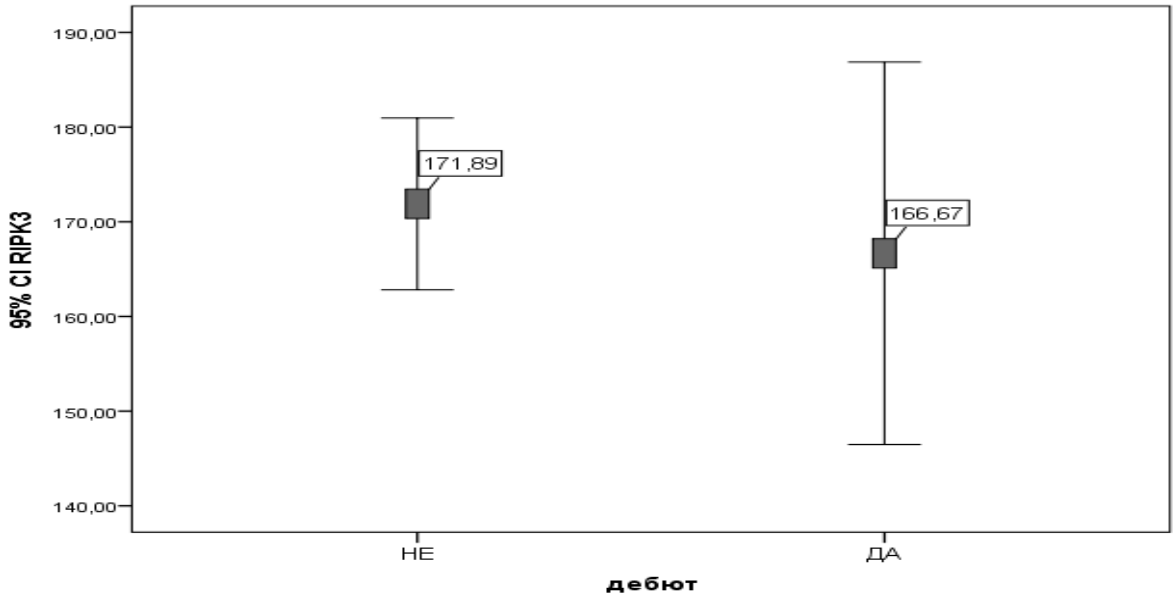


Fig. 16. Mean values of RIPK3 in patients with debut of CD

The mean number of hospitalizations of CD patients was 6.54 ± 3.48, with a minimum number of 1 and a maximum of 15. Although not statistically significant, there was a weak positive relationship between the number of hospitalizations and RIPK3 expression ($r = 0.213$; $p = 0.137$) (Fig. 17).

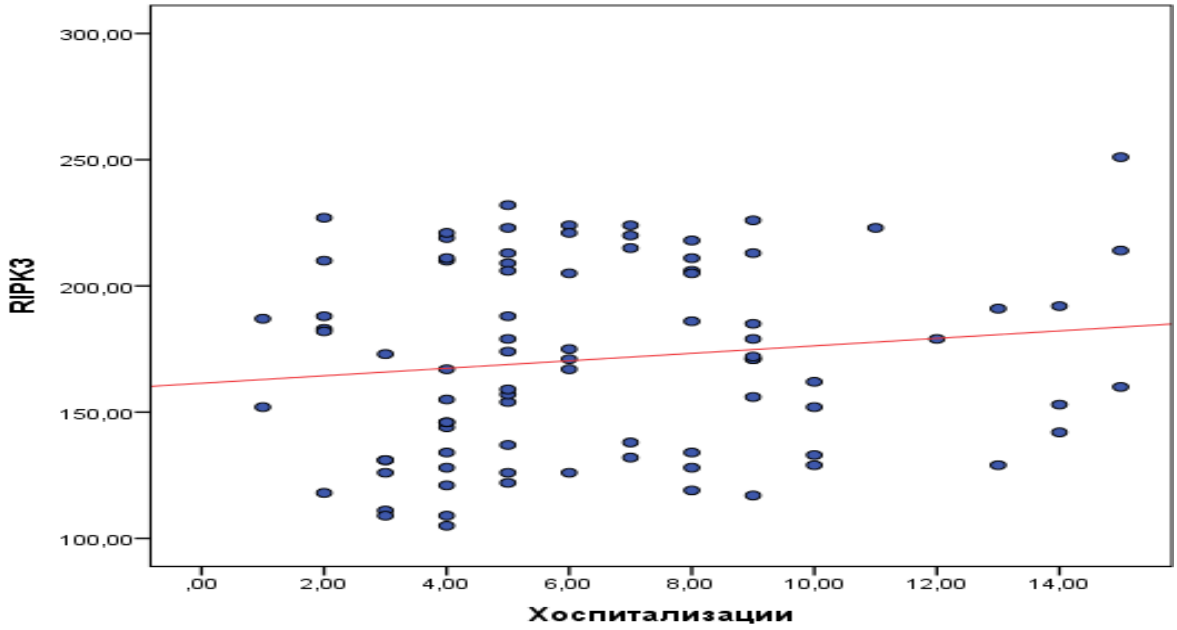


Fig. 17. Correlation analysis between the number of hospitalizations and the expression of RIPK3

There was a significant difference in the expression of RIPK3 according to the localization of CD ($p < 0.05$), with the highest expression of RIPK3 in the ileum (L1) and colon (L2) in the extensive range of the disease affecting the upper GIT (L4) - L1 + L4 (225.50) and L2 + L4 (218.00). On the other hand, involvement of only the terminal ileum (L1) is characterized by the lowest expression of the marker (158.52), while the column localization retains its position of localization characterized by high expression of RIPK3 (184.87) (Fig. 18).

There was no significant difference in the expression of RIPK3 according to the behavior of disease, and in patients with stricturing and fistulizing form there was almost no difference in the expression of the marker (Fig. 19). However, the highest expression of the marker is in phenotype B2 + B3 - stricturing with fistulizing form, followed by inflammatory form (B1).

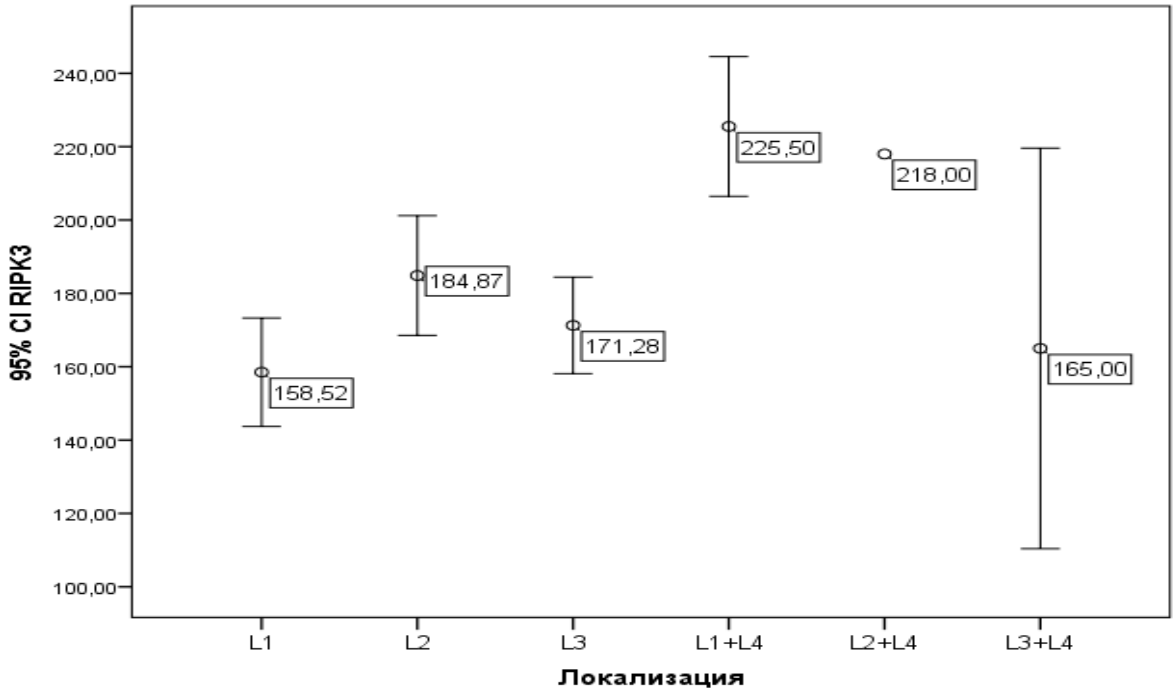


Fig. 18. Mean values of RIPK3 expression according to the localization of CD

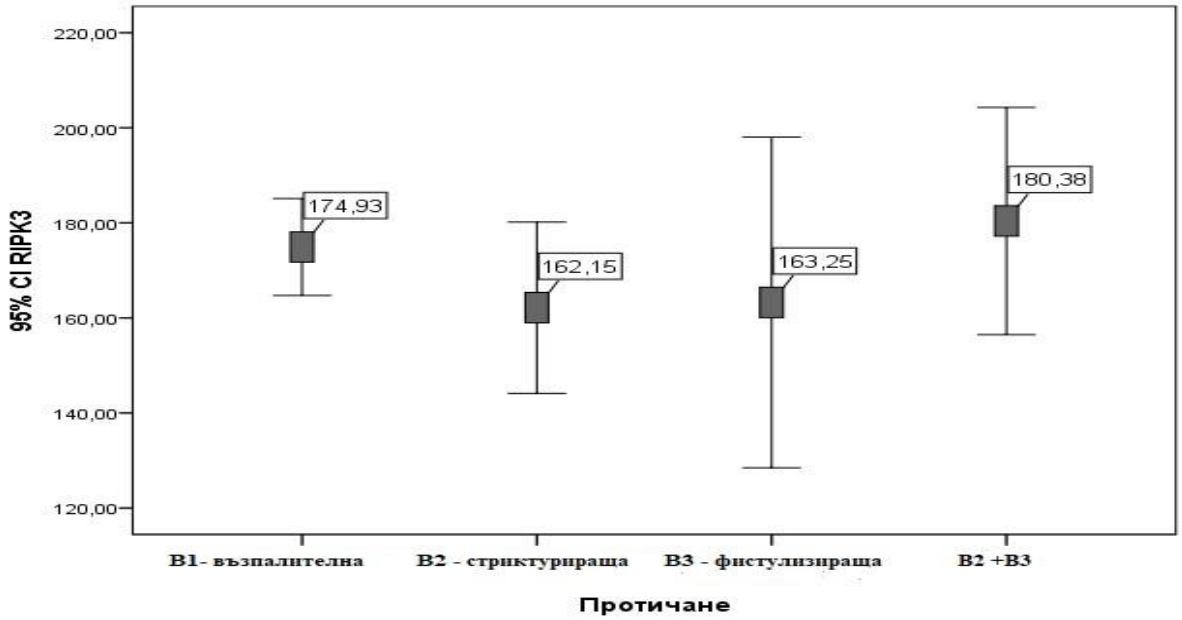


Fig. 19. Mean values of RIPK3 expression according to the behavior of CD

Perianal disease further increased RIPK3 expression, with CD patients who had concomitant perianal disease being characterized by high expression of the marker (181.85 to 167.86 for those without perianal disease; $p < 0.05$, respectively).

There is a tendency to increase the expression of RIPK3 with clinical activity in CD (CDAI) (p = 0.038), with patients in remission having the lowest expression (154.67), while patients with severe activity have overexpression of the marker (180.0) (Fig. 20).

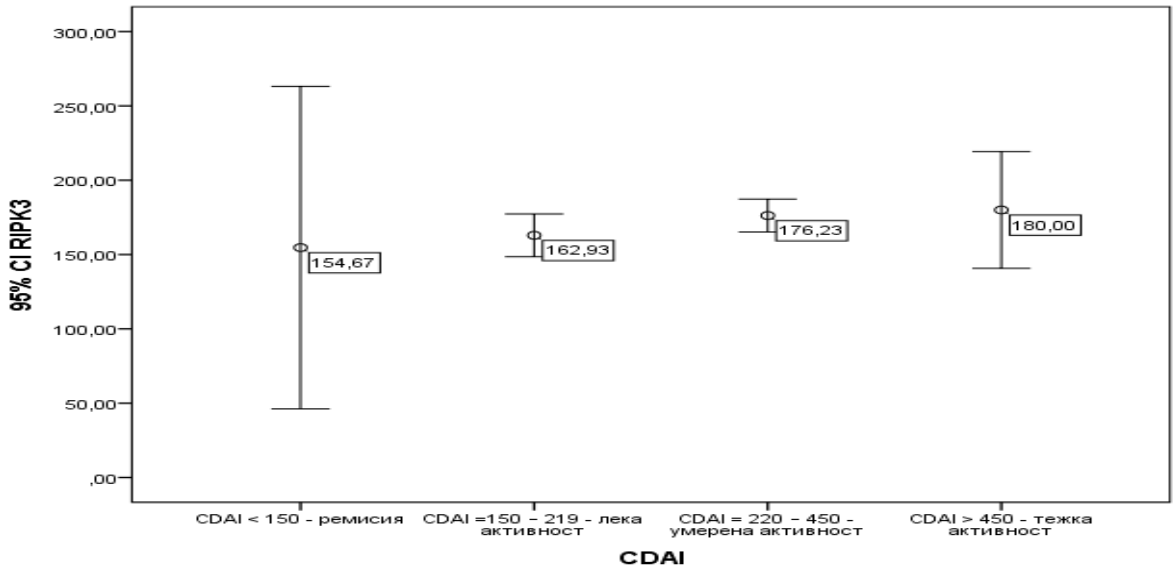


Fig. 20. Mean values of RIPK3 expression according to the clinical activity of CD

Patients who frequently relapsed (≥2 times per year) had significantly higher RIPK3 expression than those who rarely relapsed (≤1 times per year) (181.36 versus 162.50; p = 0.021, respectively) (Fig. 21). There was a proportionally weak relationship between the frequency of relapse and the expression of RIPK3 (r = 0.250; p = 0.021), which shows that with increasing relapses, the expression of the marker increases.

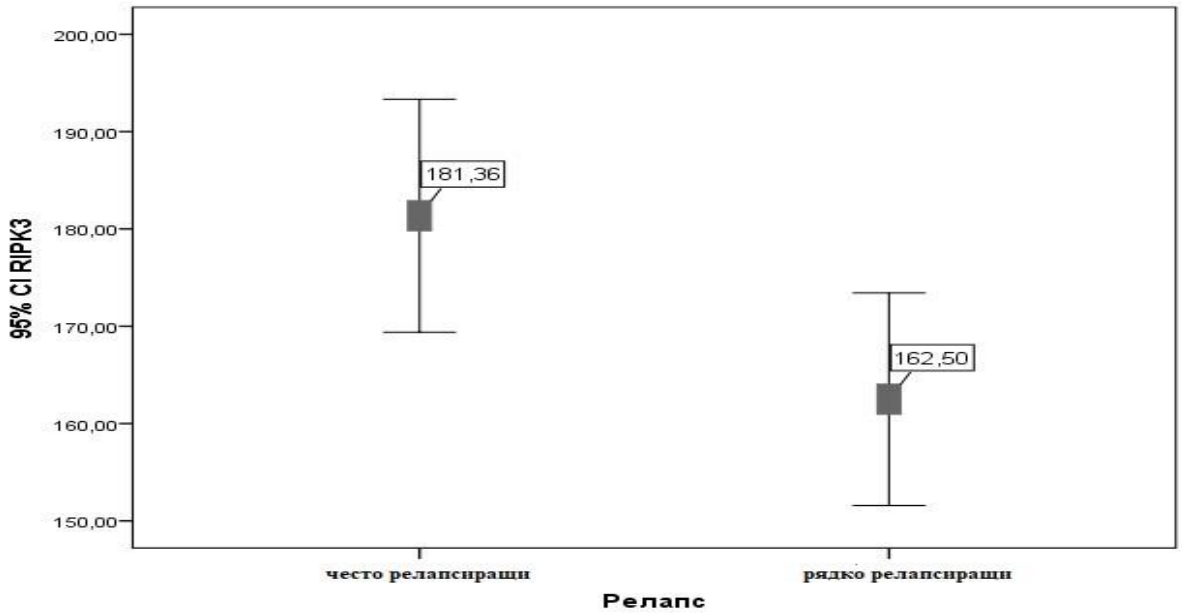


Fig. 21. Mean values of RIPK3 expression according to the frequency of relapse in CD patients

No association was found between RIPK3 expression and some clinical indicators, such as the incidence of diarrhea during hospitalization. On the other hand, rectal bleeding is associated with higher marker expression (p = 0.043) (Fig. 22).

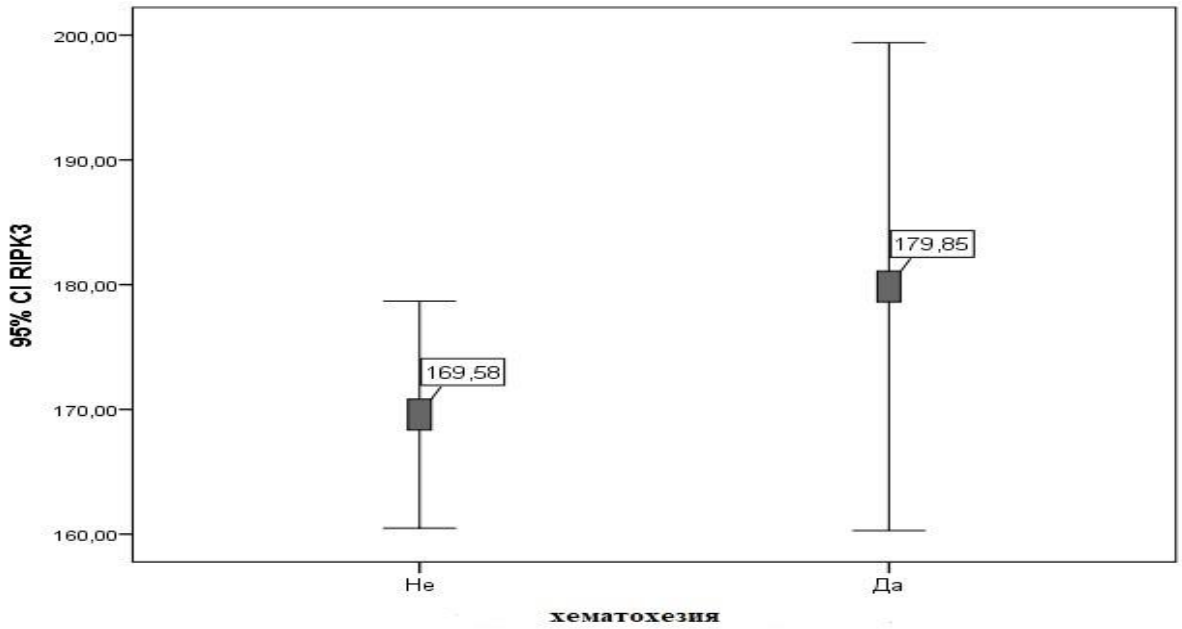


Fig. 22. Mean values of RIPK3 expression according to the presence of hematochezia

Although there was no significant difference, the presence of abdominal pain was also associated with higher expression of RIPK3 (171.88 for CD patients and abdominal pain and 166.27 for CD patients without abdominal pain, respectively). Only two CD patients with palpable abdominal formation had significantly elevated levels of nuclear marker expression (191.50 ± 41.72).

Interestingly, in patients with established weight loss, decreased expression of RIPK3 was observed (160.84 ± 39.02), while in CD patients who did not show a change in weight, the expression of the marker remained high (175.45 ± 36.79).

As shown in Table 12 with extraintestinal manifestations are 63.5% of CD patients who have a mean RIPK3 expression of 173.81 ± 39.45 (Fig. 23), which is higher than patients without extraintestinal manifestations.

The most common are joint manifestations (40.0%) and iron deficiency anemia (38.8%) (Fig. 24).

A significant difference in the expression of RIPK3 was found in the different types of extraintestinal manifestations ($p < 0.05$) (Fig. 25). Patients with cutaneous manifestations had the lowest expression of the marker (156.33), and patients with hepatic extraintestinal manifestations (hepatic steatosis) had the highest expression (182.5).

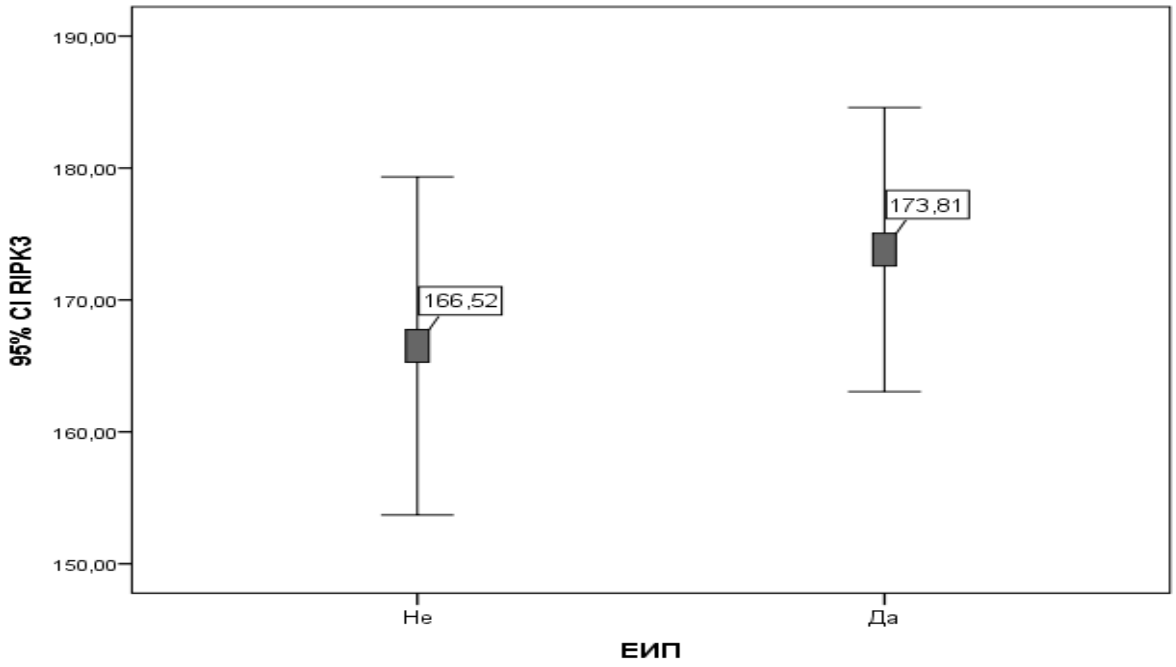


Fig. 23. Mean values of RIPK3 expression according to the presence of extraintestinal manifestations

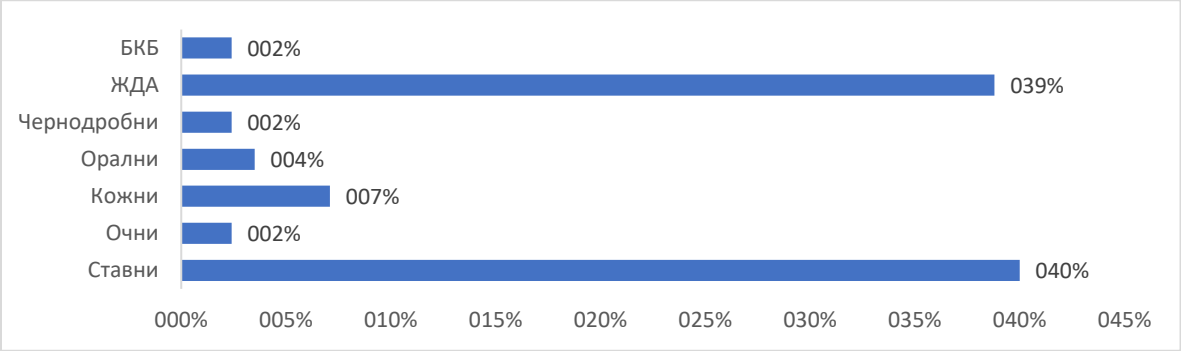


Fig. 24. Types of extraintestinal manifestations in patients with CD

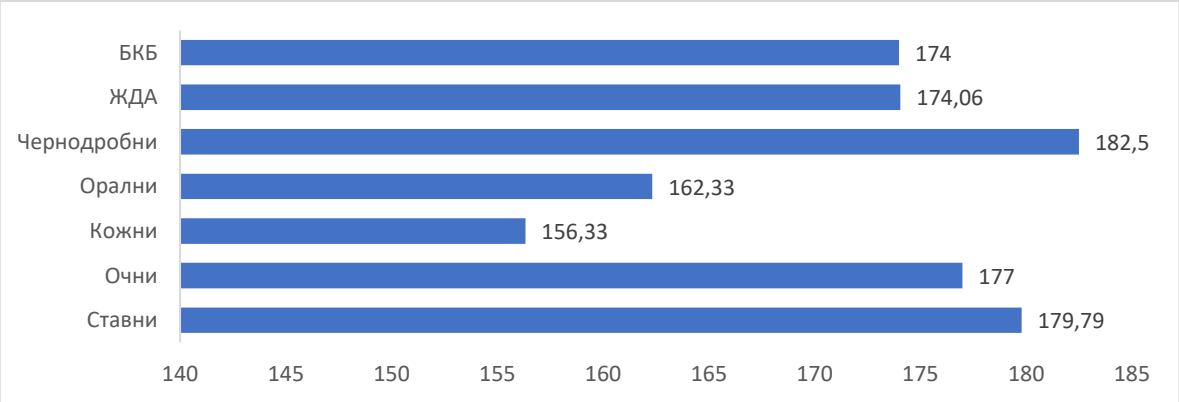


Fig. 25. Mean values of RIPK3 expression according to extraintestinal manifestations types in patients with CD

There was no significant difference in the expression of RIPK3 according to the presence or absence of thromboembolic complications, which may be due to the small number of patients (5 patients).

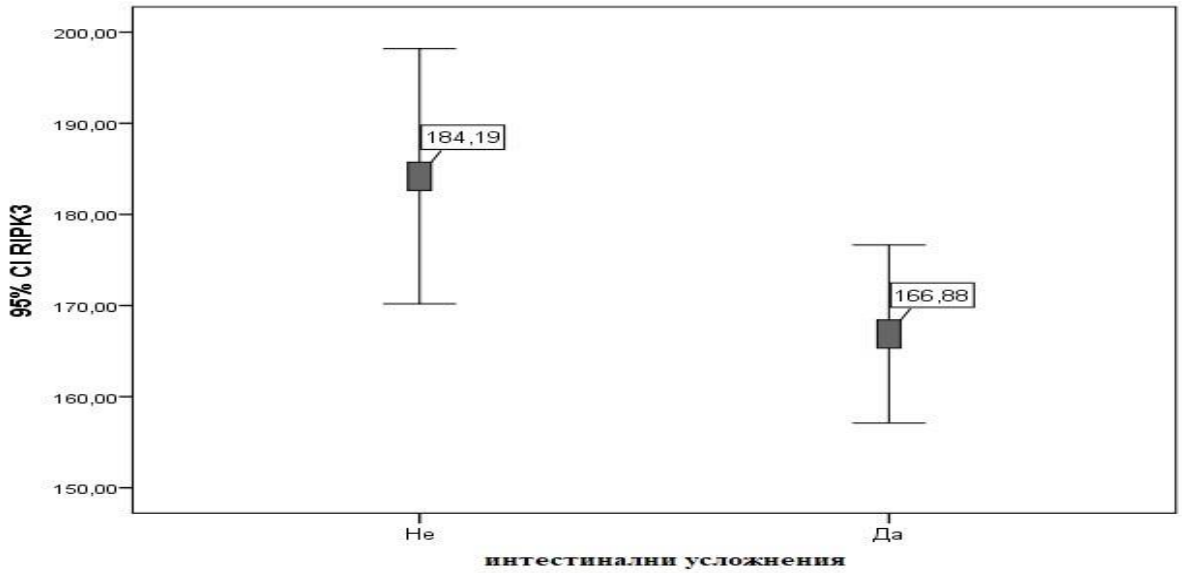


Fig. 26. Mean values of RIPK3 according to the presence or absence of intestinal complications

In patients with intestinal complications (stenosis, fistulas, abscesses, pseudopolyposis, subileus, ileus, malabsorption and others shown in Fig.27), significantly lower expression of RIPK3 was observed compared to those without complications (p = 0.048) (Fig. 26).

The most common intestinal complications are stenoses (47.1%) and fistulas (27.1%). Pelvic osteomyelitis is a manifestation in one patient (Fig. 27).

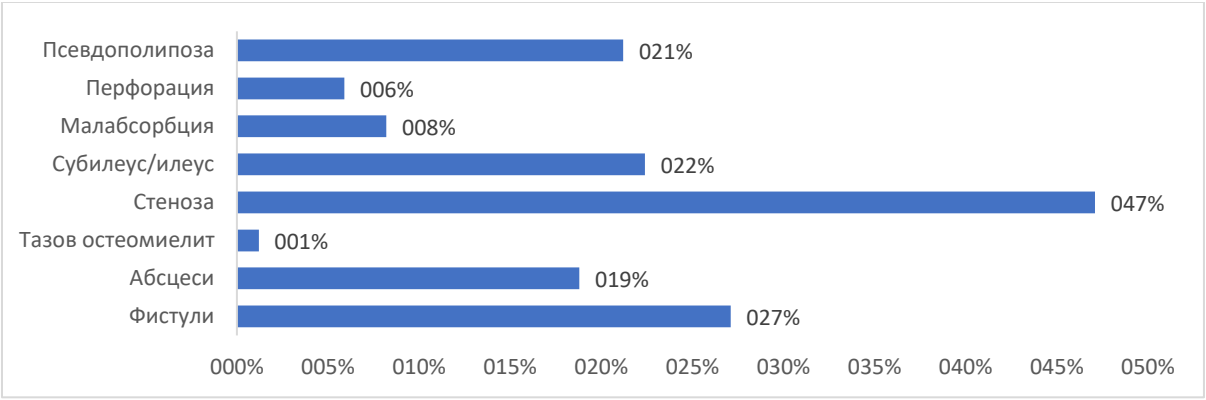


Fig. 27. Distribution of patients with CD according to the type of intestinal complications

A significant difference in the expression of RIPK3 was found in the different types of intestinal complications (p <0.05) (Fig. 28).

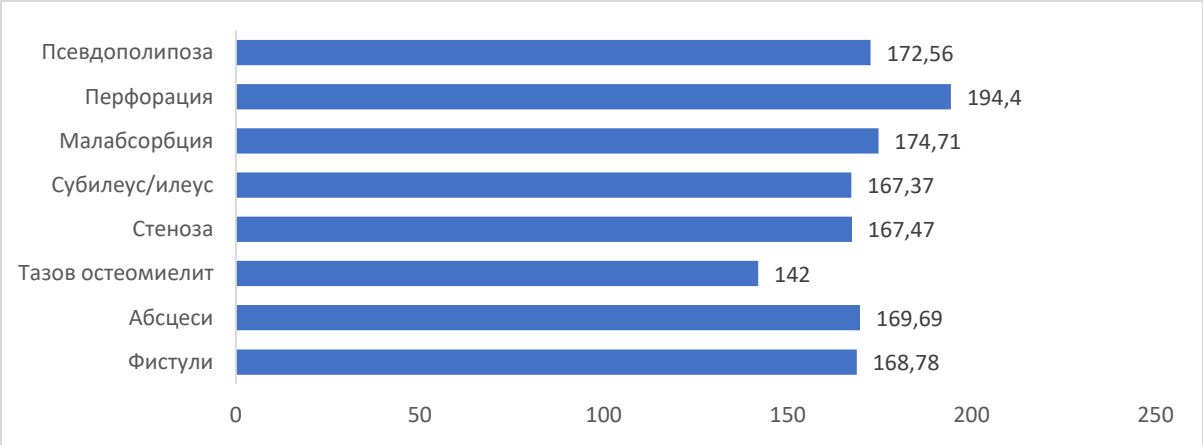


Fig. 28. Mean values of RIPK3 expression according to the type of intestinal complication

Surgical intervention underwent 30.6% of CD patients, with a mean RIPK3 expression of 168.08 ± 45.06. Patients with appendectomy in the past had the lowest levels of marker expression (156.22), while those with hemicolectomy and small bowel resection found high expression (184.14 and 183.60, respectively).

A significant difference in RIPK3 expression was found between CD patients who discontinued conventional therapy and those on treatment (p = 0.036) (Fig. 29). The group of patients without treatment includes 10 patients with the onset of CD who took antidiarrheal drugs until diagnosis.

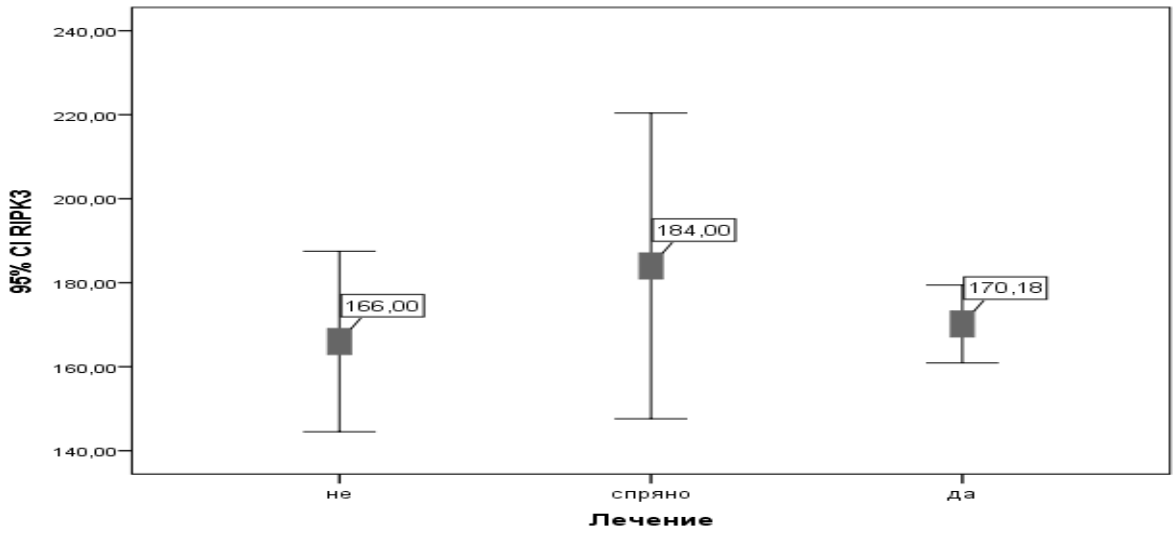


Fig. 29. Mean values of RIPK3 expression according to treatment

In Fig. 30 presents the results of the analysis of RIPK3 expression according to the type of therapy performed. The results show that CD patients on corticosteroid therapy have the

lowest expression. While treatment with immunomodulators and 5-ASA (5-aminosalicylic acid) did not show a significant difference in the expression levels of the marker.

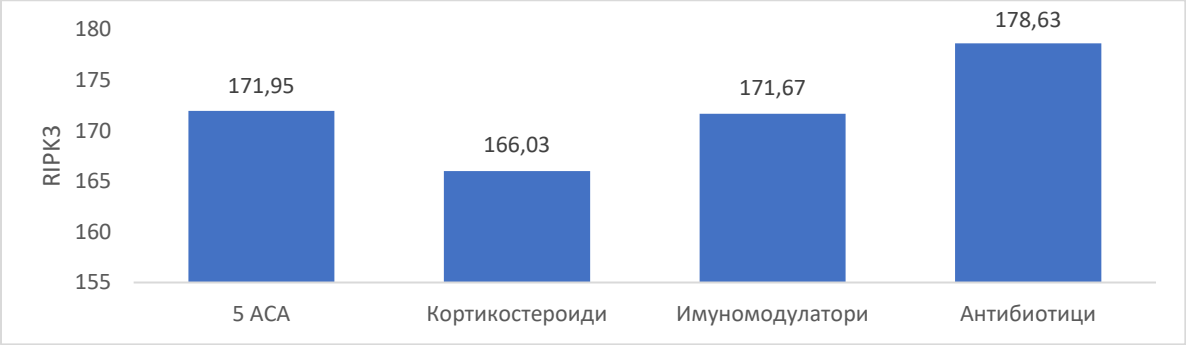


Fig. 30. Mean values of RIPK3 expression according to the type of therapy

Patients with CD who are currently on biologic therapy or on biologic therapy have overexpression of the necroptosis marker. Those not on biologic therapy had lower RIPK3 expression (p = 0.018) (Fig. 31). These are patients with debut of CD and those on conventional treatment.

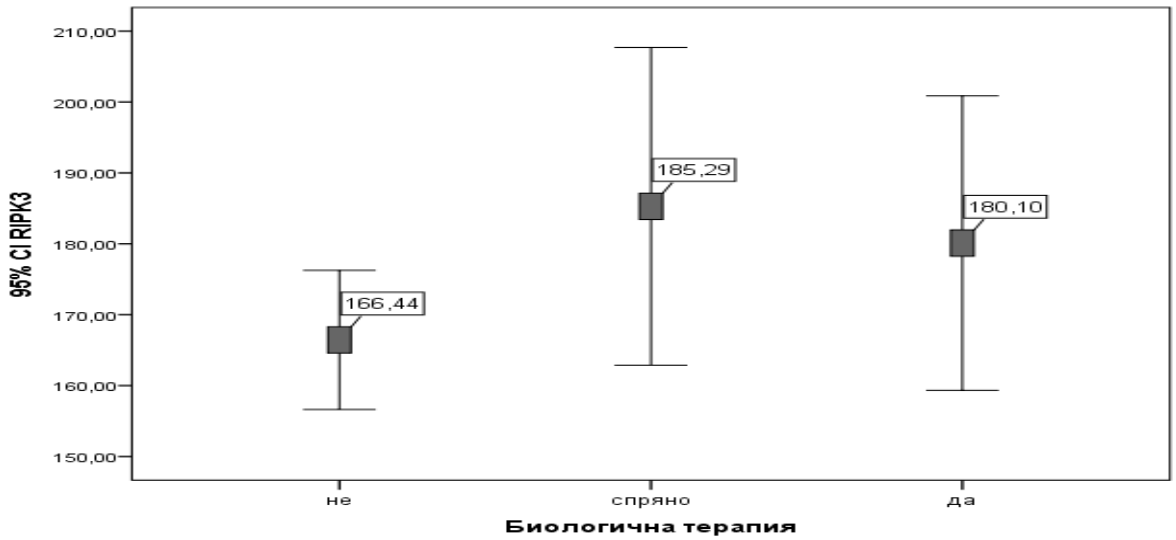


Fig. 31. Mean values of RIPK3 expression according to biological therapy

The significant proportion of CD patients (71.8%) have a different number of concomitant diseases, and the expression of RIPK3 in them is 173.51 ± 37.33. More than half of the patients have accompanying gastritis, HP (-) (62.4%) and 15.3% have hypertensive disease. Comparative analysis of RIPK3 expression according to the presence of gastritis showed a significant difference (p <0.05), with patients with chronic gastritis having higher expression of the marker (Fig. 32).

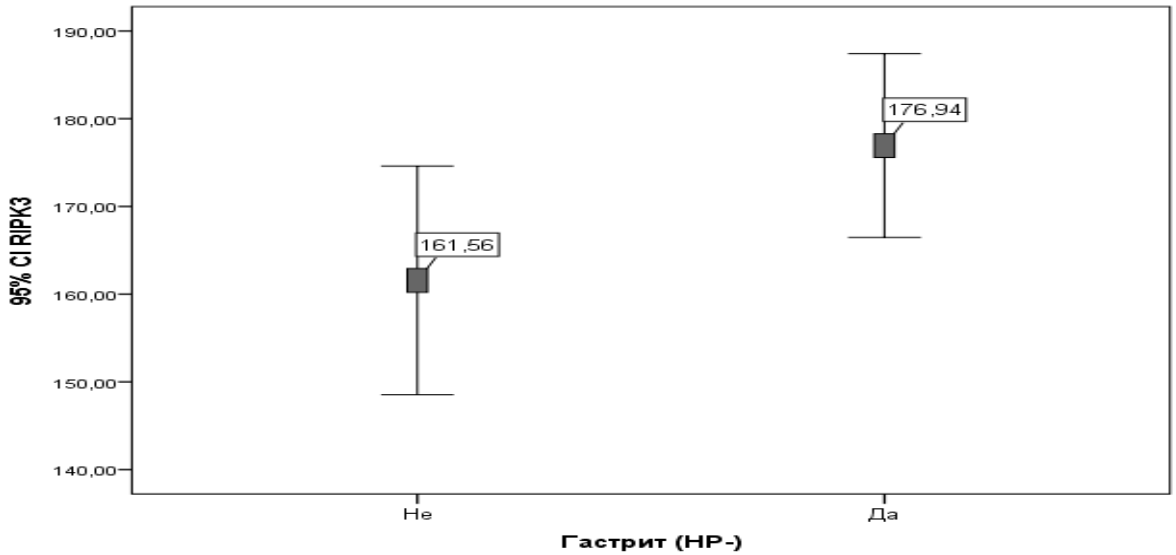


Fig. 32. Mean values of RIPK3 expression according to the the presence of gastritis HP(-)

No relationship was found between ESR and RIPK3 levels, but it can be said that in individuals with normal ESR levels just over half have low RIPK3 expression <163.5, while more than half of patients with elevated ESR levels have high expression of the marker (Fig. 33).

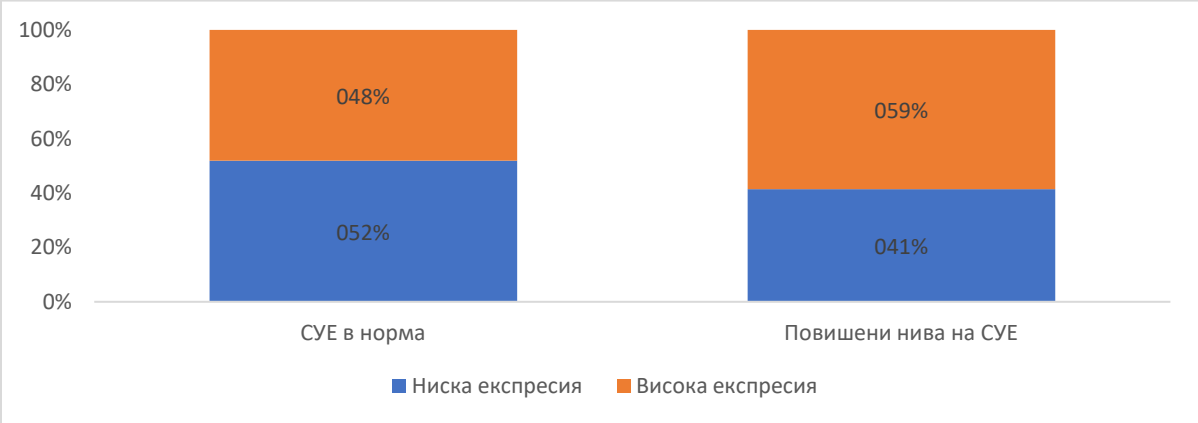


Fig. 33. Distribution of patients with CD according to ESR levels and RIPK3 expression

No dependence was also found on CRP levels and RIPK3 expression, with an increase in patients with increased expression, regardless of CRP levels. A similar trend is observed with regard to the levels of leukocytes, thrombocytes, albumin, ferritin, hemoglobin and serum iron.

No association was found between the presence of accompanying intestinal infection with Cl. Difficile and nuclear expression of RIPK3, and it can be said that patients with infection

have lower expression of the marker (144.00 and 172.65 for patients with CD without infection, respectively).

There was a significant difference and a proportionally weak relationship between the nuclear expression of RIPK3 in patients with and without endoscopic activity (r = 0.236; p = 0.031) (Fig. 34).

In 8 CD patients, the diagnosis was made intraoperatively for ileus, and the detected expression of RIPK3 in the resect was 197.5 (p = 0.038).

Another 10 patients underwent postoperative endoscopic activity, which was assessed with the Rutgeerts score. There was no significant difference in the expression of the necroptosis marker at Rutgeerts scores 1 to 3.

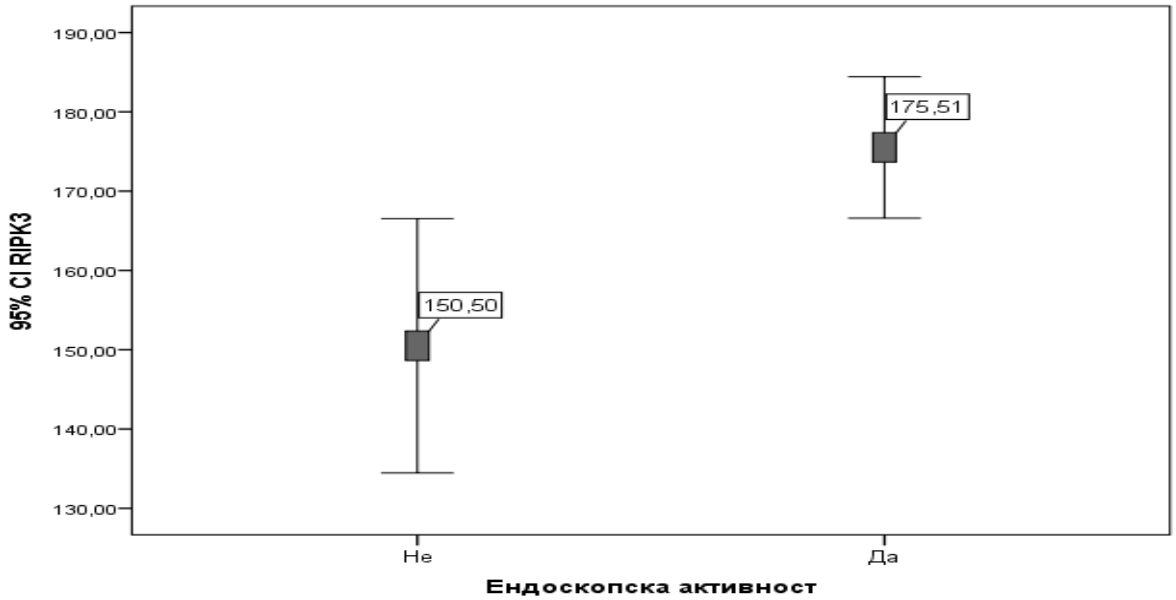
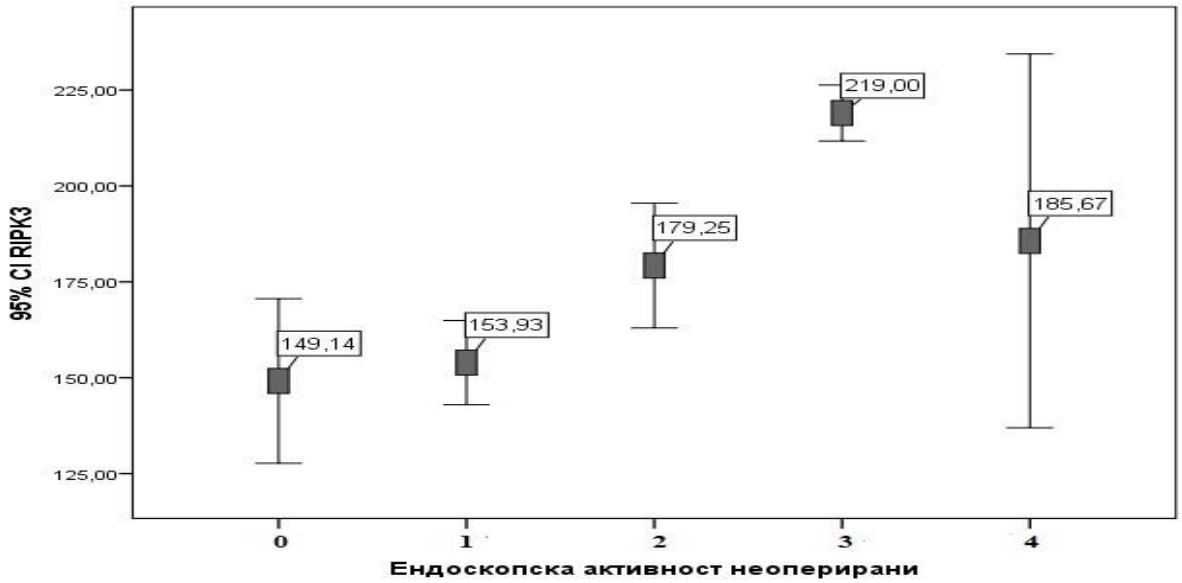


Fig. 34. Mean values of RIPK3 expression according to endoscopic activity

In the majority of patients who did not undergo surgery, endoscopic activity was assessed using the Simplified Endoscopic Mucosal Assessment System (SEMA-CD), with a significant difference in the expression of RIPK3 according to the degree p <0.001) (Fig. 35).



Фиг. 35. Mean values of RIPK3 expression according to Simplified endoscopic mucosal assessment for Crohn's disease (SEMA-CD).

According to the established results, it can be said that regardless of whether the expression of RIPK3 was examined in intraoperative material or in endoscopic biopsies, Rutgeerts score or SEMA-CD over 3 showed overexpression of the marker..

When comparing the expression of RIPK3 with the histological activity, it was found that with increasing inflammatory activity, the expression levels of the marker also increased ($p < 0.001$). In CD patients and marked histological activity, the expression values of the necroptosis marker were twice as high as in those without activity (217.57 versus 138.21, respectively) (Fig. 36).

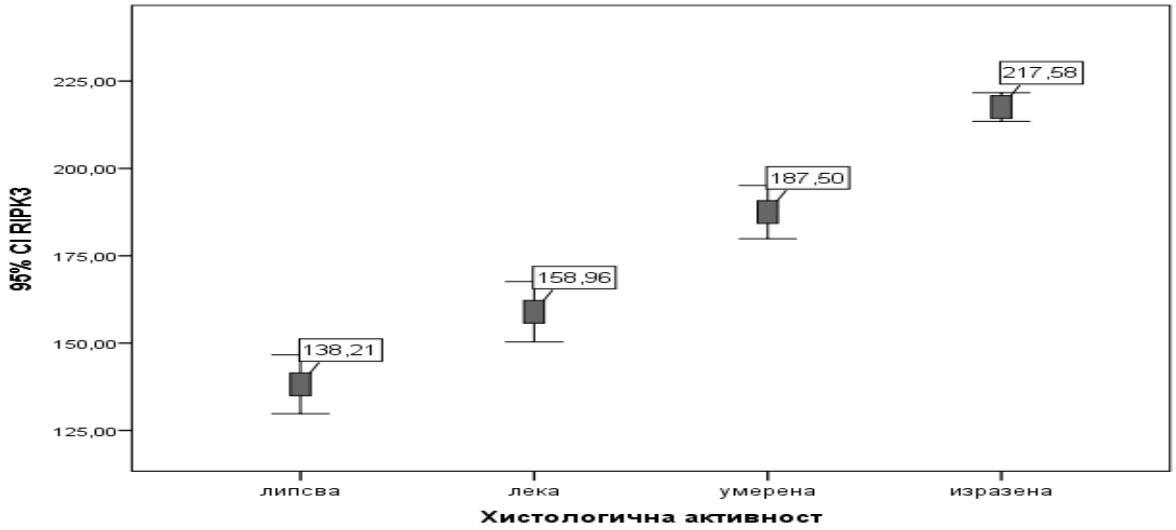


Fig.36. Mean values of RIPK3 expression according to the histological activity of patients with CD

There was a strong proportional relationship between histological activity and RIPK3 expression (r = 0.874; p <0.001), with 76.4% of cases of high expression of the necroptosis marker being associated with high inflammatory activity in CD patients.

In the case of colon localization of CD, the analysis of histological samples shows that with the increase of the percentage of cryptite the expression of the marker for necroptosis also increases (r = 0.464; p <0.001) (Fig. 37).

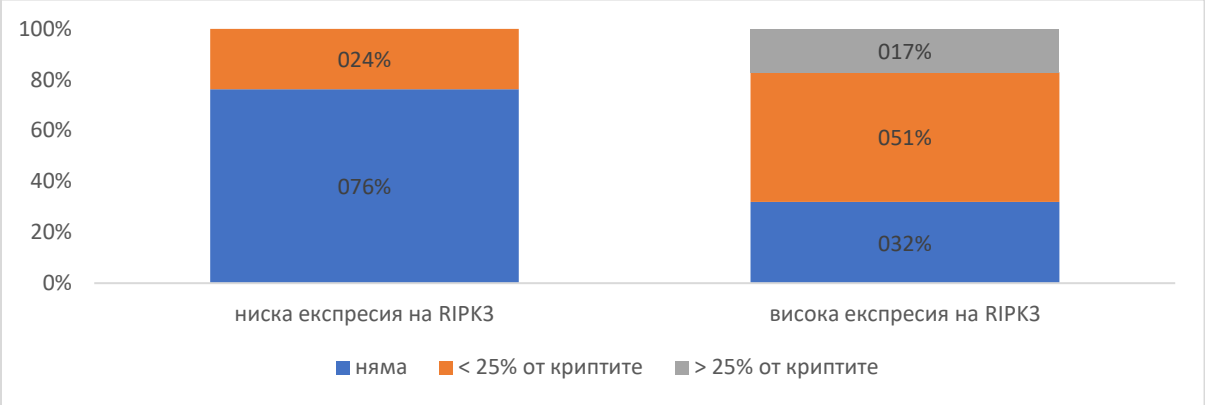


Fig. 37. Expression of RIPK3 according to the percentage of crypt involvement

A similar trend is found for crypt abscesses (Fig. 38). RIPK3 expression correlated proportionally with the percentage of crypt abscesses detected (r = 0.325; p = 0.002).

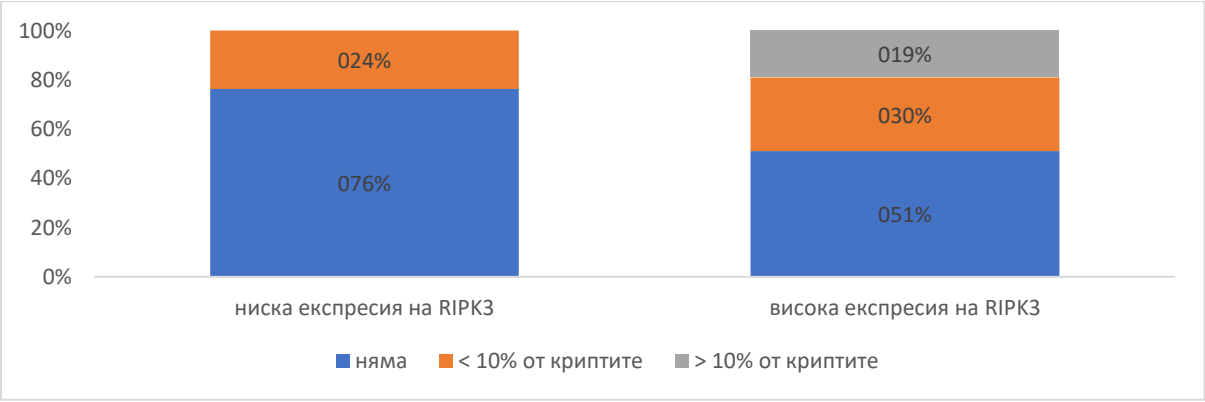


Fig. 38. Expression of RIPK3 according to the percentage of crypt abscesses

A strong relationship between erosions and high RIPK3 expression was found (r = 0.597; p <0.001) (Fig. 39).

With regard to granulomas, no difference in RIPK3 expression was found.

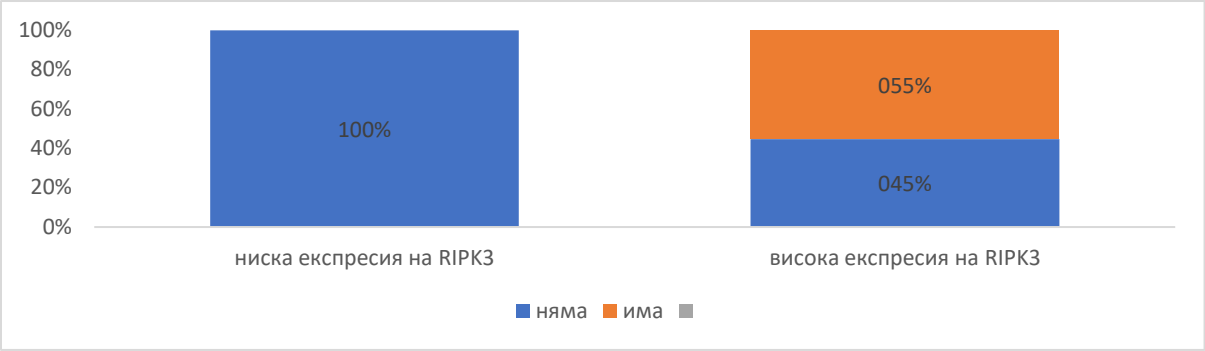


Fig. 39. Expression of RIPK3 according to the presence of erosions

4.3. Study of the level of RIPK3 expression in endoscopically altered areas in UC patients and comparison with clinical, laboratory, endoscopic and histological indices of activity.

The analysis of the relationship between RIPK3 expression and the age of UC patients and the years of the disease did not show an association between the indicators.

There was no significant difference in the expression of RIPK3 according to age at debut of UC, with a tendency to increase expression with increasing age (Fig. 40).

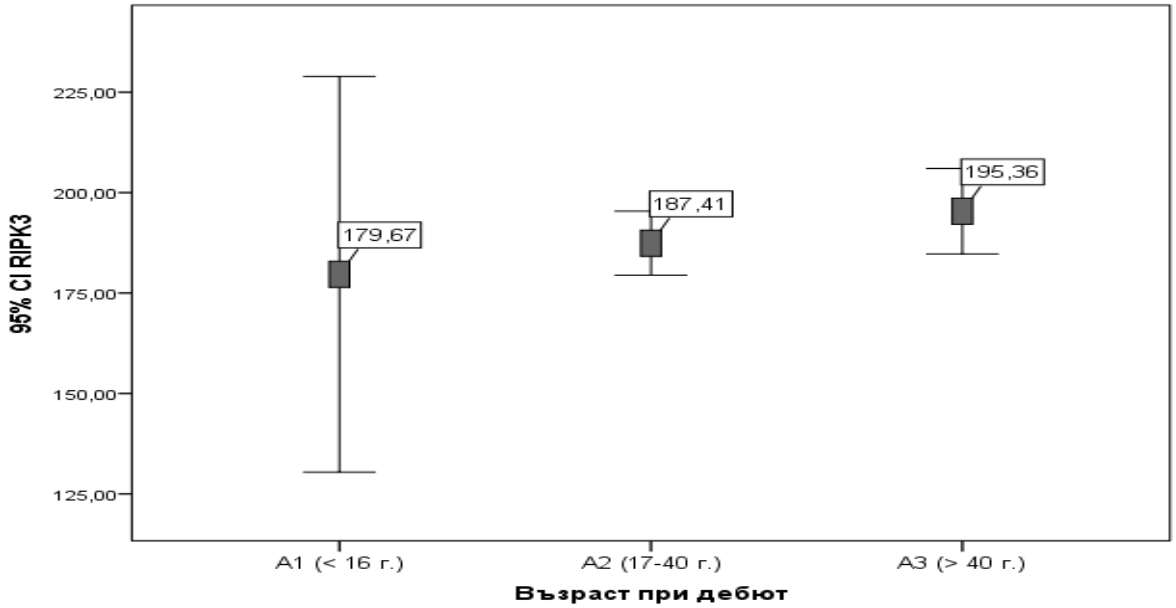


Fig. 40. Mean values of RIPK3 expression according to the age group of CD patients with debut

No association was found between the necroptosis marker and gender, and the expression of the marker was 189 in both.

Of the patients studied, only 14 (16.5%) had the debut of UC, and in these patients a slightly higher expression of RIPK3 was observed (Fig. 41).

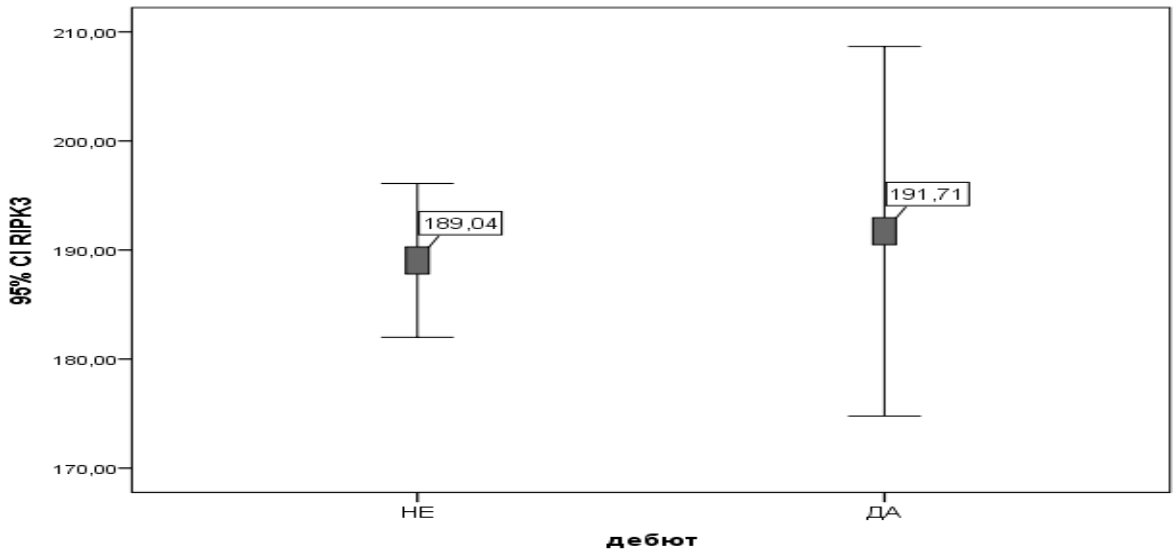


Fig. 41. Mean values of RIPK3 expression in patients with debut of UC

The mean number of hospitalizations of UC patients was 4.99 ± 2.93, with a minimum number of 1 and a maximum of 15. There was no relationship between the number of hospitalizations and RIPK3 expression in this group of patients..

No difference in RIPK3 expression was found according to the UC extent, as with E1 extent there is only one patient, with marker expression for necroptosis (186.0). The difference in RIPK3 expression between UC patients with E2 and E3 was insignificant (194.53 for E2 and 185.39 for E3, respectively), but the expression was high in both cases..

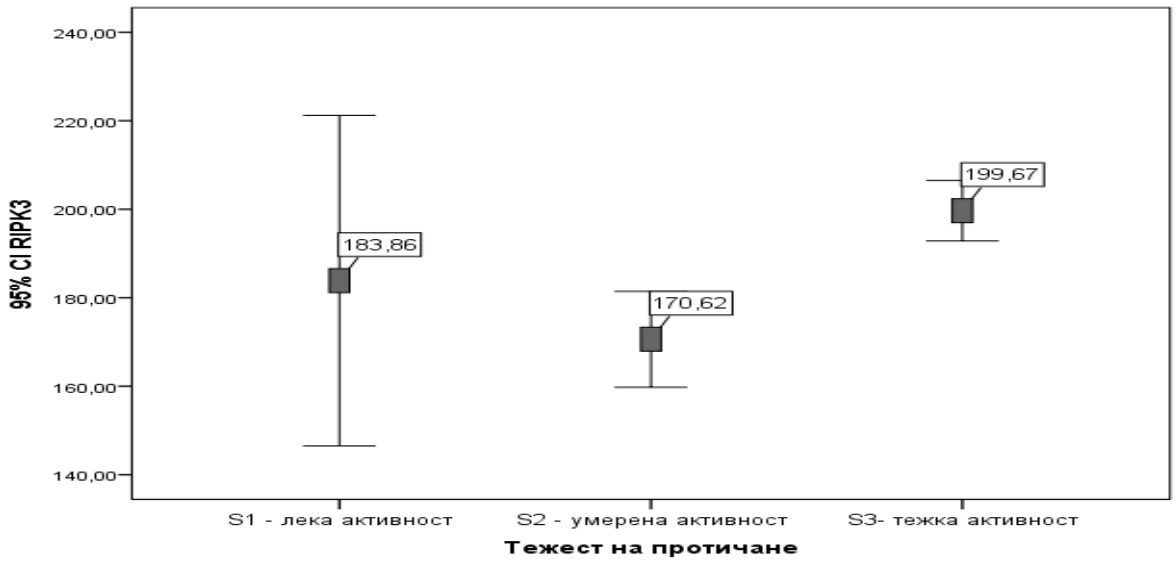


Fig. 42. Mean values of RIPK3 expression according to the severity of the UC

A significant difference in the expression of RIPK3 was found with respect to the severity of UC (S), estimated by the Montreal classification (p <0.001). (Fig. 42). A directly proportional moderate relationship between disease severity and RIPK3 expression (r = 0.354; p = 0.001) was also found, with severe disease characterized by overexpression of the marker (199.67).

Moderate proportional dependence was also found in the analysis of RIPK3 expression and UC activity, measured by the total Mayo score (r = 0.481; p <0.001). RIPK3 expression increased with increasing disease activity (p <0.001), with patients with severe disease activity having overexpression of the marker (211.71) (Fig. 43).

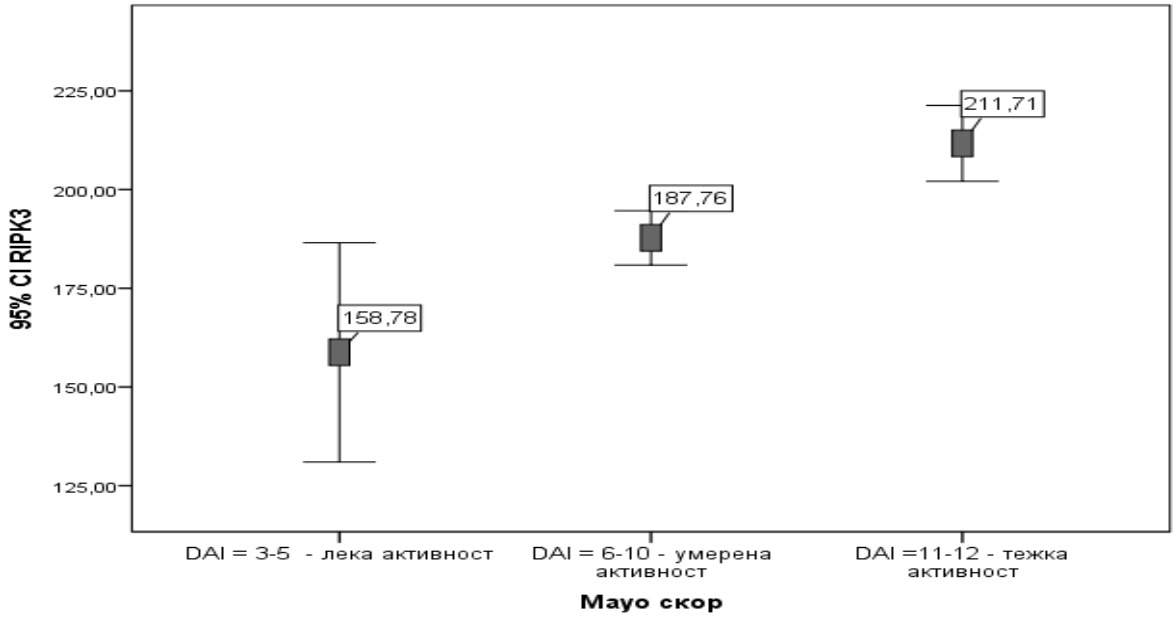


Fig. 43. Mean values of RIPK3 expression according to total Mayo score for UC activity (DAI, disease activity index)

The study of endoscopic activity assessed by the endoscopic Mayo score showed the same trend as the index of disease activity (Fig. 44). A directly proportional moderate relationship between RIPK3 expression and the degree of endoscopic activity was found (r = 0.363; p = 0.001), with patients with severe endoscopic activity having high marker expression (198.27).

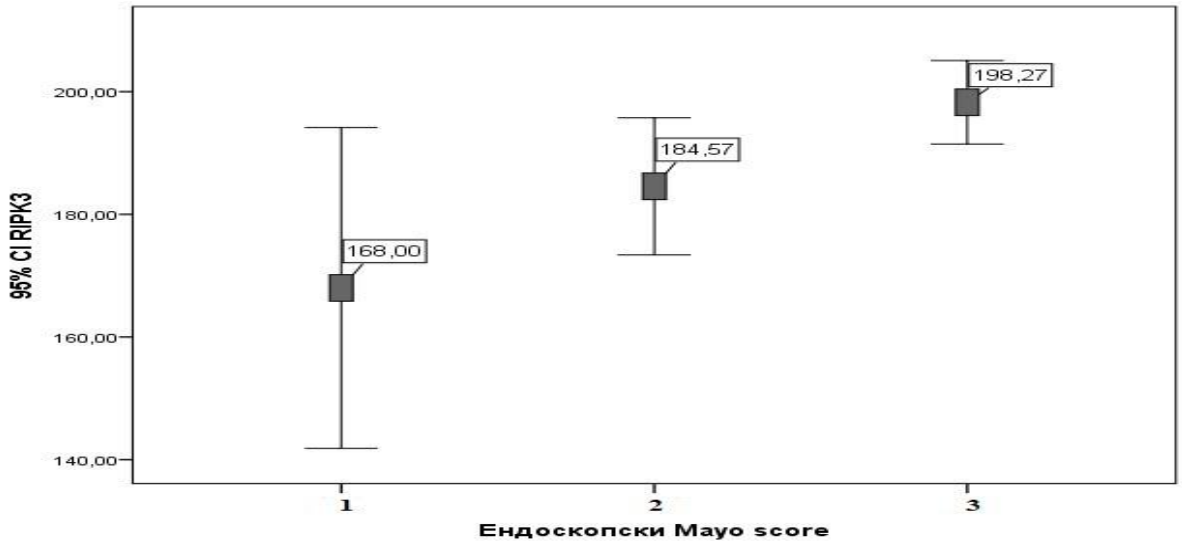


Fig. 44. Mean values of RIPK3 expression according to the endoscopic activity of UC

Patients who rarely relapsed (≤1 time per year) had significantly higher RIPK3 expression than those who frequently relapsed (≥2 times per year) (195.29 to 179.35, respectively; p = 0.016) (Fig. 45). There was an inversely weak relationship between the frequency of relapses in UC patients and the expression of RIPK3 (r = -0.261; p = 0.016), which shows that the increase in the frequency of relapses decreases the expression of the marker.

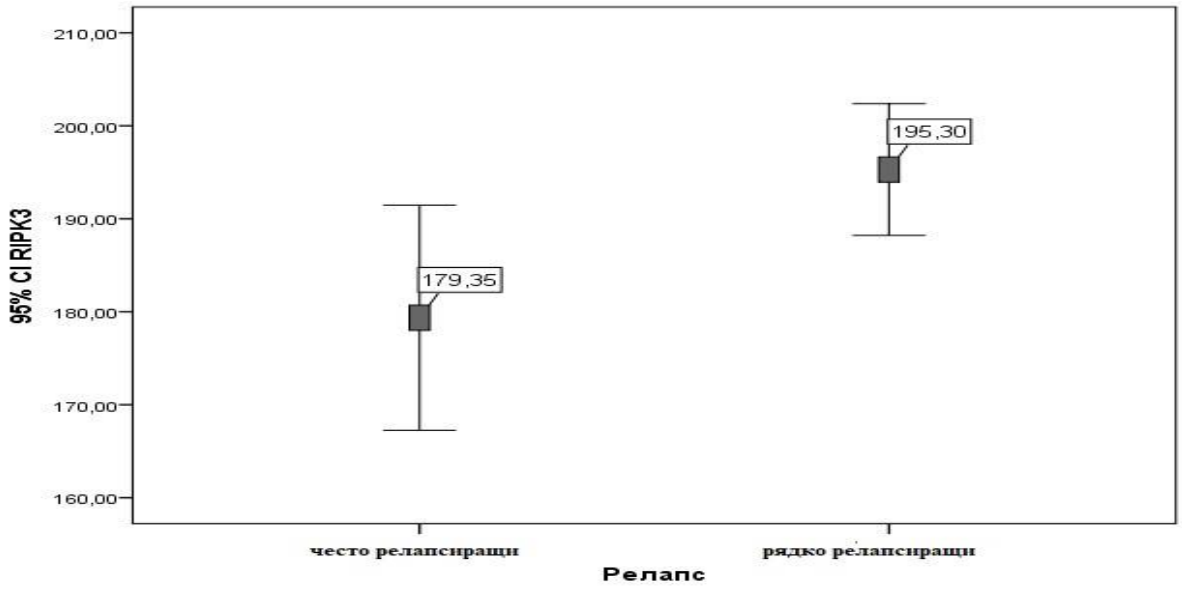


Fig. 45. Mean values of RIPK3 expression according to the frequency of relapses in UC patients

When comparing the expression of RIPK3 with the histological activity, it was found that with increasing inflammatory activity, the expression levels of the marker also increased

(p <0.001). In UC patients and marked histological activity, the expression values of the necroptosis marker were significantly higher than those in patients without activity (212.13 to 143.40, respectively) (Fig. 46).

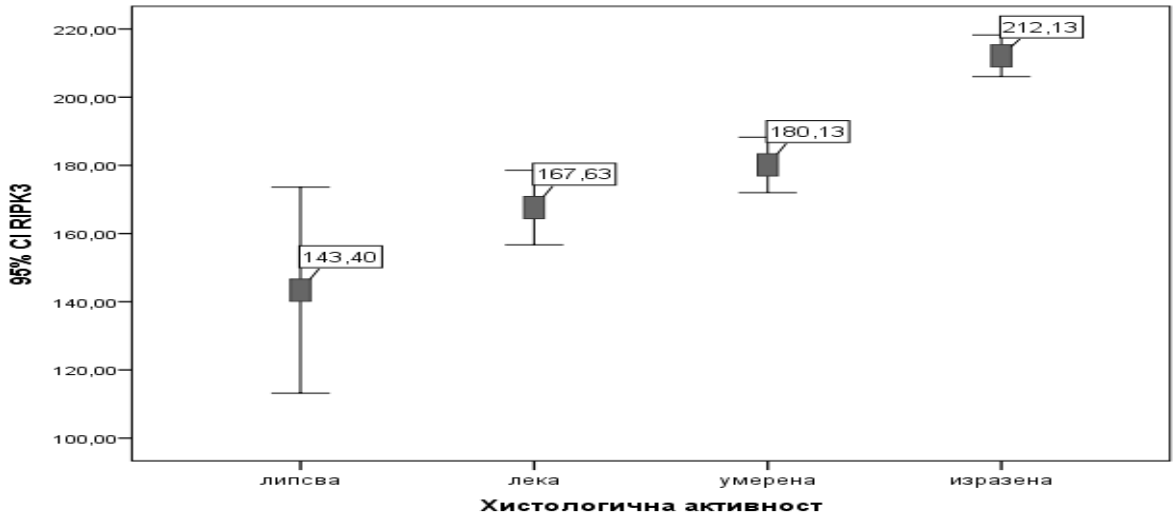


Fig.46. Mean values of RIPK3 expression according to the degree of inflammatory activity of UC patients

There was a strong proportional relationship between histological activity and RIPK3 expression (r = 0.735; p <0.001), with 54.0% of cases of high expression of the necroptosis marker being associated with high inflammatory activity in UC patients.

Analysis of histological samples showed that as the percentage of cryptite increased, so did the expression of the necroptosis marker (r = 0.342; p <0.001) (Fig. 47).

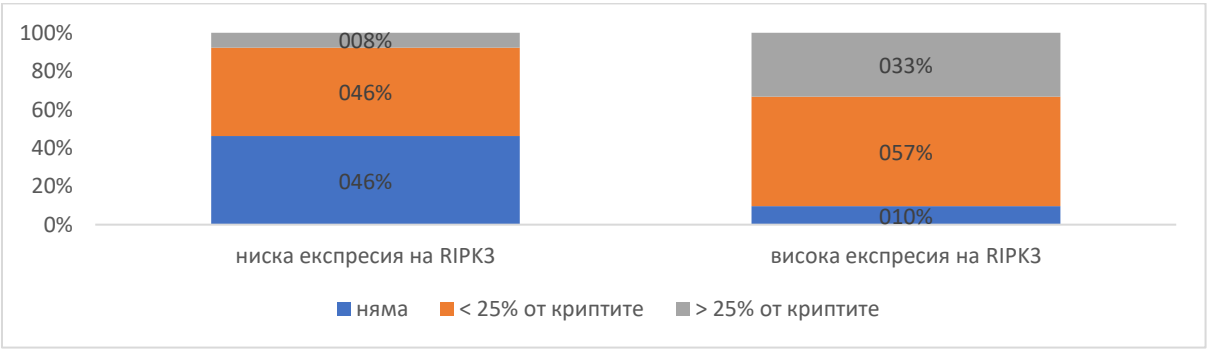


Fig. 47. Expression of RIPK3 according to the percentage of crypt involvement

No dependence on crypt abscesses and RIPK3 expression was found.

A moderate relationship between erosions and high RIPK3 expression was found (r = 0.316; p <0.001) (Fig. 48).

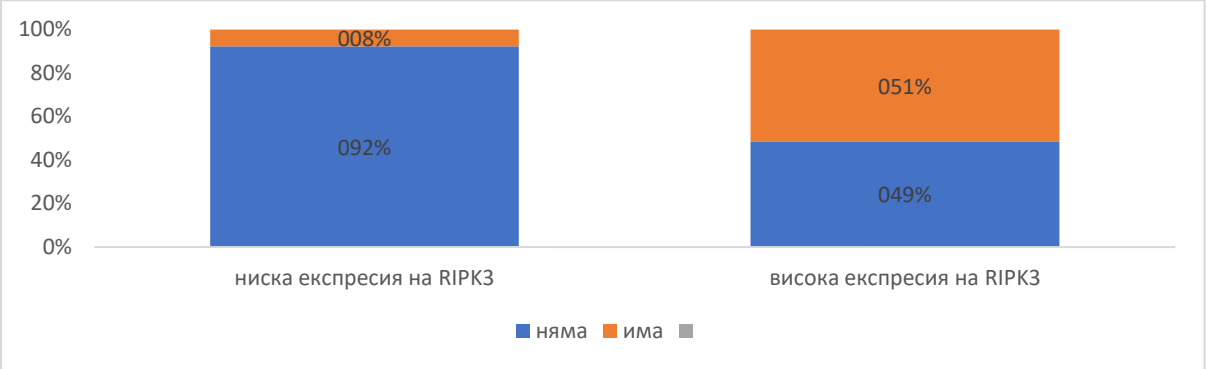


Fig. 48. Expression of RIPK3 according to the presence of erosions

No association was found between RIPK3 expression and the incidence of diarrhea during hospitalization. On the other hand, hematochezia is associated with higher marker expression (p = 0.028) (Fig. 49).

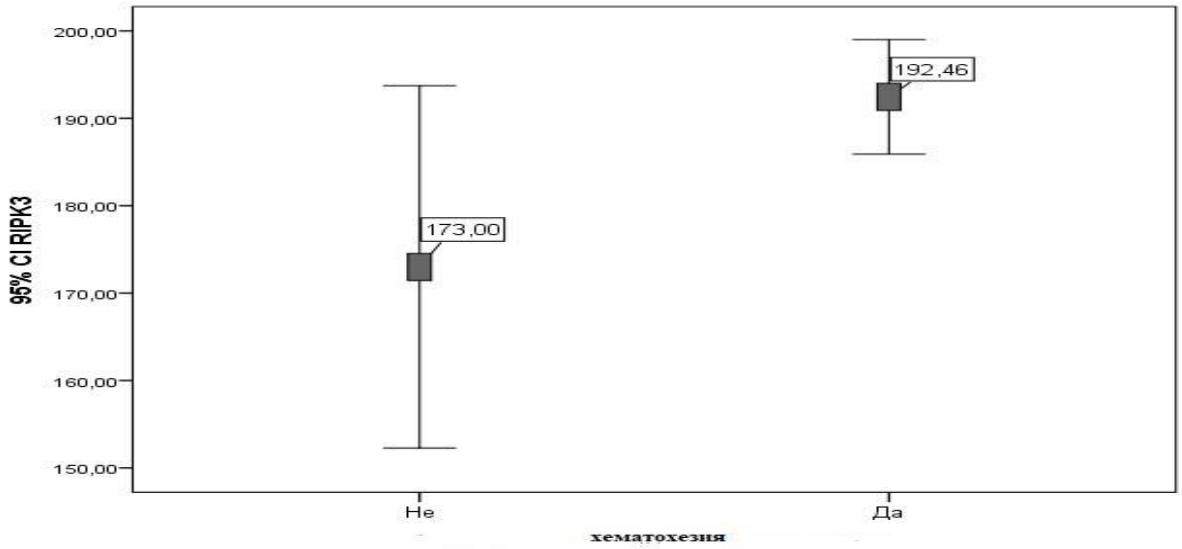


Fig. 49. Mean values of RIPK3 expression according to the presence of hematochezia

There was no significant difference in the expression of RIPK3 and the presence of abdominal pain.

In weight loss, there was no difference in RIPK3 expression in UC patients, which remained high (191.47 for weight loss patients and 188.40 for non-weight loss patients, respectively).

As shown in Table 12 with extraintestinal manifestations were 60.0% of UC patients, with a mean expression value of 184.41 ± 28.19. The most frequent are the joint manifestations (21.2%) and iron deficiency anemia (IDA) (44.7%) (Fig. 50).

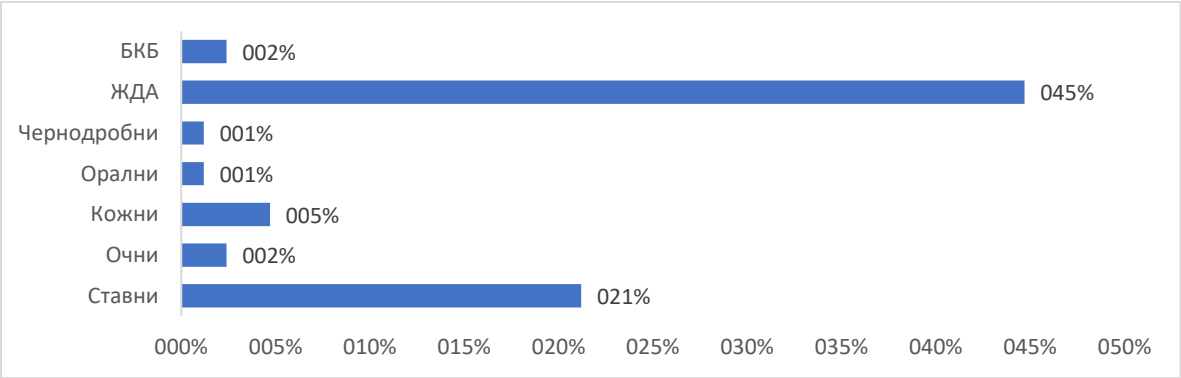


Fig. 50. Types of EIM in UC patients

No significant difference was found between the expression of RIPK3 in the different types of extraintestinal manifestations (Fig. 51) in UC patients.

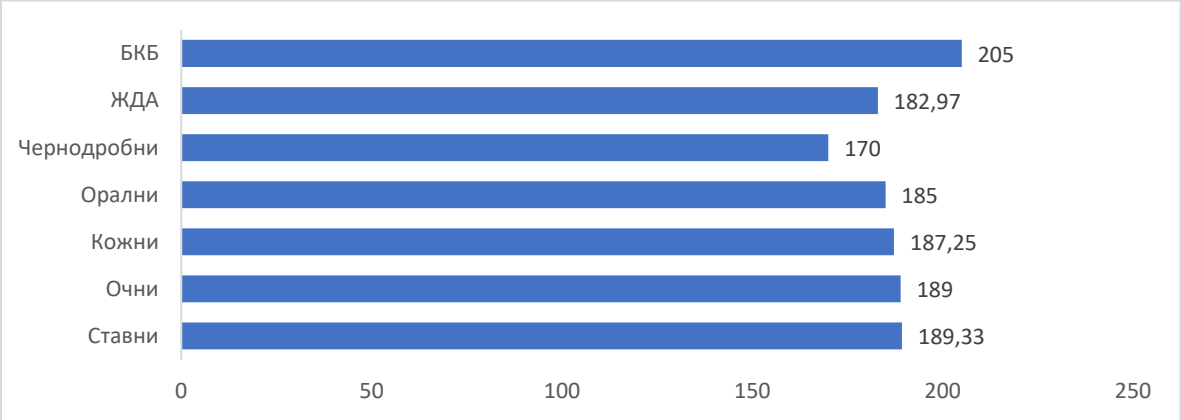


Fig. 51. Mean expression of RIPK3 according to EIM types in UC patients

There was no significant difference in the expression of RIPK3 according to the presence or absence of thromboembolic complications, which may be due to the small number of patients (1 patient).

No difference in RIPK3 expression was observed in patients with intestinal complications compared to those without complications (Fig. 52).

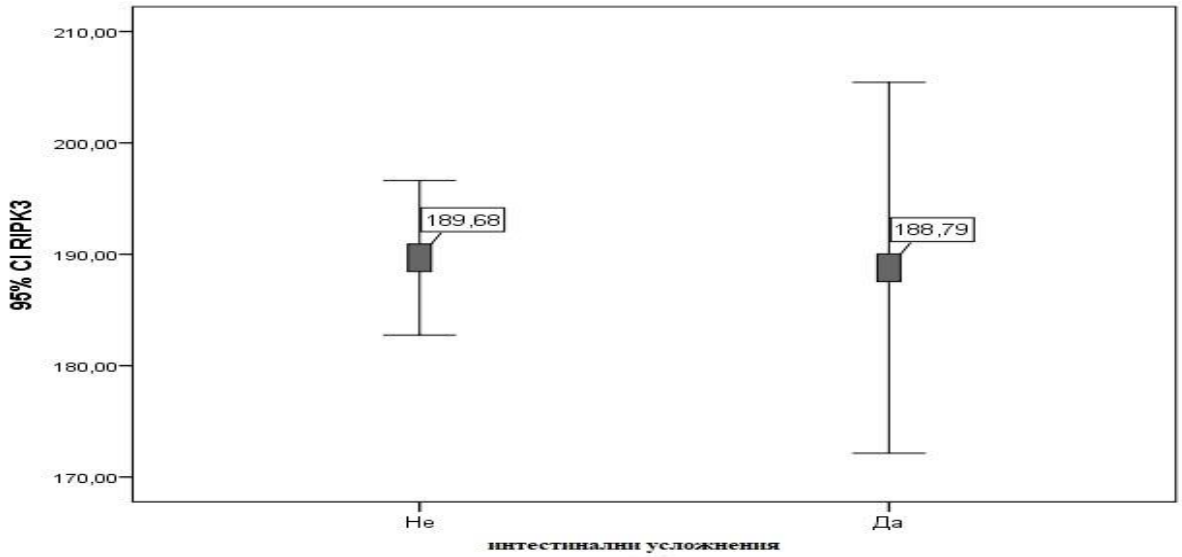


Fig. 52. Mean values of RIPK3 according to the presence or absence of intestinal complications

Only pseudopolyposis occurs in UC patients from intestinal complications (21.2%)

Only 3.5% (three) underwent surgery in the past (appendectomy), with an average RIPK3 expression of 210.33 ± 23.16.

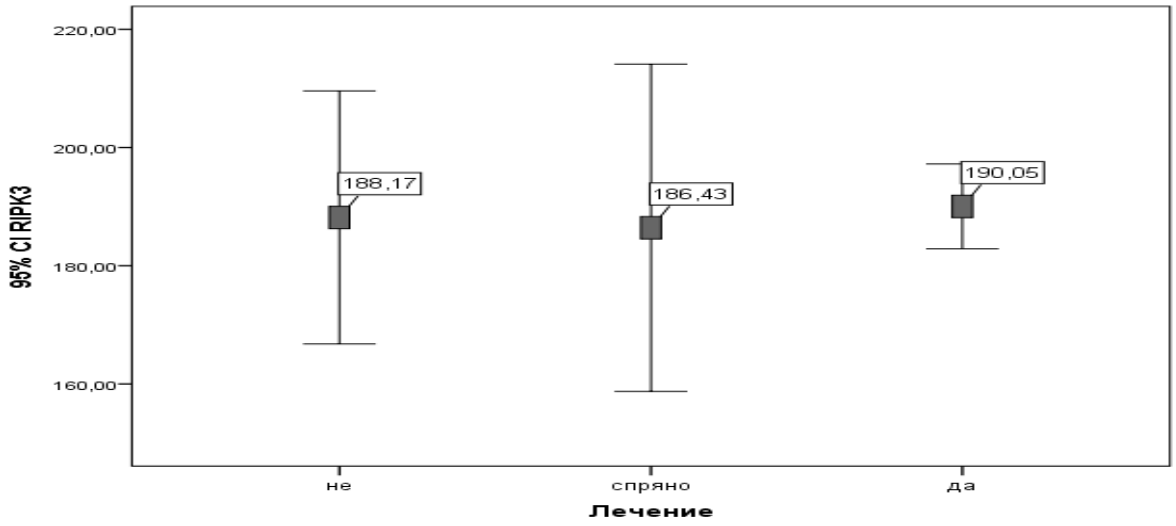


Fig. 53. Mean values of RIPK3 expression according to treatment

There was no significant difference in RIPK3 expression between UC patients who were not currently receiving treatment (with debut of UC) or those with ongoing or discontinued therapy (Fig. 53).

In Fig. 54 presents the results of the analysis of RIPK3 expression according to the type of therapy performed. The results show that UC patients on antibiotic therapy have the lowest expression (164.75). While treatment with immunomodulators and corticosteroids did not show a significant difference in the expression levels of the marker.

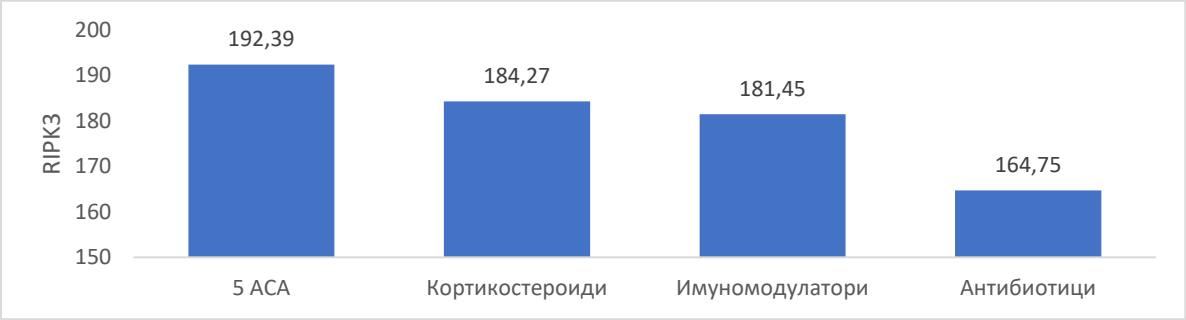


Fig. 54. Mean RIPK3 expression by type of therapy performed

There was no difference in the expression of RIPK3 according to the conduct of biological therapy in UC patients. (Fig. 55).

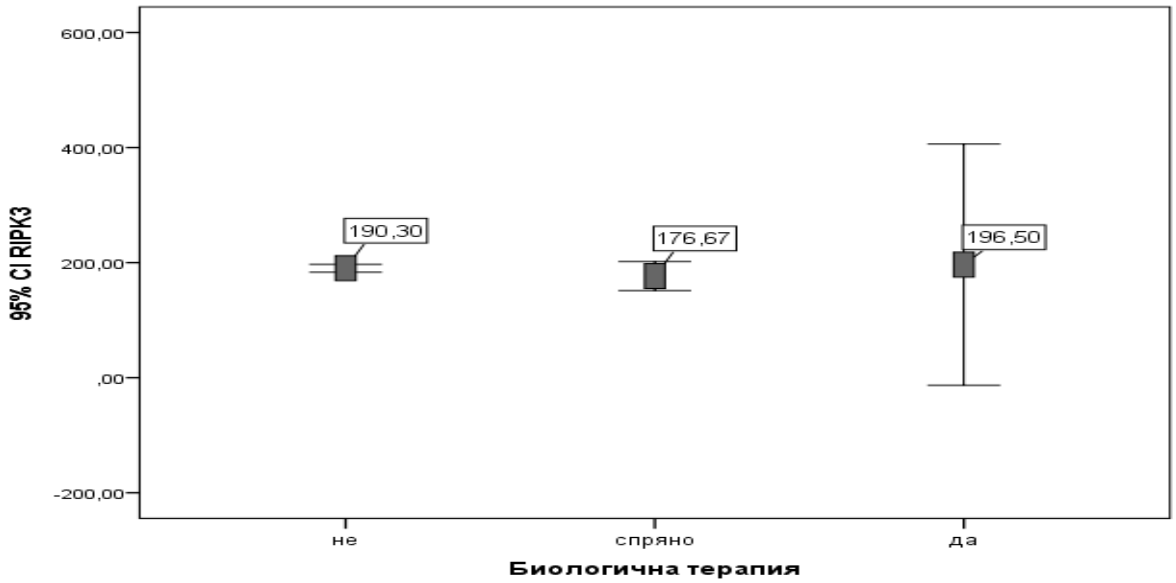


Fig. 55. Mean RIPK3 expression values according to biological therapy

No relationship was found between ESR and RIPK3 levels. No dependence was also found on CRP levels and RIPK3 expression, with predominant patients with increased expression, regardless of CRP levels. A similar trend is observed with regard to the levels of leukocytes, thrombocytes, albumin, ferritin, hemoglobin and serum iron.

There is a weak inverse relationship between the presence of accompanying intestinal infection with Cl. Difficile and nuclear expression of RIPK3 ($r = -0.214$; $p = 0.05$), and it can

be said that patients with Cl. Difficile infection have a lower expression of the marker (174.00 and 192.03 for UC patients without infection, respectively).

4.4. Comparison of RIPK3 expression levels in IBD patients and healthy controls

A significant difference was found between the mean values of RIPK3 expression in IBD patients and healthy controls (p = 0.001) In IBD patients, overexpression of RIPK3 (180.3) was found compared to controls (158.93) (Fig. 56).

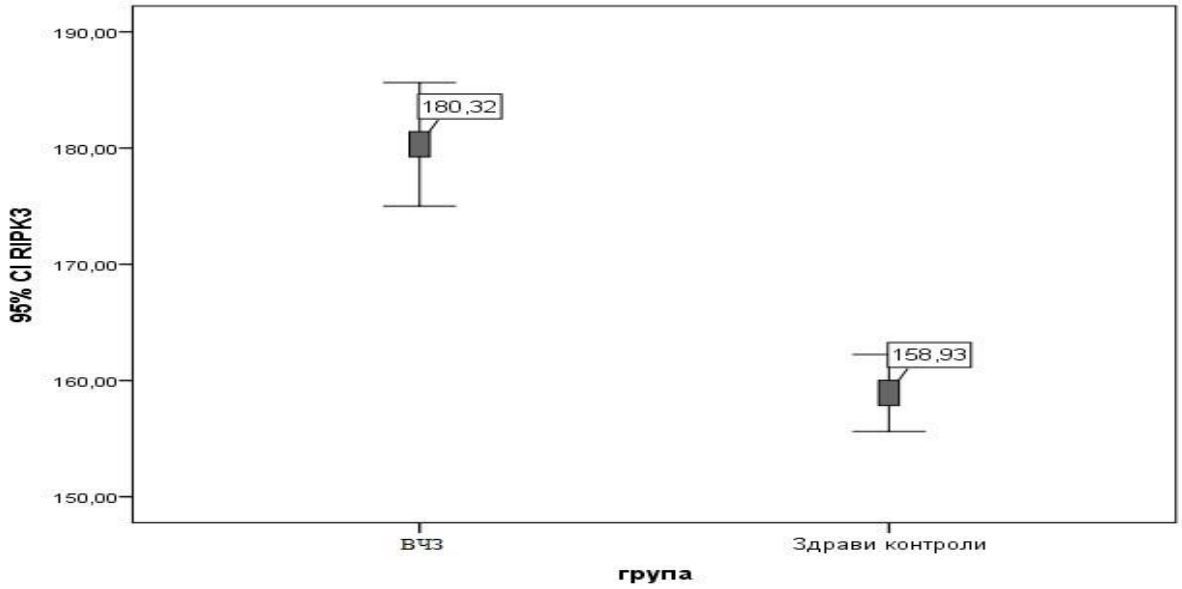


Fig. 56. Mean value of RIPK3 expression in the study groups

An additional analysis of RIPK3 expression in IBD patients and healthy controls found that high marker expression resulted in a 4.14-fold higher risk of high IBD activity (RR = 4.15 (2.01-8.57); p <0.001).

A significant difference in the expression of RIPK3 was also found in terms of gender and study group, as in men and women the expression of the marker was higher in IBD patients (p = 0.001) (Fig. 57). However, in both groups, RIPK3 expression values were slightly lower in women than in men.

In both study groups, no relationship was found between RIPK3 expression and age.

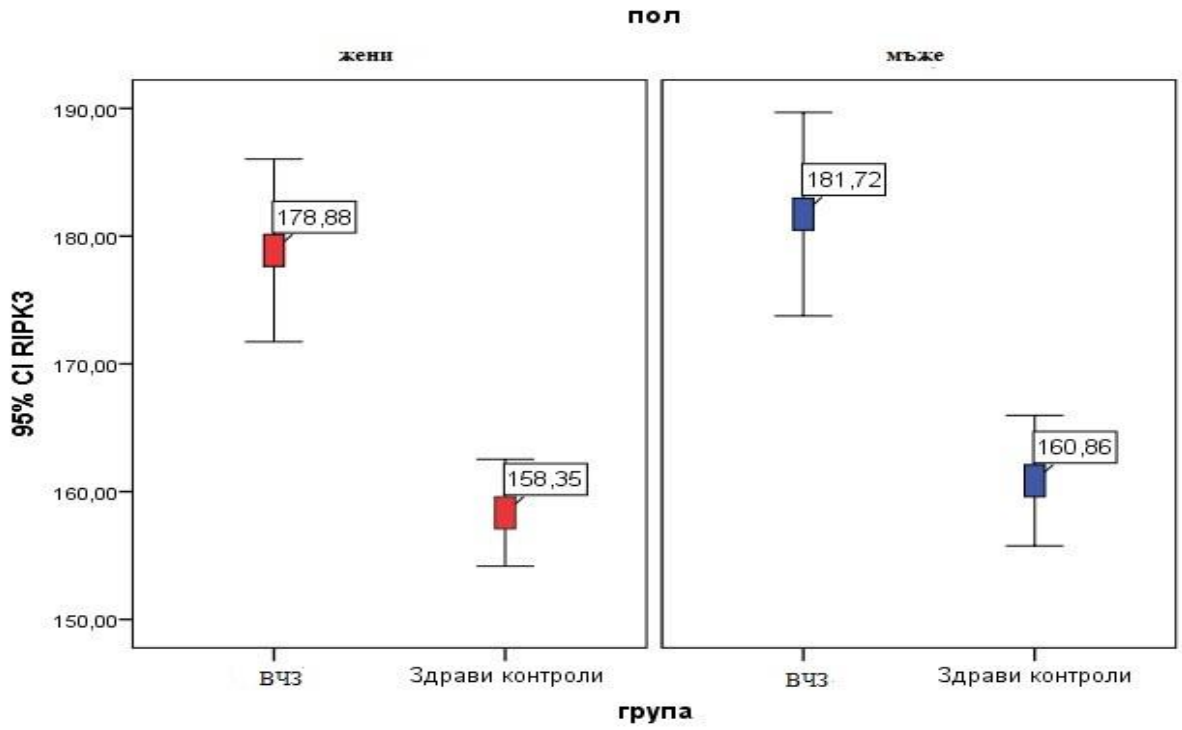


Fig. 57. Mean values of RIPK3 expression according to sex and study group

In a study of nuclear expression of RIPK3 in patients with debut of disease, marker levels were found to be significantly higher than in healthy controls ($p < 0.001$) (Fig. 58). It is noteworthy that the expression of the marker in IBD patients, whether are with debut or already with a known disease, remains high, which indicates that the marker for necroprotosis is mainly influenced by the presence of inflammation.

Similar results were found for relapse, where the mean values of marker expression were significantly higher in IBD patients compared to healthy controls ($p < 0.001$) (Fig. 59).

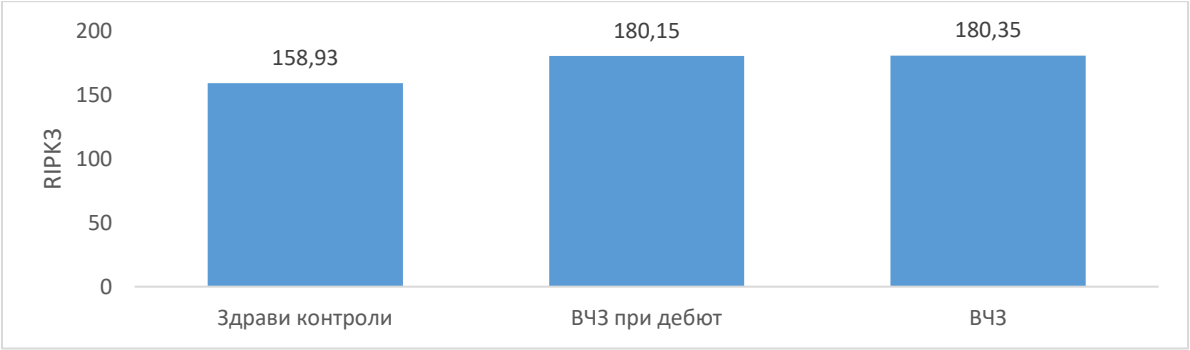


Fig. 58. Mean values of RIPK3 expression according to study groups and debut of the disease

Fig. 59. Mean values of RIPK3 expression according to study groups and relapses

According to the conducted comparative analysis of the expression of RIPK3 according to the studied group and the degree of inflammatory activity, a significant difference was found ($p < 0.001$) (Fig. 60). Inflammatory activity is associated with increased expression of the necroptosis marker compared to healthy controls, and suppression of inflammatory activity by drug treatment leads to a significant decrease in expression.

Fig. 60. Mean values of RIPK3 expression according to the studied groups and the degree of inflammatory activity

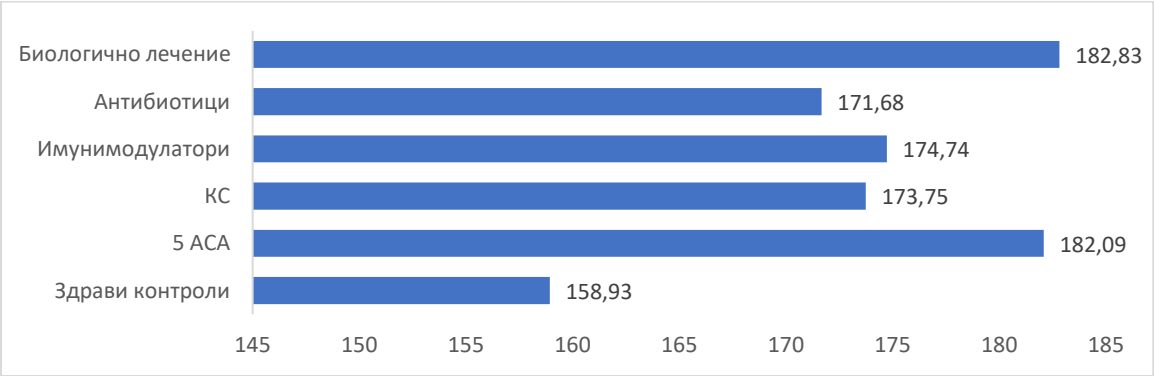


Fig. 61. Mean values of RIPK3 expression according to study groups and treatment

The results of the analysis show that regardless of the type of treatment performed, it is not possible to achieve such suppression of RIPK3 expression in IBD patients that it is close to

that of healthy controls (p <0.001) (Fig. 61). The lowest values of marker expression were observed with the use of antibiotics (171.68). On the other hand, RIPK3 expression remains high during 5-ASA treatment and biologic therapy. Because biologic therapy is used in patients with moderate to severe disease who have not responded to conventional therapy, the presence of increased expression of the necroptosis marker suggests that even this most potent therapy is not yet available. able to control inflammation and reduce RIPK3 expression levels to levels as in healthy controls or remission of the disease.

4.5. Comparison of RIPK3 expression levels in patients with Crohn's disease and ulcerative colitis

The comparative analysis of the expression levels of the necroptosis marker in CD and UC patients showed a significant difference (p = 0.001), with UC patients showing higher levels of expression compared with CD patients (189.48 versus 171.15, respectively). (Fig. 62).

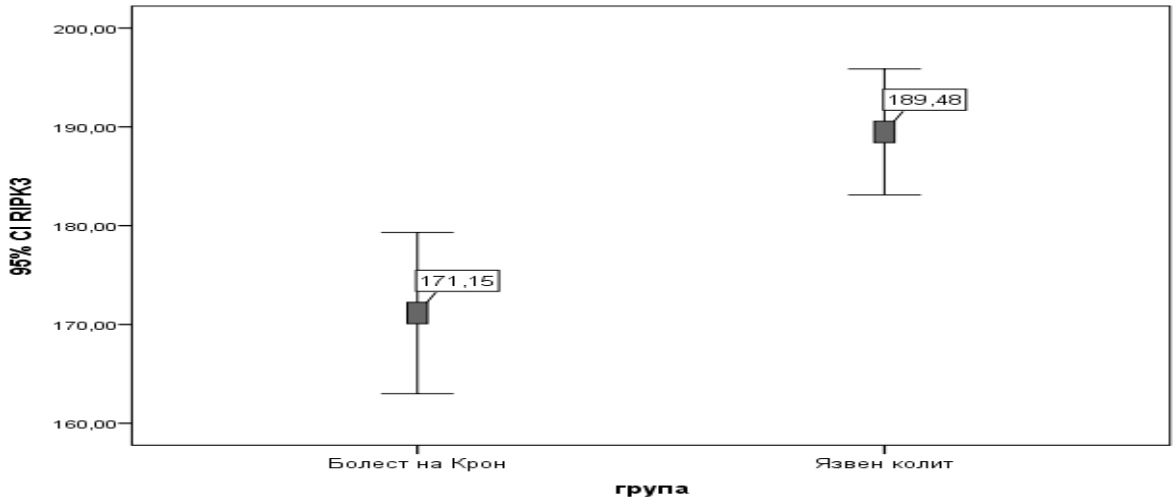


Fig. 62. Mean values of RIPK3 expression according to the study group

There was a moderate relationship between RIPK3 expression and UC (r = 0.321; p <0.001), with the presence of necroptosis increases the risk of UC more than 4-fold (OR = 4.47 (2.160-9.284); p <0.001).

A significant difference was found between the expression of the necroptosis marker, the study groups of patients and the sex (p <0.001) (Fig. 63). On the other hand, it was found that in women the expression of RIPK3 correlated moderately with UC (r = 0.345; p = 0.001), in men the dependence was also moderate (r = 0.300; p = 0.005). Interestingly, in women high expression is associated with more than 5 times higher risk of UC (OR = 5.44 (1.797-16.469); p <0.001), while in men this risk is almost 4 times higher (OR = 3.88 (1,452-10,402); p <0.001).

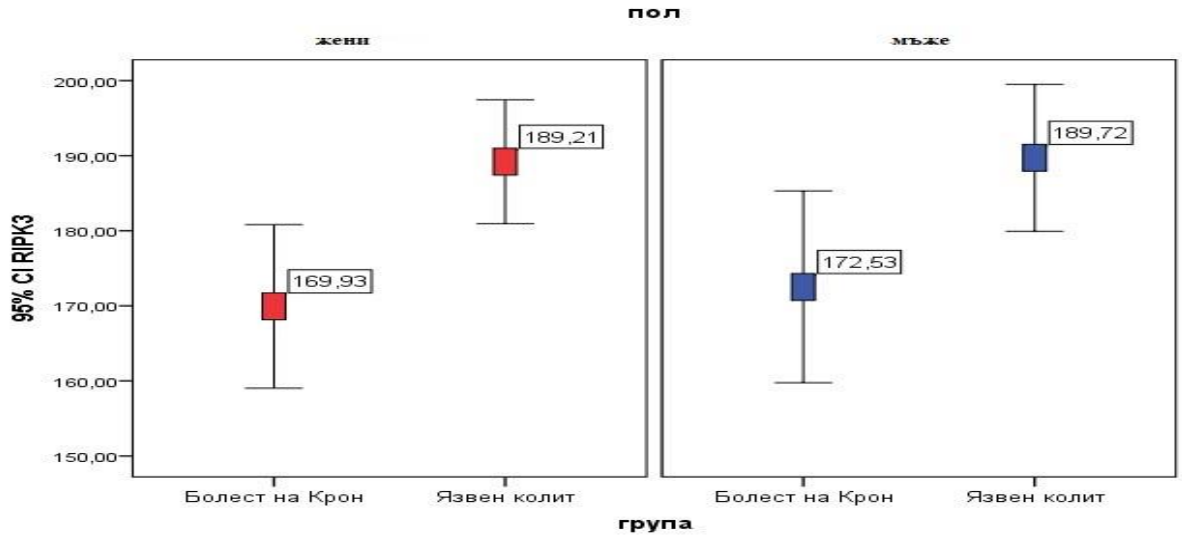


Fig. 63. Mean values of RIPK3 expression according to study group and sex

No relationship was found between RIPK3 expression and age according to the study group.

The study of necroptosis marker expression in patients with debut showed a significant difference between UC and CD patients ($p < 0.001$), with UC patients debuting with overexpression of RIPK3 (Fig. 64).

A significant difference in the expression of RIPK3 between patients with relapsed CD and relapsed UC was found in the group of those who rarely relapse ($p < 0.001$), with expression significantly lower in patients with CD (162.5 versus 195.3, respectively). (Fig. 65).

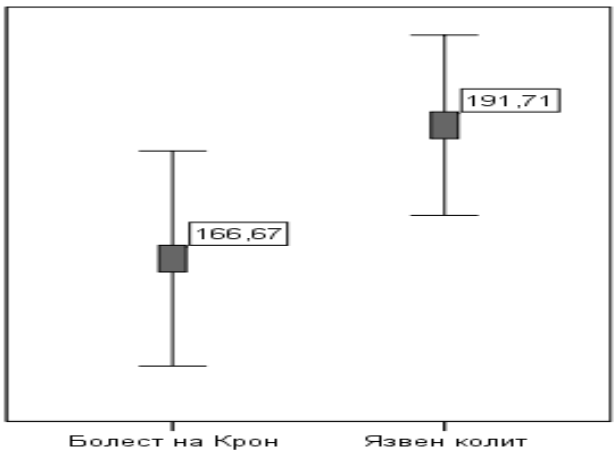


Fig. 64. Mean values of RIPK3 at debut according to the study group of patients

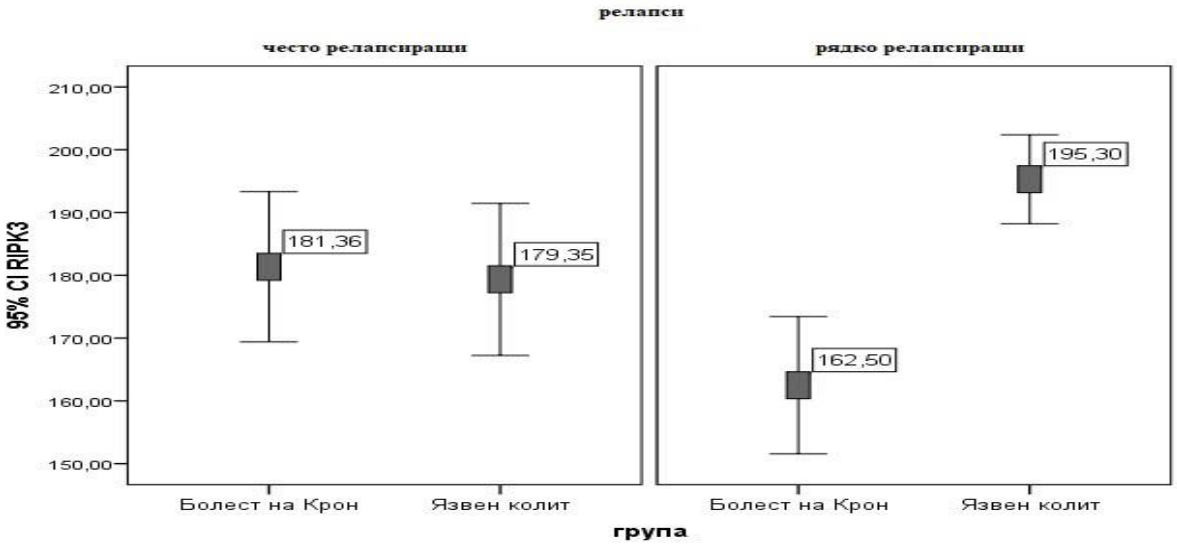


Fig. 65. Mean values of RIPK3 expression in relapse according to the study groups

The study of RIPK3 expression according to age at debut between the studied groups of patients, a significant difference was found in patients in group A2 ($p < 0.01$) and A3 ($p < 0.01$), where the expression of the marker was significantly higher in UC patients (Fig. 66).

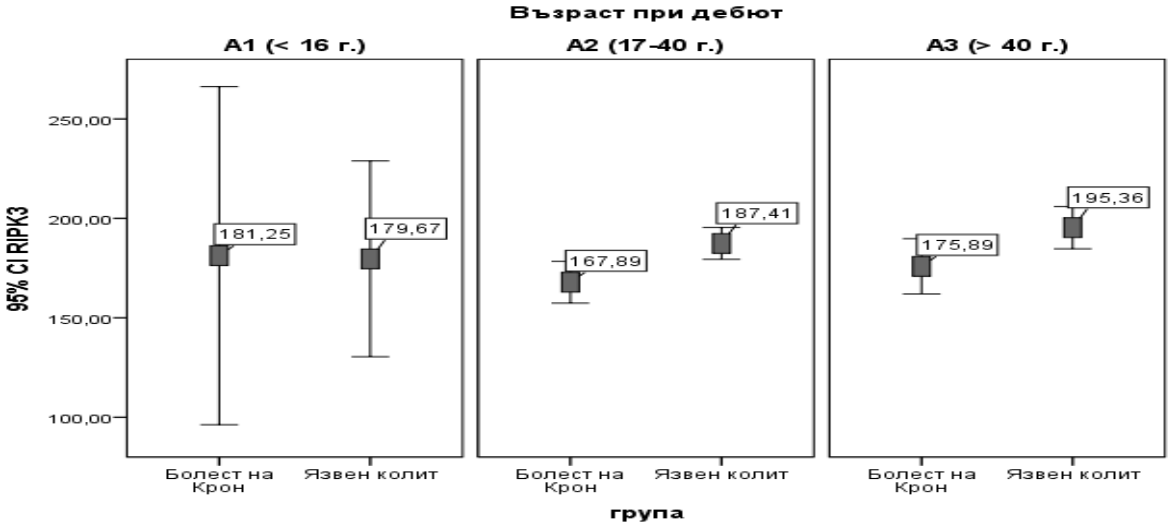


Fig. 66. Mean values of RIPK3 expression according to the studied patient groups and age at debut

In both diseases, an increase in RIPK3 expression was observed with an increase in disease activity (Fig. 67). The results show that patients in remission and with low activity show expression of the marker close to that of healthy controls, with no difference between the two diseases. In patients with moderate and severe activity, higher expression is found in UC patients.

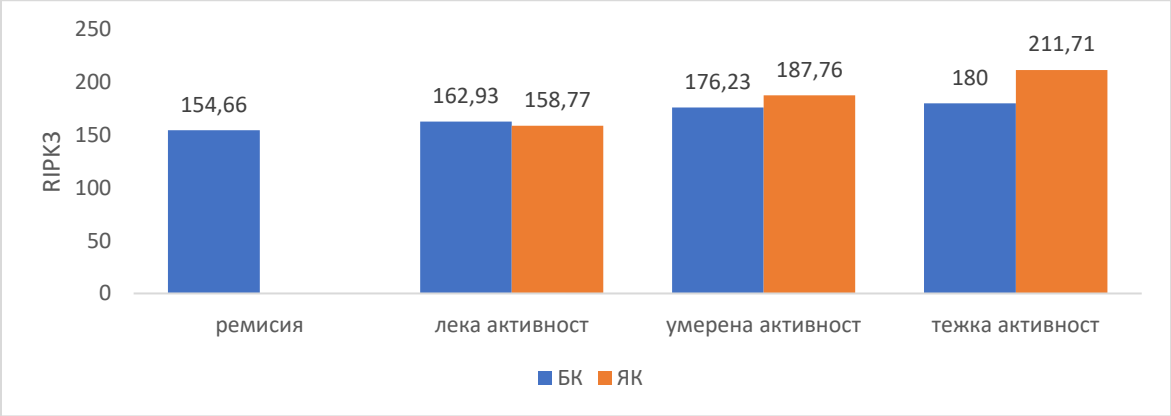


Fig. 67. Mean values of RIPK3 expression according to the studied patient groups and disease activity

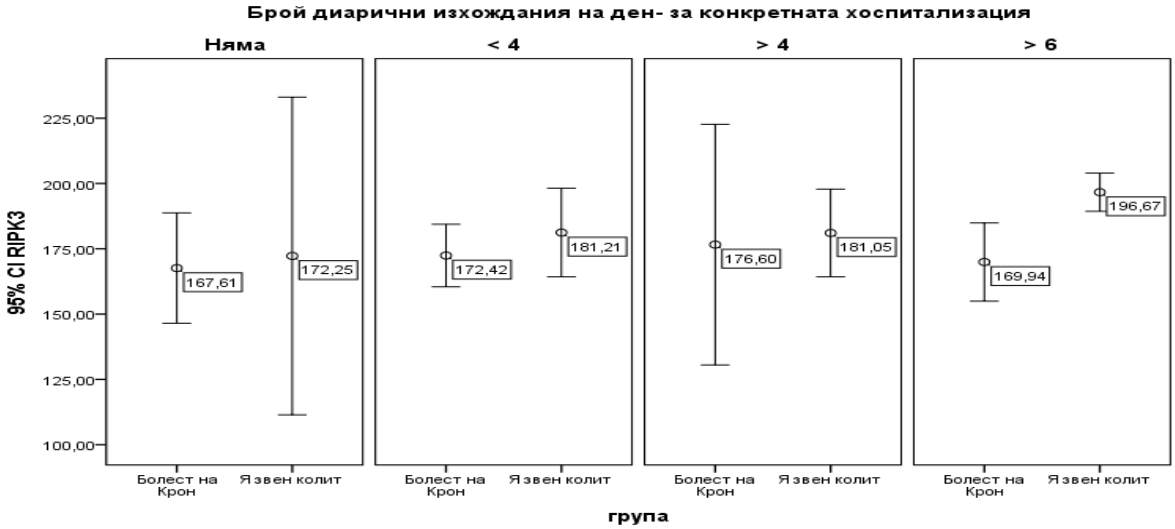


Fig. 68. The study of the mean values of RIPK3 expression according to the number of stools and study groups

According to the data presented in fig. 68, the comparative analysis of RIPK3 expression between the studied groups found that in UC patients the expression of the marker is increased, with a significant difference found in the number of diarrheal stools up to 4 (respectively 172.42 for CD and 181.21 for UC; $p < 0.05$) and in more than 6 stools (169.94 for CD and 196.67 for UC, respectively; $p < 0.01$).

In the presence of hematochezia, there was also a significant difference in the expression of RIPK3, where the values in UC patients were significantly higher (179.85 for CD and 192.46 for UC; $p < 0.01$, respectively) (Fig. 69)

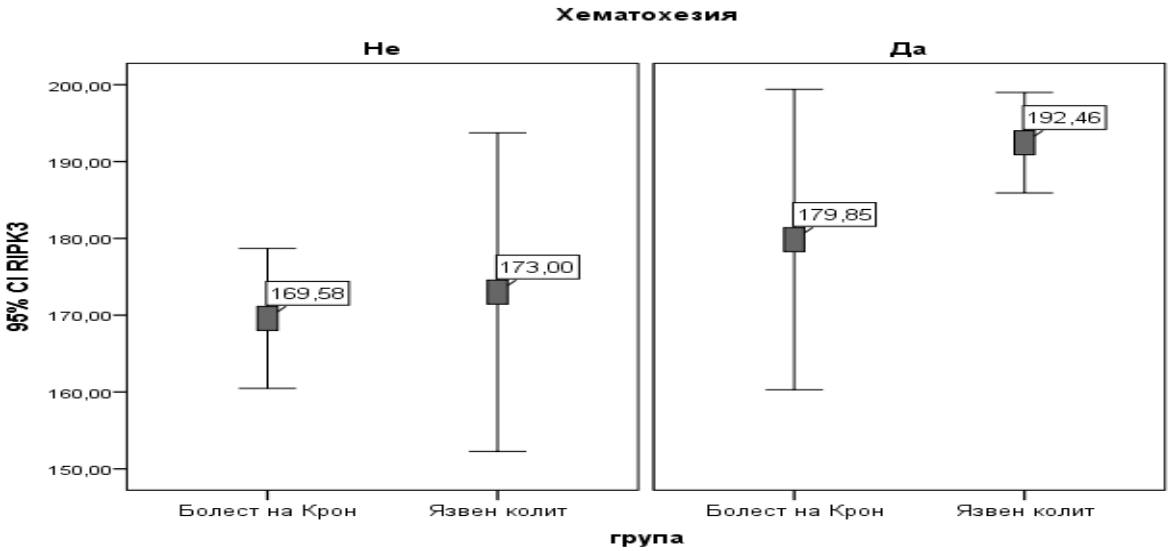


Fig. 69. Mean values of RIPK3 expression according to the studied patient groups and the presence of hematochezia

Regarding to the abdominal pain (Fig. 70), the comparative analysis of the expression results between the two studied groups of patients showed a significant difference in the expression of the marker, which was significantly higher in UC patients ($p < 0.001$).

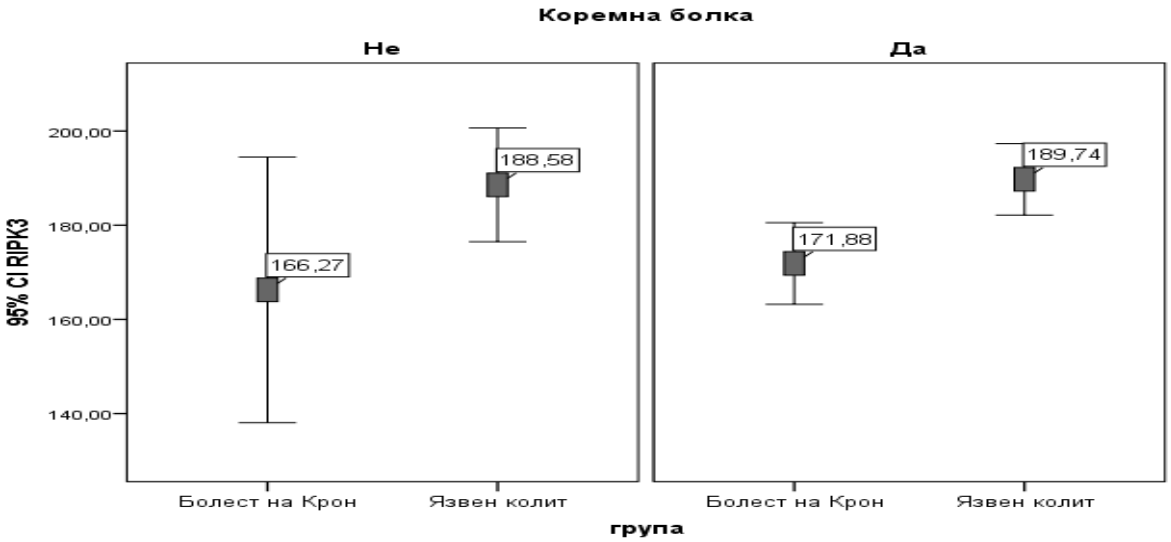


Fig. 70. Mean values of RIPK3 expression according to the studied patient groups and the presence of abdominal pain

Weight loss reveals a difference in both directions (Fig. 71). Significantly higher expression of RIPK3 was found in UC patients compared with CD patients (191.47 for UC and 160.84 for CD, respectively; $p < 0.001$). Weight loss in CD patients is associated with decreased

expression of the necroptosis marker, while in UC patients an inverse relationship is found - expression increases with weight loss.

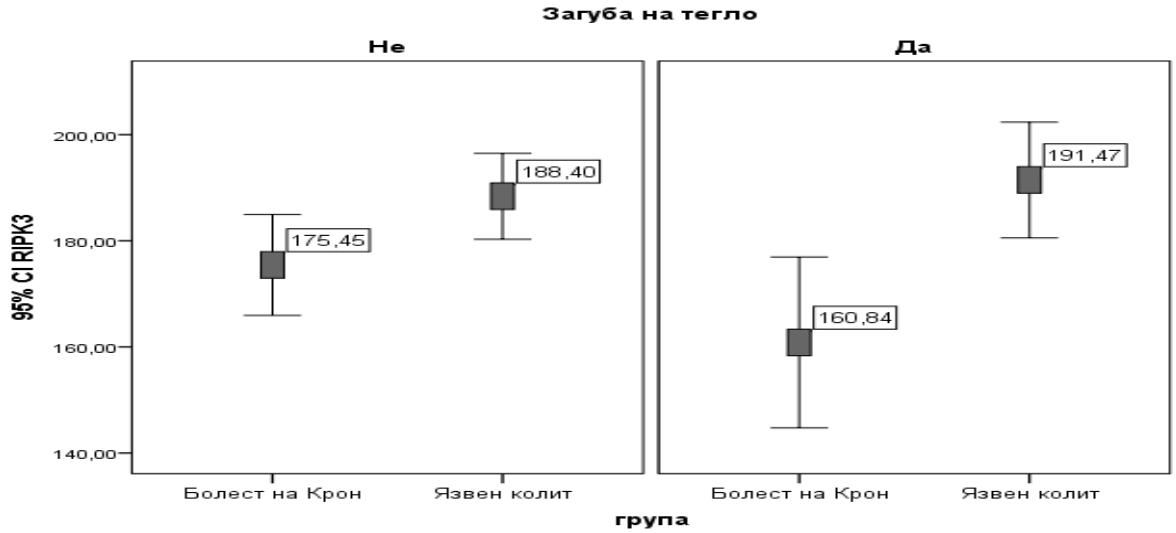


Fig. 71. Mean values of RIPK3 expression according to the studied patient groups and weight loss

The EIM also showed a difference in the expression of RIPK3 in both directions (Fig. 72). On the one hand, there is a significant difference in the absence of EIM, where the expression of the necroptosis marker is significantly higher in UC patients (197.09) than those with CD (166.52) ($p < 0.001$). On the other hand, the presence of EIM in CD patients is associated with an increase in RIPK3 expression, while in UC patients there is a significant decrease in.

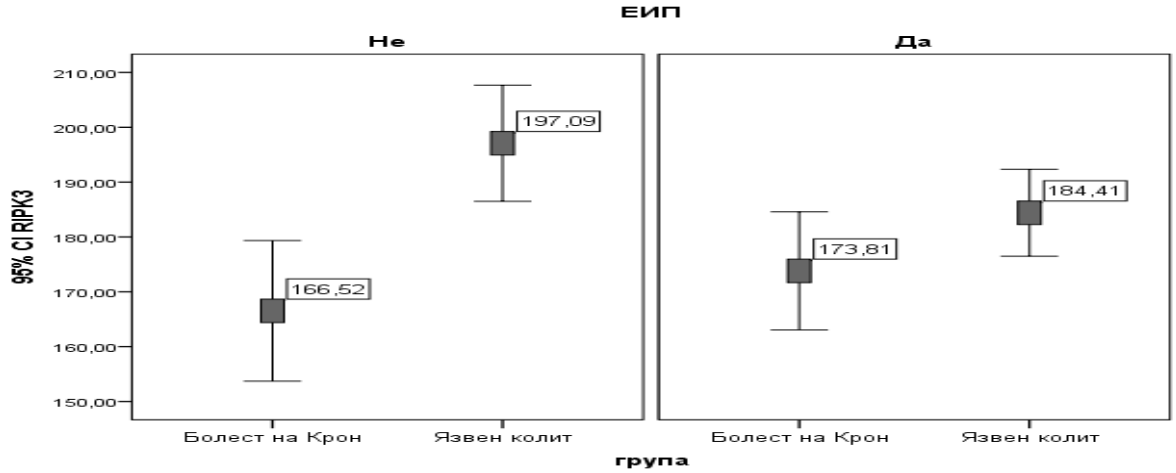


Fig. 72. Mean values of RIPK3 expression according to patient groups studied and the presence of EIM

The study of thromboembolic complications did not show a significant difference in RIPK3 expression between the study groups (Fig. 73).

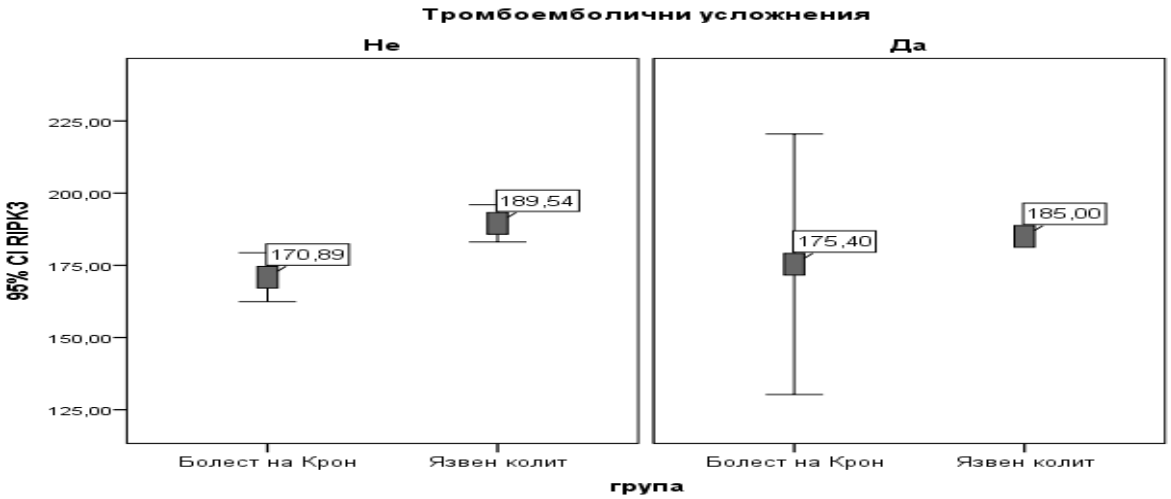


Fig. 73. Mean values of RIPK3 expression according to the study groups and the presence of thromboembolic complications

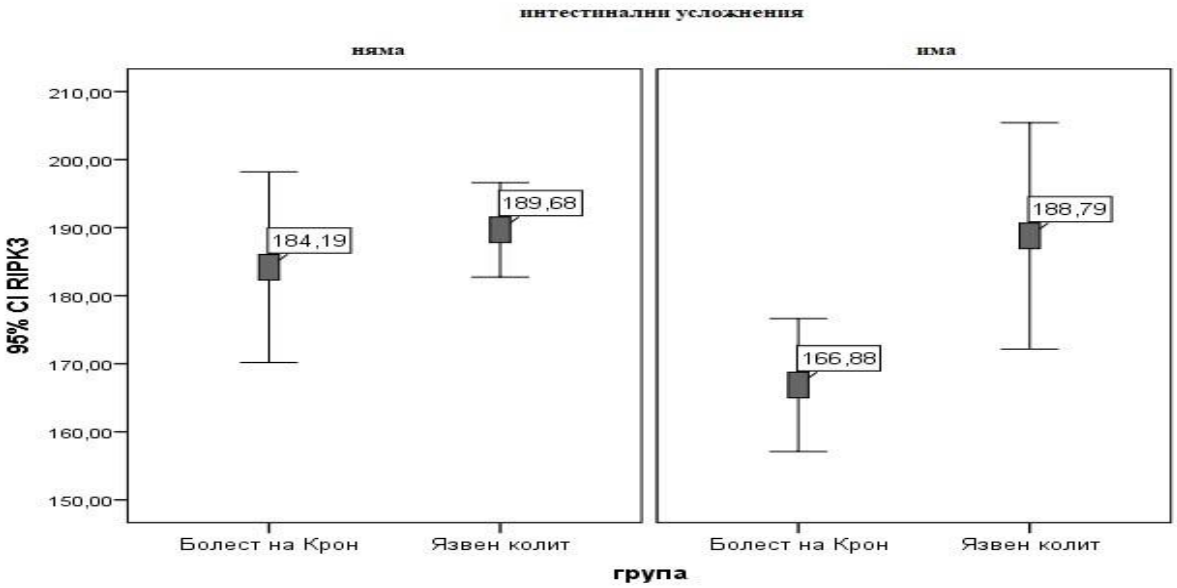


Fig. 74. Mean values of RIPK3 expression according to the studied patient groups and the presence of intestinal complications

Regarding to intestinal complications, there was a significant difference in the expression of RIPK3 between CD and UC patients ($p < 0.001$) (Fig. 74), as the values in the second group of patients were significantly higher (166.88 for CD and 188.79 for UC, respectively).). This difference is due to the decrease in the expression of the necroptosis marker, which is observed in the occurrence of intestinal complications in CD patients.

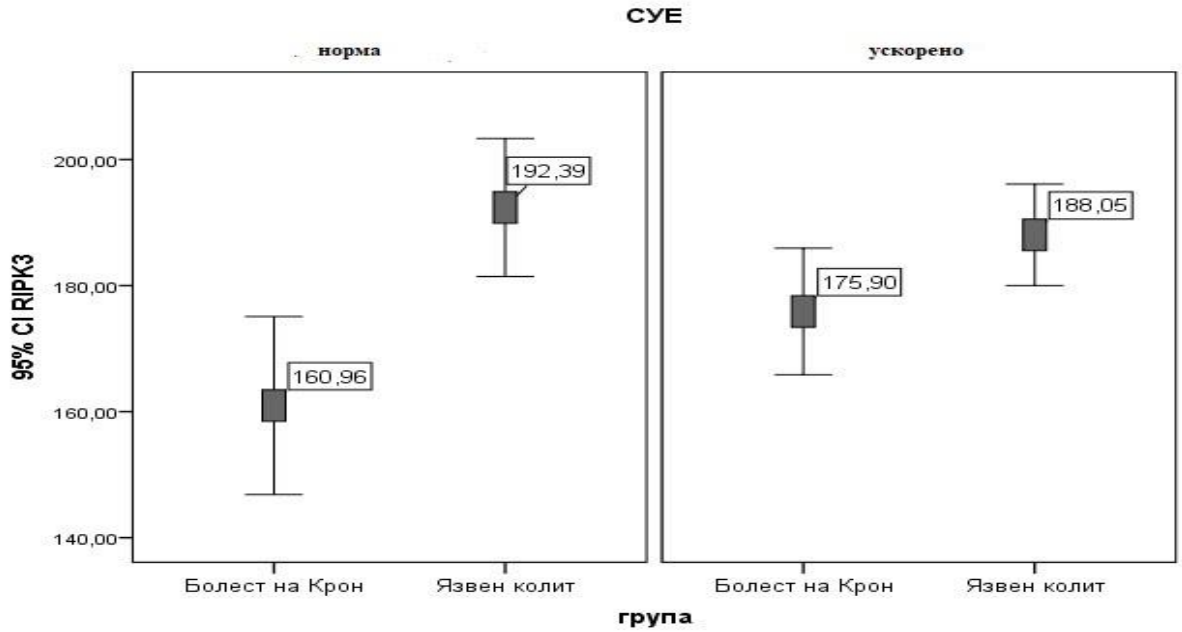


Fig. 75. Mean values of RIPK3 expression according to patient groups and ESR levels

Significant differences were also found with regard to laboratory parameters such as ESR (Fig. 75). In patients with normal ESR, there was a significant difference in RIPK3 expression ($p < 0.001$), with RIPK3 expression being significantly higher in UC than in CD patients (192.39 for UC and 160.96 for CD), respectively. On the other hand, in accelerated ESR there is a high expression of RIPK3 in the group of CD patients.

In UC patients, RIPK3 expression is increased regardless of CRP levels. A significant difference was found in CD patients ($p < 0.05$) (Fig. 76), as the expression of the marker was significantly lower in CD patients not only in the group of patients with normal CRP levels (178.21 for CD and 189.95 for UC, respectively), but also in the group of patients with elevated serum CRP levels (169.12 for CD and 189.32 for UC, respectively). This difference becomes even more significant with decreasing RIPK3 expression in CD patients with high serum CRP levels.

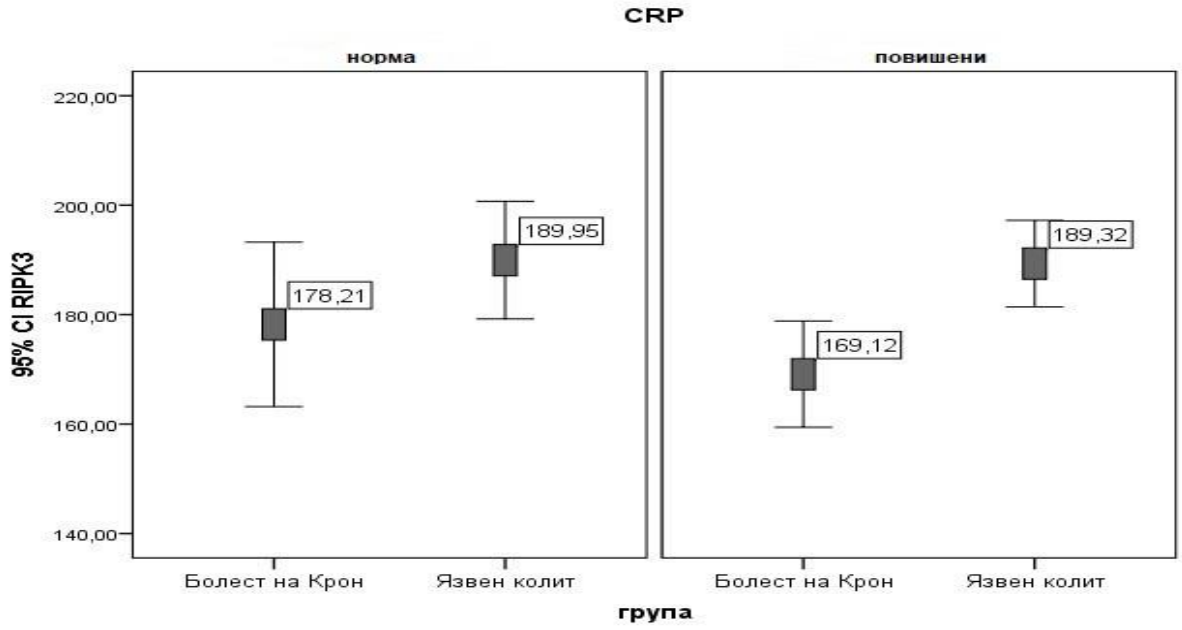


Fig. 76. Mean values of RIPK3 expression according to the studied patient groups and serum CRP levels

The study of RIPK3 expression according to serum leukocyte levels (Leu) showed that a significant difference was observed both in the group of patients in normal levels ($p < 0.05$) and in those with elevated leukocyte value ranges ($p < 0.05$). 0.05) (Fig. 77). In both groups of patients, high expression of the marker was observed at high white blood cell counts, but in CD patients the expression of RIPK3 was lower than in UC patients.

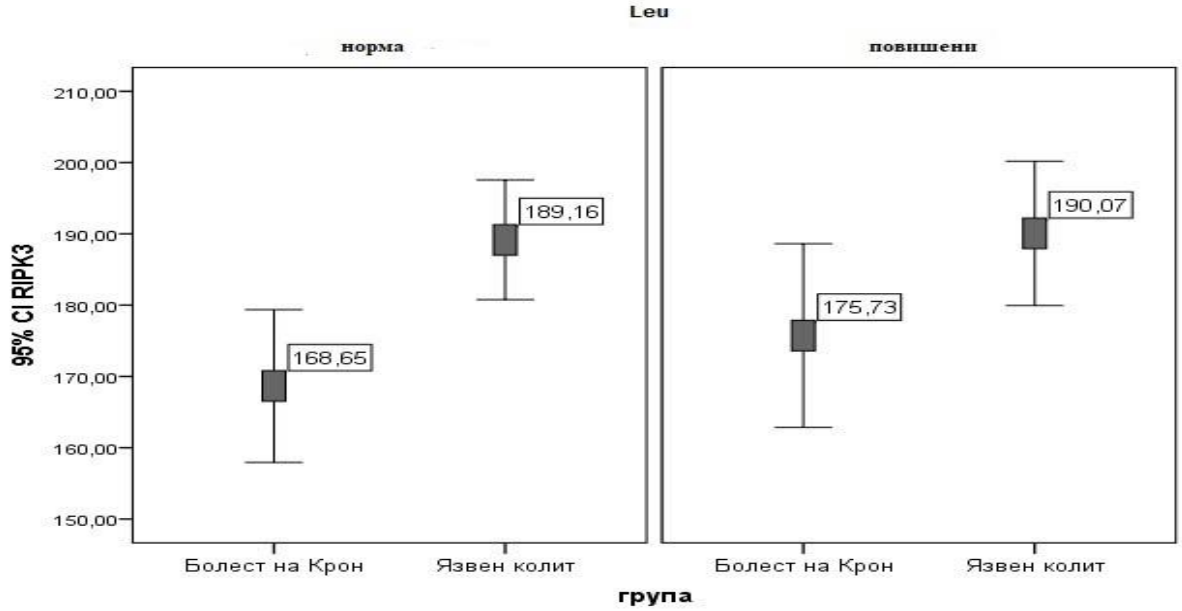


Fig. 77. Mean values of RIPK3 expression according to the studied patient groups and leukocyte count

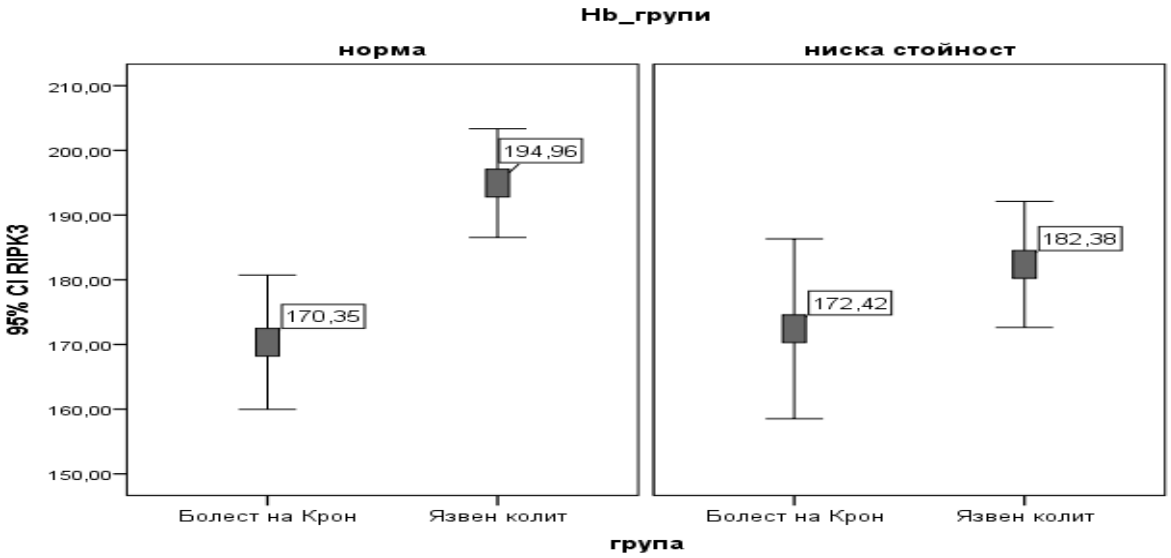


Fig. 78. Mean values of RIPK3 expression according to patient groups and serum hemoglobin levels

The study of RIPK3 expression according to serum hemoglobin (Hb) levels revealed a significant difference both in the group of patients in normal ($p < 0.05$) and in those with low hemoglobin levels ($p < 0.05$) (Fig. 78). In patients with CD, the expression of RIPK3 is high at low serum hemoglobin levels, and compared to UC patients, it is lower.

Another indicator that shows the same trend as serum hemoglobin levels is serum iron (Fe), with a significant difference observed in both the normal group of patients ($p < 0.05$) and those with reduced levels. of serum iron ($p < 0.05$) (Fig. 79). In CD patients, high expression of the marker is observed at low serum iron levels, but in them the expression of RIPK3 remains lower than in UC patients.

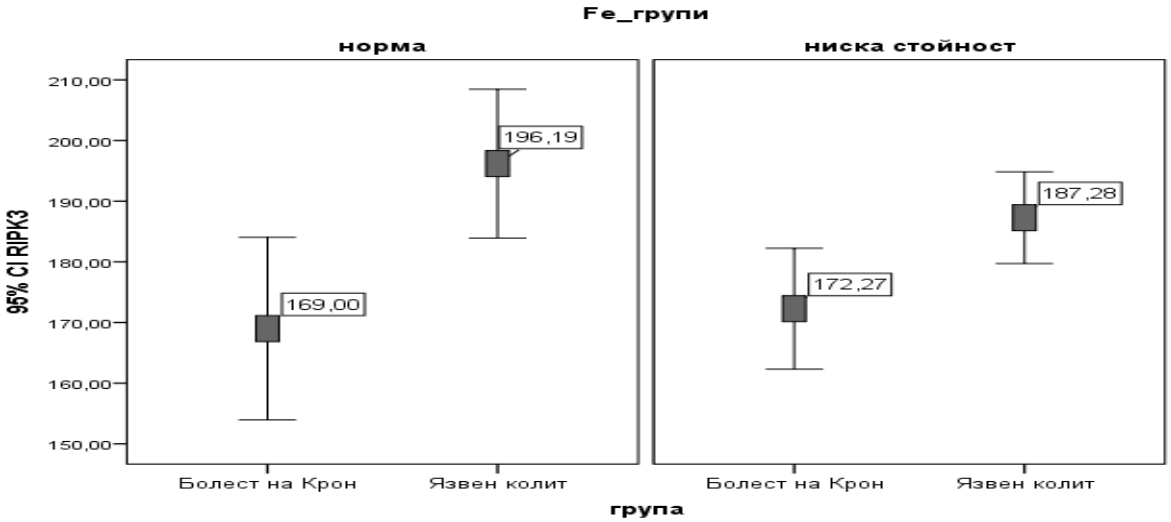


Fig. 79. Mean values of RIPK3 expression according to patient groups and serum iron levels

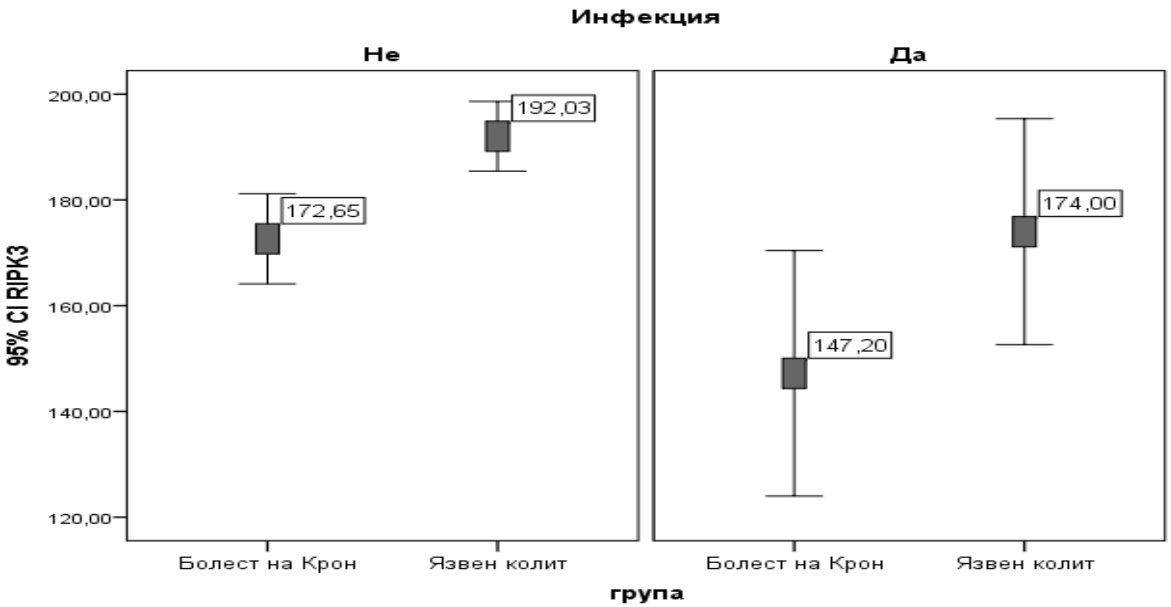


Fig. 80. Mean values of RIPK3 expression according to the studied patient groups and the presence of Cl. Difficile infection

The presence of Cl. Difficile infection in both groups of patients was associated with low RIPK3 expression (p <0.01), which was more significant in CD patients (Fig. 80). There was a significant difference in the expression of the necroptosis marker in the group of patients without infection and in the group of patients with infection (p <0.001).

This may be due to the fact that the inflammation associated with necroptosis is immunomediated, which is different from the inflammation caused by Cl. Difficile infection. The different molecular mechanisms of the two types of inflammation may explain the low values of RIPK3 in Cl. Difficile infection.

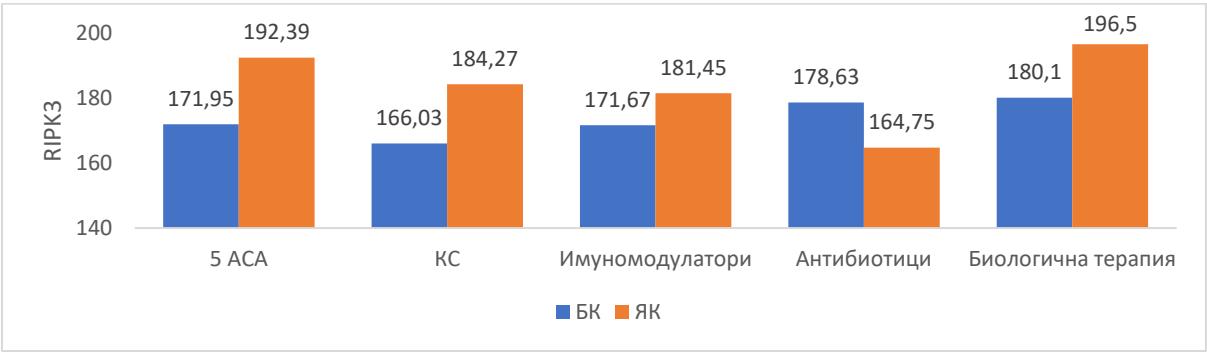


Fig. 81. Mean values of RIPK3 expression according to the studied patient groups and the type of treatment

From the point of view of the conducted treatment, a significant difference in the expression of RIPK3 was observed in all types of treatment ($p < 0.05$) (Fig. 81). Treatment with 5-ASA, corticosteroids, immunomodulators and biologic therapy has been shown to increase marker expression in UC patients. The lowest values of RIPK3 expression are found in patients with CD treated with corticosteroids and in UC patients treated with antibiotics, and in these groups the expression is close to that of healthy controls.

4.6. Establishing the potential of RIPK3 levels as a prognostic marker for the progression and development of severe disease in IBD patients

To determine the potential of nuclear expression of RIPK3 as a prognostic marker for progression and development of severe disease in IBD patients, a risk analysis was performed in both CD patients (Table 15) and in UC patients (Table 16).

Table 15. Evaluation of high expression of RIPK3 in CD patients as a risk factor

Indicator	HR (95% CI)	P value
Hospitalizations	1.056 (0.994-1.121)	0.046
Hematochezia	2.123 (0.404-4.539)	< 0.001
Intestinal complications	3.831 (2.335-6.287)	< 0.001
Accompanying diseases	2.349 (1.451-3.802)	0.001
Operation	2.158 (1.347-3.456)	0.001

According to the results presented in table. 15 it can be said that high expression of the necroptosis marker is associated with an increased risk of intestinal complications in CD patients (HR = 3.831).

Patients with UC and high RIPK3 expression have an increased risk of high disease activity (HR = 21.470), as well as clinical manifestations with hematochezia (HR = 5.00) (Table 16).

Table 16. Evaluation of the high expression of RIPK3 in UC patients as a risk factor

Indicator	HR (95% CI)	P value
Hematochezia	5.00 (1.313-19.046)	< 0.001
Number of diarrheal bowel movements	1.411 (1.131-1.759)	0.002
Operation	1.829 (0.512-3.567)	0.002
Infection	2.851 (1.533-5.304)	0.001
Disease activity	21.470 (0.010-46201.564)	< 0.001

In patients with CD, moderate and severe activity (CDAI> 220) have been associated with increased expression of RIPK3 in patients over 40 years of age (192.0) (p = 0.035) (Fig.82).

High expression of the marker for necroptosis was also found in patients with severe CD who underwent surgery (180.42 to 153.66; p <0.05).

The high expression of the studied marker in patients with CD was associated with frequent relapses and severe disease activity (184.04 to 169.41; p = 0.048).

Concerning endoscopic activity, severity of CD and RIPK3 expression, conflicting results have been found. In patients with CDAI <220, no significant difference in marker expression was observed with respect to endoscopic activity. While in patients with CDAI> 220 RIPK3 expression was significantly higher in patients with endoscopic activity (p = 0.027) (Fig.83).

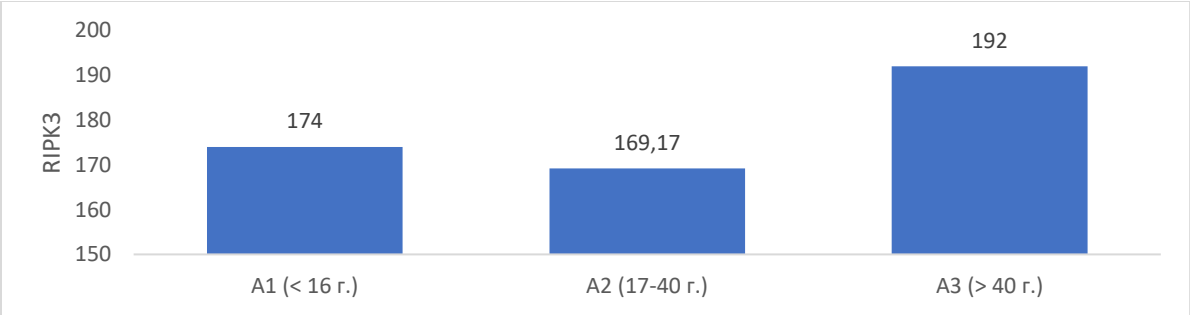


Fig. 83. Mean values of RIPK3 expression in patients with severe disease according to age at debut

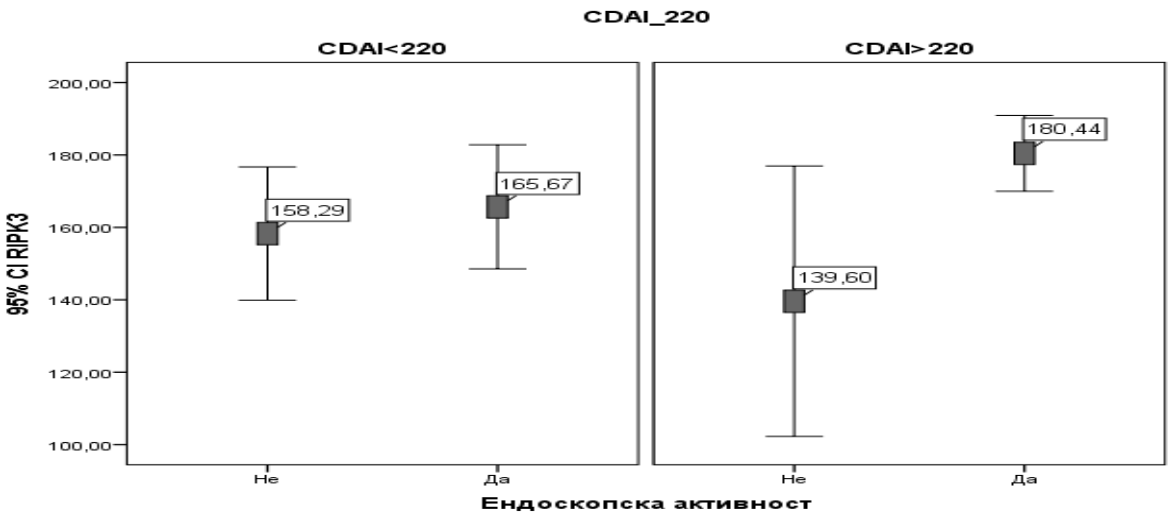


Fig. 83. Mean values of RIPK3 expression according to disease severity and endoscopic activity

Analysis of necroptosis marker expression according to clinical and histological activity showed the same trend. Expression increases with increasing activity (p = 0.035) (Fig. 84)

In severe clinical activity of CD, the high expression of the necroptosis marker is associated with ileal localization with involvement of the upper GI tract (L1 + L4) (p=0.045) (Fig. 85).

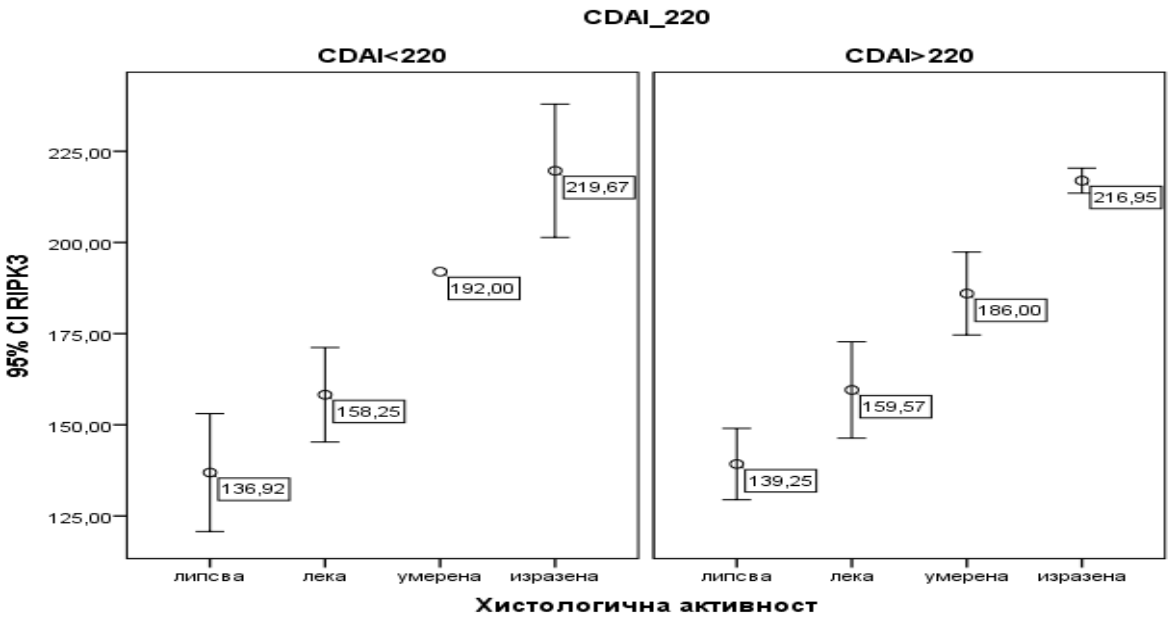


Fig. 84. Mean values of RIPK3 expression according to disease severity and histological activity

Although no significant difference was found, the highest expression of RIPK3 was in patients with severe clinical activity, stricturing with fistulizing disease (B2 + B3) (180.38), and those with inflammatory disease (B1) (174.92). In isolated involvement, no difference was found in the stricturing (162.15) and fistulizing form (163.25). In isolated involvement, no difference was found in the stricturing (162.15) and fistulizing form (163.25).

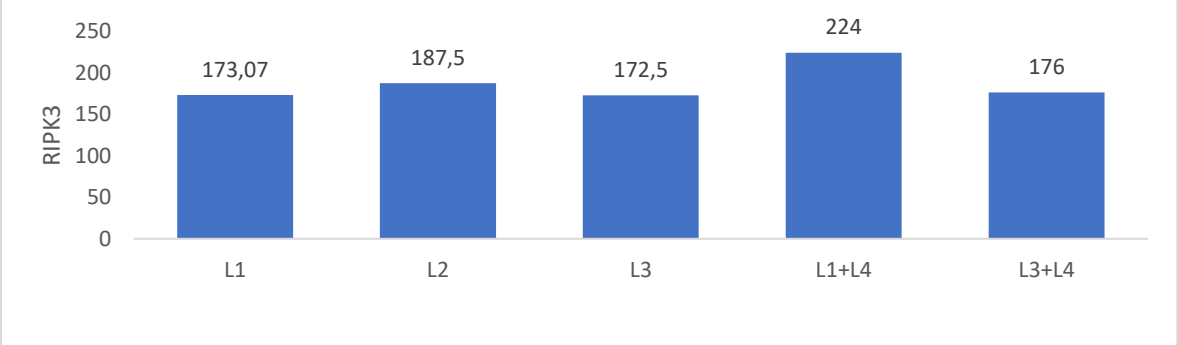


Fig. 85. Mean values of RIPK3 expression in CDAI> 220 and by localization

The evaluation of the expression of the necroptosis marker according to laboratory parameters and the severe activity of CD revealed increased expression of RIPK3 at low albumin levels (respectively 192.57 to 123.50; p <0.01), low hemoglobin levels (respectively 174.79 to 166.11) and increased CRP levels (respectively 174.23 to 158.91; p <0.05).

The literature often describes that appendectomy in the past was a protective factor for the development of UC, and in the present study found that in patients with UC surgery (appendectomy in the past) is associated with moderate and severe disease and increased expression of RIPK3 (p <0.001) (Fig.86).

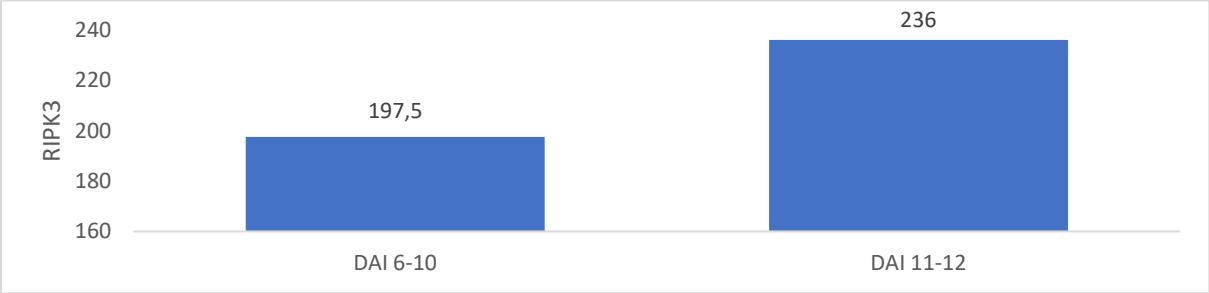


Fig. 86. Mean values of RIPK3 expression according to DAI and surgery

In contrast to CD, in patients with UC, increased expression of the necroptosis marker was associated with increased disease activity and rare relapses (p <0.01) (Fig. 87).

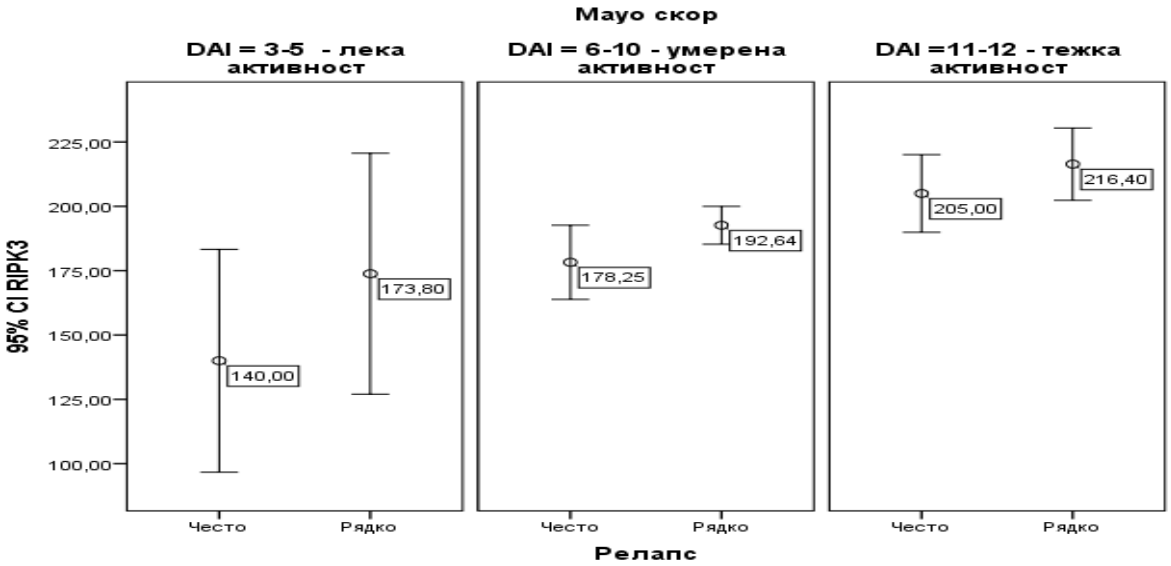


Fig. 87. Mean values of RIPK3 expression according to DAI and relapse rate

In terms of endoscopic activity, a direct relationship was also found between disease activity and expression of the necroptosis marker (r = 0.481; p <0.001) (Fig. 88).

A directly proportional moderate dependence was also found on the histological activity and expression of RIPK3 according to disease activity (DAI) in UC (r = 0.482; p <0.001) (Fig. 89).

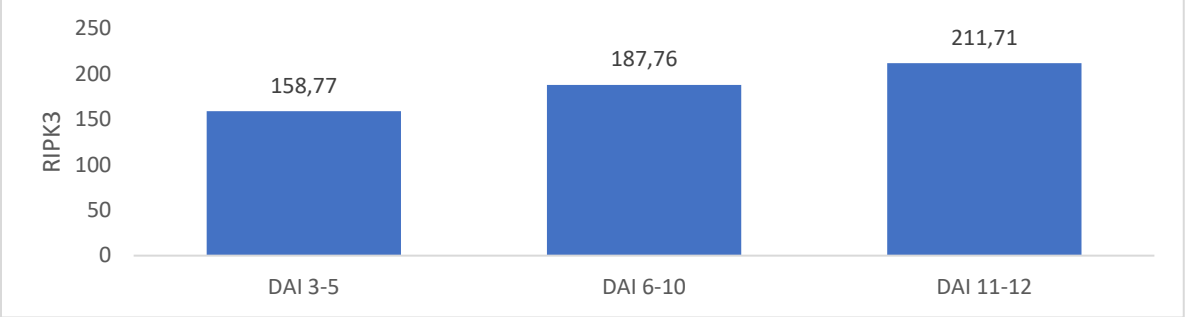


Fig. 88. Mean values of RIPK3 expression according to DAI and endoscopic activity

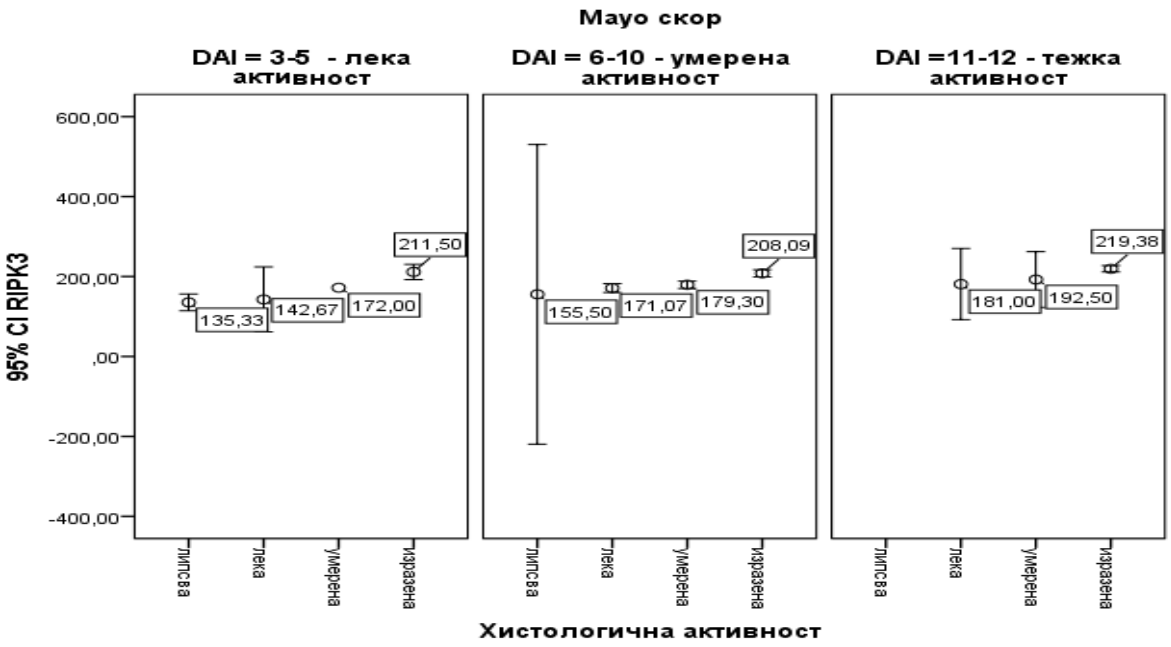


Fig. 89. Mean values of RIPK3 expression according to DAI and histological activity

There was also a significant difference in RIPK3 expression according to UC activity and low albumin levels (p <0.001), with severe disease is associated with low albumin levels and high expression of the necroptosis marker (208.17 at 129.00, respectively). Similar results were reported in the analysis of iron deficiency anemia, where severe disease was again associated with the presence of iron deficiency anemia and high RIPK3 expression (203.71 versus 137.80, respectively; p <0.001).

Low hemoglobin levels in patients with UC were associated with severe disease (DAI 11-12) and high RIPK3 expression (203.71 versus 158.77, respectively; p <0.001).

Severe UC activity is associated only with elevated CRP levels and overexpression of the necroptosis marker (211.71).

Overexpression of RIPK3 in patients with UC can be considered as a risk factor for severe disease and the development of intestinal complications (215.80 to 155.57, respectively; p <0.001).

One of the most common intestinal complications in patients with UC is pseudopolyposis, which is associated with moderate and severe activity (180.66 and 226.25, respectively) and is characterized by high expression of RIPK3.

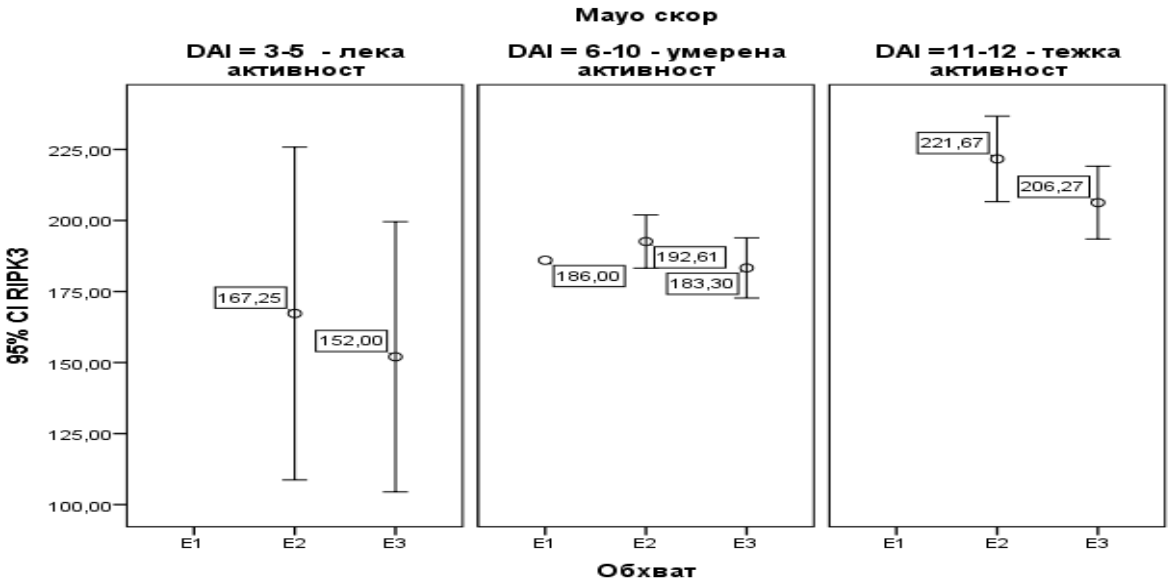


Fig. 90. Mean values of RIPK3 expression ccording to DAI and UC extent

From the point of view of the extent, the expression of the marker for necroptosis was established in the extent (E2), both in moderate and in severe UC activity (Fig. 90).

According to the presented results for the expression of RIPK3 in UC and CD patients, it can be said that there are some variations, but the main trend remains, namely that, increased expression of the necroptosis marker is associated with severe disease and risk of disease progression.

DISCUSSION

Despite advances in medicine, the pathogenesis of IBD is still unclear.

Several studies in recent years have reported that necroptosis in the intestinal epithelium is an important factor contributing to uncontrolled inflammation and defects in the intestinal barrier in IBD. (Günther C et al., 2013; Negroni A et al., 2015, Pasparakis M, Vandenabeele P, 2015). The RIPK3 protein has been identified as a key molecule required for the necroptosis pathway, and its expression correlates with the sensitivity of cells to undergo necroptosis (He S et al., 2009).

There are few data in the scientific literature on the role of necroptosis in IBD in humans. Most studies have examined the expression of RIPK3 in experimental animal models, making it difficult to compare results. Despite the growing number of publications examining RIPK3 expression in IBD patients, no established and validated cut-off levels for RIPK3 have been reported in CD and UC patients. The only guidelines in the study of IBD patients are the determination of increased or decreased expression in endoscopic intestinal biopsies taken from inflamed and non-inflamed areas, and comparing them with healthy controls (Günther C et al., 2013, Negroni A et al., 2017, Pierdomenico M et al., 2014, Wu T et al., 2019, Zhou M al., 2021). Other limitations in comparing the results are the different methods of analysis and the small samples.

Based on these limitations, were determined the cut-off values, the accuracy of RIPK3 for distinguishing IBD patients from healthy controls, as well as for distinguishing CD from UC, the specificity, sensitivity and positive and negative predictive value.

When determining the cut-off values of nuclear expression of RIPK3 for distinguishing healthy controls from IBD patients, we found a cut-off - 163.5, and for distinguishing UC patients from CD patients, we found a cut-off - 185.5.

A comparison of tissue expression of RIPK3 in IBD patients and healthy controls in the present study revealed elevated values in IBD patients. The results correspond to similar reports in the scientific literature (Günther C et al., 2013, Negroni A et al., 2017, Pierdomenico M et al., 2014, Wu T et al., 2019, Zhou M al., 2021), such as the authors do not give specific cut-off values or average values of the measured indicators.

In a study of RIPK3 expression in CD patients, it was found that the values in the inflamed areas were significantly higher than in the healthy controls. Our results correspond to

those of Günther C et al. (Günther C et al., 2011). In 2011, the team found increased expression of RIPK3 in inflammatory lesions of the terminal ileum through experimental studies in mouse models. In vivo results were also confirmed in the analysis of histological samples of terminal ileum in patients with active CD, with a high expression of RIPK3 in inflamed areas compared to healthy controls.

Similar results were reported by Pierdomenico et al. (Pierdomenico M et al., 2014). In 2014, the team examined 63 children with IBD - Crohn's disease (33) and ulcerative colitis (30) in activity, as well as 20 children with functional gastrointestinal disorders, normal endoscopy and histology, which serve as healthy controls. They examined endoscopic biopsies of inflamed and non-inflamed areas in CD and UC and found that Western blots had significantly increased levels of RIPK3 and MLKL in endoscopically active areas compared to healthy controls and non-inflamed areas, with comparable levels of markers in the latter. those of healthy controls. Biopsies were further analyzed by examining caspase-8, which was found to inhibit necroptosis by TNF stimulation. The results revealed a significant reduction in caspase-8 ($p < 0.05$) in the inflamed ileum in CD compared with controls. Decreased expression was also observed in the inflamed areas of the colon in CD and UC. To analyze the link between necroptosis and inflammation in CD and UC, they examined the gene expression of IL-8, a reliable marker of intestinal inflammation. The results found that IL-8 was significantly elevated in inflamed areas in IBD pediatric patients compared to non-inflamed areas and healthy controls, confirming that necroptosis and inflammation in the intestinal epithelium are closely related events. In their study, they did not find a correlation between RIPK3 expression and disease activity, or an association with therapies.

This is in contrast to the analyzes of the present study, which reveal a direct relationship between RIPK3 expression and clinical activity (assessed by CDAI). There is a similar trend when comparing the marker with endoscopic and histological activity, as overexpression of the marker is associated with severe clinical, endoscopic and histological activity of CD.

A proportional relationship was also found between the number of hospitalizations in CD patients and the expression of RIPK3. In addition, patients with frequent relapses have significantly higher marker expression than those with rare relapses.

There are no data in the available scientific literature on the changes in the expression of RIPK3 according to the localization, the disease behavior and the applied treatment in CD.

In the present study, it was found that the highest expression of the marker is in the ileum (L1) and colon (L2) in the extensive range of the disease affecting the upper GIT (L4) - L1 + L4 and L2 + L4. On the other hand, isolated involvement of the terminal ileum (L1) is characterized by the lowest expression of the marker, while colonic localization (L2) retains its position of localization, characterized by high expression of RIPK3.

Several studies have reported that upper GI involvement in CD is associated with poor prognosis, frequent recurrences, and the need for surgery (Cosnes J et al., 2002; Crocco S et al., 2012; Kim OZ et al., 2018, Moon JS et al., 2020, Sun XW et al., 2019). Chow et al. (Chow DK et al., 2019) found that in an analysis of the Asian population, patients with CD with upper GI involment have a more severe course of the disease, including strictures, fistulas, and the risk of prolonged hospitalization. This suggests that the L4 phenotype is a predictor of severe disease and requires more aggressive treatment than those without upper GIT (Cosnes J et al., 2002). However, there is controversy about the relationship between upper GI tract involvement and poor prognosis (Greuter T et al., 2018), which is most likely due to the lack of a standardized definition and the low frequency of screening fibrogastroduodenoscopy in the past (Greuter T et al. ., 2018).

In this regard, further analysis in the present study found that regardless of intestinal localization, patients with accompanying HP (-) gastritis who did not have the well-known criteria for assuming upper GIT (L4) involvement had high RIPK3 expression. This suggests that the presence of HP (-) gastritis may be an indicator of the severity of changes in the lower departments of the GIT in CD, which may serve to change the approach to treatment of patients, possibly including additional therapy as part of personalized approach.

Regarding the disease behavior, it was found that the highest expression of the marker is in patients with phenotype B2 + B3 - stricturing with fistulizing disaease, followed by the inflammatory (B1). It has been repeatedly pointed out in the scientific literature that the presence of fistulizing and stricturing disease predicts a more severe course, with patients with B3 phenotype having a weak response to immunomodulators (Lecomte T et al., 2003, Papi C et al., 2007).

In the present study, it was further found that patients with accompanying perianal disease are characterized by overexpression of RIPK3. Beaugerie et al reported that CD patients who have perianal disease at debut have a significant risk of a complicated course of the disease over the next 5 years (Beaugerie L et al., 2005). Another study confirms these results, adding that the stricturing disease is a marker of poor prognosis (Loly C et al., 2008).

Given the above results, it can be concluded that high expression of RIPK3 has a predictive value in CD in terms of increased risk of complicated course - development of perianal disease, stricturing and fistulizing disease, and severe activity.

Analysis of the results of this study did not establish a relationship between RIPK3 expression and CRP levels in CD and UC patients. It is known that serum CRP levels do not always correlate with IBD activity, and there may be normal values for both active CD and UC (Denis MA et al., 2007, Florin TH et al., 2006, Lewis JD, 2011, Vermeire S et al., 2006).

Clinical and population-based studies have shown that CD patients, with debut at a younger age, have a wider range and more complicated course, which is associated with a higher risk of developing fistulas and corticosteroid dependence (Gupta N et al., 2006 , Solberg IC et al., 2007). Etchevers et al. report that debut at a younger age is an independent predictor of aggressive disease (Etchevers MJ et al., 2009). In the present study, CD patients with the debut in the lowest age group (A1 <16 g) were found to have higher levels of marker expression than others, which is associated with high disease activity. Thus, our results confirm the consensus that young age is a predictor of severe disease in CD.

Increased expression of RIPK3 in inflamed tissues in UC, similar to that of Pierdomenico (Pierdomenico M et al., 2014), has been reported in several other studies. In 2019, Wu et al. analyzed endoscopic biopsies from inflamed and non-inflamed areas in 22 patients with active UC and 19 healthy controls. Immunohistochemical analysis revealed that RIP3 and MLKL expression was increased in inflamed areas of UC patients compared to non-inflamed and healthy controls. In addition, RIP3 and MLKL levels in inflamed areas are significantly positively associated with activity, including disease (Mayo score) and endoscopic activity (Modified Baron score) (Wu T et al., 2019).

The analysis from the present study showed similar dynamics. Examination of RIPK3 expression in endoscopically altered areas of the UC revealed a significant difference in expression compared to healthy controls, with UC patients having overexpression of the marker (189.4) and subjects in the control group having low expression (158.9).

Like Wu et al., we found a proportional relationship between RIPK3 expression and disease severity as assessed by the total Mayo score as well as the Montreal classification, with severe disease characterized by overexpression of the marker.

Another study confirms the current results and those of Wu et al. Lee et al in 2020 examined the expression of RIPK3 and MLKL in 11 patients with UC and found that the

expression of both markers and IL-17A were greatly increased in endoscopically altered areas of the colon compared to unaffected ones. In addition, in the experimental model of DSS-induced (dextran sulfate sodium) colitis in mice, there was overexpression of RIPK3 and MLKL compared with the control group (Lee S. H. et al., 2020).

Another group of scientists analyzed the expression of caspase-8, RIPK1, RIPK3 and MLKL in biopsies of IBD patients, as well as in the experimental model of DSS-induced colitis in mice. The results show a significant decrease in caspase-8 and a significant increase in RIPK1, RIPK3 and MLKL in inflamed colon tissues compared to levels in neighboring non-inflamed tissues (Zhou M al., 2021). In the present study in IBD patients, RIPK3 expression in affected tissues showed similar results.

Duan et al., In a study of patients with active UC, found that the levels of RIPK3 expression in the colon were directly proportional to the UC severity. Immunohistonymic analysis showed that the level of RIP3 expression in the intestinal tissue of patients with severe UC was higher than that of patients with moderate UC ($P < 0.01$). Subsequently, Duan et al., found that the use of necrostatin (Nec-1), a necroptosis inhibitor, can alleviate intestinal pathology and histological activity in mice with DSS-induced colitis. In addition, genetic deficiency of RIP3 inhibits the secretion of inflammatory cytokines (IL-16, IL-17 and IFN-γ) and TNF-α-induced ROS production (Duan, C et al., 2022)..

The present study also revealed a directly proportional moderate relationship between the severity of UC, as assessed by the total Mayo score, and the expression of RIPK3 ($r = 0.481$; $p < 0.001$). The same trend is observed when comparing the immunohistochemical analysis for necroptosis with the endoscopic and histological activity of UC. A directly proportional moderate relationship between RIPK3 expression and the degree of endoscopic activity assessed by the endoscopic Mayo score ($r = 0.363$; $p = 0.001$) was found. When comparing the expression of RIPK3 with histological activity, a strong proportional relationship was found ($r = 0.735$; $p < 0.001$), with the expression values of the necroptosis marker being significantly higher in UC patients with pronounced histological activity compared to those without activity (respectively 212.13 to 143.40).

An additional analysis of RIPK3 expression in IBD patients and healthy controls found that high marker expression correlated with the presence of IBD compared to healthy controls ($r = 0.398$; $p < 0.001$) and was associated with a 4.14-fold higher risk of high IBD activity (RR = 4.15 (2.01-8.57); $p < 0.001$).

Similar to the cited authors, this study showed increased expression of RIPK3 in IBD compared to expression in healthy controls. This proves that regardless of the way of selection of the subjects, the study period and the different population of patients, increased expression of RIPK3 is observed in Bulgarian patients, which gives grounds for increased expression of RIPK3 to enter the profile of patients with active IBD.

In the analysis of IBD patients and accompanying infection with Cl. Difficile found that the presence of Cl. Difficile in CD and UC is associated with low RIPK3 expression ($p < 0.01$), which is more significant in patients with CD. This may be due to the fact that the inflammation associated with necroptosis is immunomediated, which is different from the inflammation caused by Cl. Difficile infection. The different molecular mechanisms of the two types of inflammation may explain the low values of RIPK3 in Cl. Difficile infection. Further studies are needed in this area to establish the potential of RIPK3 as a marker to distinguish IBD from infectious enterocolitis..

For the first time worldwide, RIPK3 expression levels were compared between CD and UC patients, which showed a significant difference ($p = 0.001$), with patients with UC having higher expression levels than CD patients. In the evaluation to differentiate patients with CD from UC patients according to the expression of RIPK3, it was found that high expression is associated with more than 2 times more likely IBD patients to have UC (RR = 2.61 (1.41-4.85)); <0.05). From the presented results it can be concluded that the tissue expression of RIPK3 can be used as a marker to distinguish CD from UC.

After analysis of the expression of RIPK3 in IBD patients, it was found that RIPK3 is a complex important marker for the assessment of inflammation in IBD, as well as a risk factor for severe clinical activity associated with hematochezia (UC > CD), increased diarrhea bowel movements (UC), infectious complications (UC), intestinal complications (CD > UC) and surgical interventions (CD > UC).

CONCLUSION

The results of the analysis of the modern medical literature show that the role of RIPK3 as a marker of necroptosis in the course of inflammatory diseases is increasingly being studied. There are few data in the literature on the role of RIPK3 in IBD, which is limited to single studies in IBD patients, and the results of these studies are mainly aimed at demonstrating the relationship between necroptosis marker expression and the presence of inflammatory activity.

Due to the lack of validated values of necroptosis marker expression and better assessment of the prognostic role of the marker, cut-off values were set to distinguish IBD patients from healthy controls, as well as CD and UC patients. These results served as a guide for the direction and intensity of expression according to the considered characteristics and indicators in the studied groups. Thus, according to the established cut-off values, it was proved that IBD patients have increased expression only of healthy controls, and UC patients are characterized by overexpression of the marker..

The results of the analyzes give us reason to accept Hypothesis 1, namely that there is a significant difference in the expression of RIPK3 in IBD patients, where the studied indicator can be used as a marker to distinguish CD from UC and as a prognostic marker for development of severe disease and progression.

Increased expression of RIPK3 was found in IBD patients due to the active inflammatory process, and regardless of the treatment used at the time of follow-up, none showed that the mean expression of the marker was reached in healthy controls.

There are different profiles of patients with CD and UC regarding RIPK3 expression. In CD patients, the high expression of the marker for necroptosis correlates with young age at debut (<16 years), increased number of hospitalizations, extensive range (upper GIT with ileum / colon), stricturing with fistulizing disease (B2 + B3), the presence of perianal disease, moderate and severe clinical activity (CDAI> 220), frequent relapses, presence of endoscopic and histological activity, and discontinuation of treatment (conventional and biological).

In UC patients, elevated RIPK3 expression correlates with disease in debut over 40 years, debut disease, severe clinical and histological activity, rare relapses and haematochezia.

The analysis of RIPK3 expression in different therapeutic regimens revealed that against the background of treatment there are no levels of the necroptosis marker close to those in

remission or healthy controls, which may be a prerequisite for optimizing the treatment strategy as a first step towards a personalized approach in IBD patients.

Due to its unique nature, the current study also has some limitations related to the small number of patients, the current assessment, the lack of validated values and proven methodologies to make the results comparable to other studies. In order to validate the obtained results, it is necessary to conduct an extensive study among IBD patients and their follow-up over time.

FINDINGS

1. In patients with IBD, high expression of RIPK3 in inflamed tissues was observed compared to healthy controls..
2. The expression of RIPK3 differs significantly in CD and UC patients and healthy controls, with the highest expression of the marker found in UC patients.
3. High expression of RIPK3 in CD patients is associated with age at debut up to 16 yeras (A1), increased number of hospitalizations, upper GIT involvement in combination with colonic (L2) or ileal involvement (L1), stricturing with fistulizing form of the disease (B2 + B3), accompanying perianal disease, clinical activity of the disease (CDAI> 220), frequent relapses, the presence of hematochezia, high endoscopic and histological activity, discontinuation of conventional and biological treatment.
4. High expression of RIPK3 in UC patients is associated with debut, debut of disease over 40 years of age, rare relapses, hematochezia, severe disease activity as assessed by the Montreal Classification and the overall Mayo score, marked endoscopic and histological activity..
5. A comparative analysis of RIPK3 expression between IBD patients and healthy controls showed that the necroptosis marker was more elevated in men, in patients with severe inflammatory activity, and in 5-ASA or biologic therapy.
6. The lack of a significant difference in the expression of RIPK3 between IBD patients with debut and those with known disease, as well as the lack of difference between frequent and rare relapses indicates that the expression of the marker is mainly influenced by the presence of inflammation.
7. A comparative analysis of RIPK3 expression between CD and UC patients showed that UC expression was significantly higher in terms of gender, debut, clinical activity, bowel movements, hematochezia, abdominal pain, and thromboembolic complications.
8. The direction of RIPK3 expression differs between CD and UC patients in terms of patient age, incidence of relapses, weight loss, the presence of extraintestinal manifestations, accelerated ESR, the presence of iron deficiency anemia, decreased serum iron levels.
9. Increased expression of RIPK3 in CD patients is a prognostic factor for predicting the frequency of relapses, clinical manifestations with hematochezia, the occurrence of intestinal complications, accompanying diseases and future surgical interventions.

10. Increased expression of RIPK3 in UC patients is a prognostic factor that predicts clinical manifestations with hematochezia, increased incidence of diarrhea, future surgical interventions, high incidence of infections and high disease activity.
11. In IBD patients, high expression of the necroptosis marker (RIPK3) is associated with severe disease, marked clinical, endoscopic and histological activity, lack of response to treatment and poor prognosis associated with complications and surgical interventions.

CONTRIBUTIONS

Contributions of a theoretical nature

1. For the first time in Bulgaria a detailed and comprehensive review of the scientific literature on the use of RIPK3 as a marker for IBD.
2. A detailed analysis of the available literature data on the role of RIPK3 in patients with IBD has been made..
3. An in-depth analysis of RIPK3 expression was performed according to the characteristics of CD and UC patients, showing the differences between the two groups of patients.

Practical contributions

1. Cut-off values have been established to differentiate RIPK3 expression between healthy controls and patients with IBD, as well as between CD and UC patients.
2. A detailed analysis of the expression of RIPK3 in CD and UC patients was performed, identifying the specific characteristics of the marker for each group of patients..

Contributions of original character

1. For the first time nationally, study of the nuclear expression of RIPK3 in patients with IBD has been performed..
2. For the first time nationally and globally, the relationship between RIPK3 expression and the clinical and endoscopic activity of IBD patients has been demonstrated.
3. For the first time nationally and globally the role of RIPK3 expression as a marker for differentiating IBD patients has been studied.
4. For the first time nationally and globally RIPK3 expression has been evaluated as a prognostic marker for the development of severe disease and progression.

PUBLICATIONS RELATED TO THE DISSERTATION

1. Panayotova E., Atanasova A. The role of RIPK3 expression in the intestinal mucosa in inflammatory bowel disease. IV National Conference with International Participation - "Innovation in Public Health", Sofia, September 17-18, 2020, collection of abstracts p. 70, ISBN 978-619-7452-10-5
2. Panayotova E., Atanassova A., Programed necrosis and inflammatory bowel disease. Folia Medical I 2020 Vol.62 I Suppl.1 ISSN 0204-8043
3. Panayotova E., Atanasova A. Necroptosis - a possible trigger for the occurrence of inflammatory bowel disease. Varna Medical Forum, item 11, 2022.
4. Panayotova E., Atanasova A. Necroptosis - an inflammatory model of cell death. Varna Medical Forum, item 11, 2022.
5. Panayotova E., Atanasova A. Expression of RIPK3 among a group of Bulgarian patients with chronic inflammatory bowel disease. Varna Medical Forum, item 11, 2022.

Participation in scientific forums:

1. Panayotova E., Atanasova A. The role of RIPK3 expression in the intestinal mucosa in inflammatory bowel disease. IV National Conference with International Participation - "Innovation in Public Health", Sofia, September 17-18, 2020, poster session
2. Panayotova E., Atanasova A. Programmed necrosis and VCHZ. Jubilee Scientific Conference "Medicine of the Future", Plovdiv, 29-31.10.2020, poster session

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