UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

DOCTORAL SCHOOL

Mihail-Virgil BOLDEANU

CLINICAL, EPIDEMIOLOGICAL AND IMMUNOLOGY STUDY OF INFLAMMATORY BOWEL DISEASES

THESIS SUMMARY

PhD Coordinator:

Prof. univ. dr. Tudorel CIUREA

Craiova

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LIST OF ABBREVIATIONS

MMP	Matrix metalloproteinases
IL	Interleukin
CRP	C reactive proteine
ELISA	Enzyme Linked Immunosorbent Assay
IBD1	Identity By Descent
NOD2/CARD 15	Nucleotide-binding Oligomerization Domain 2 / Caspase-Activating Recruitment Domain 15
GWAS	Genome-wide association studies
OCTN	Organic cation transporter
DLG	Drosophila Large Discs Homologue
MDR	Multidrug resistance
ICAM	Moleculele de adeziune intercelulară
TGF β	Transforming growth factor beta
TNF α	Tumor necrosis factor alpha
CCR	Chemokine receptor
CD	Cluster designation
ROC	Receiver-operator characteristic

INTRODUCTION

Inflammatory Bowel Diseases (IBD) are in general terms chronic, idiopathic inflammatory conditions , whose primary effect is observed in the gastrointestinal tract, but can also have serious extraintestinal manifestations. BII are progressive inflammatory disease, recurrent, unexplained, particularly affecting young people, socio-professional active. Influence quality of life leading to chronic disability and higher costs of health systems.

BII are the condition that can occur at any time throughout life, from childhood to adulthood, although there are studies that show that over 80% of cases are diagnosed at present in the second or third decade of life. IBD incidence and prevalence are increasing worldwide, especially in Western countries is closely related to socio-economic progress.

Elucidation of immunologic and genetic factors with an important role in the pathogenesis of these diseases, indicates several points where inflammatory cascade can be interrupted, resulting in the possibility of targeted therapies for IBD. Thus, recent discoveries have led to new therapeutic agents that regulate the activation of the cellular immune response and inflammation of the bowel (biological therapy with *antibodies to tumor necrosis factor alpha*, *anti-TNF* associated immunosuppressants). This biological therapy changed patient management, establishing new therapeutic targets leading to mucosal healing and change the natural history of the disease. All these advances in recent years in the field of immunology and genetics, along with new minimally invasive endoscopic techniques and methods of diagnosis, helps in accurate definition of disease phenotype and in shaping new classification systems which contain besides IBD clinical classification and newly discovered genetic and serological data.

The proposed research theme is topical and has a special scientific interest because a complex analysis in terms of epidemiological and serological phenotype of patients with IBD. Originality of the study is on determining the serological profile of patients, the association between dynamics serum concentrations of serological markers and different clinical phenotypes of disease and the presence of correlations between IBD activity in adults and serum levels of MMP. Due to the high sensitivity and specificity, serological markers analyzed (MMP-3, MMP-9 and IL-13) can be considered as an alternative or additional to CRP, ESR and other inflammatory indices of disease, in diagnostic of IBD and prognostic purposes in predicting disease progression and therapeutic orientation.

Keywords: ELISA, matrix metalloproteinases, interleukins, ROC curve, diagnostic accuracy.

I. CURRENT STATE OF KNOWLEDGE

Chapter I - "Epidemiology of inflammatory bowel disease" presents recent data on the incidence and prevalence of these entities, as influences of age, gender, differences in racial/ethnic these epidemiological parameters and evolution of IBD in the world and Romania. Geographical variations were observed IBD and have found a gradient north-south and west-east of the incidence and prevalence of these diseases worldwide.

Chapter II - "Etiopathogenesis of inflammatory bowel disease" reviews the main pathogenic mechanism involved in IBD. The exact causes and etiopathogenic mechanisms leading to tissue breakdown are extremely complex and incompletely known so far. In the last decade has intensified scientific research of these diseases were brought several theories/hypotheses to explain the mechanisms sufficient progress etiopathogenesis and appeared to accept as valid the following hypothesis: BII is an

inappropriate immune response that occurs in susceptible individuals genetically, as a result of complex interactions between environmental changes induced evolution progress of society, constantly different intestinal microflora and intestinal immune system hyperactivation individual. However determine final loss of homeostasis and barrier function of the intestinal mucosa, immune-mediated tissue injury and clinical manifestations. The experimental data and clinical observations, obtained by studying altered immune mechanisms and intestinal inflammation in animal models, which was induced and caused a disease model, contributed to the understanding of the pathogenesis of IBD. These altered immune mechanisms are based on innate or acquired defects of the immune system, with the shutter trigger intestinal microflora.

Chapter III - "Genetic susceptibility to inflammatory bowel disease" presents data on the role of genetic factors and the main susceptibility genes involved in the pathogenesis of IBD.

In parallel with research in immunology, an important contribution to the study of IBD pathogenesis had the field of molecular genetics. Epidemiological studies of population and migration significant differences in the incidence and prevalence of IBD in terms of geographic and ethnic distribution. These observations led to the formulation of *new hypotheses*, such as the possible role of *genetic factors* in the pathogenesis of IBD. To prove this hypothesis were originally developed family aggregation studies, followed by studies on monozygotic twins and dizygotic twins. Followed, since 2000 genetic linkage studies of cohorts of patients related that led to the identification IBD1 locus on chromosome 16 and the first discovery of susceptibility genes for CD, the gene NOD2/CARD15. But the most important advances in molecular genetics have been moving from analysis of candidate gene to the association studies throughout the entire genome (*GWAS*), which allowed the identification of thousands of polymorphisms in the human genome, and over 160 new susceptibility genes for IBD.

Since 1996 genetic linkage studies, the human genome scan using microsatellite

DNA markers that have contributed to the identification of several susceptibility regions for CD located on several chromosomes 1, 3, 4, 5, 6, 7, 10 , 12, 14, 16, 19, X. Subsequently, the regions / loci on chromosomes 16, 12, 6, 14, 5, 19 and 1 have changed since IBD1 to IBD7, only IBD1, IBD2, IBD3 and IBD4 loci were replicated in studies (Table 1).

Region	Location	Gene involved
IBD 1	Chromosome 16	**NOD2/CARD15**, IL-4R, CD11B
IBD 2	Chromosome 12	Receptors of vit. D, STAT 6, Interferon γ
IBD 3	Chromosome 6	Major histocompatibility complex (MHC)
IBD 4	Chromosome 14	Receptors of Ly T (TCR)
IBD 5	Chromosome 5	**OCTN**, **DLG5**, **MDR1**, IL-6, CD14
IBD 6	Chromosome 19	Tromboxane A2, Leukotrienes B4, ICAM-1
IBD 7	Chromosome 1	TGFβ, Receptors of TNFα
IBD 8	Chromosome 16	-
IBD 9	Chromosome 9	Receptors of chemokines (CCR-5, CCR-9), IL-12

Table 1. Regions and important genes involved in IBD (Adapted from Zheng CQ) [1].

In **Chapter IV**, entitled **"Serological markers in inflammatory bowel disease"** are presented the most important serological markers studied in IBD and their correlations with specific disease phenotypes.

MMPs are a family of endopeptidases Zn^{2+} and Ca-dependent, considered to be the most powerful proteases controlling homeostasis of extracellular matrix proteins, including regulation of cell function, growth, cell division, regulation of innate and acquired host defense and control of extracellular matrix synthesis.

In recent years many studies aimed at understanding MMPs involvement in intestinal

homeostasis, and their role and function in IBD. More MMPs (MMP-1, -2, -3, -8, -9, -12, MT-MMP-1) have been reported to be involved in the pathogenesis of colitis induced in a variety of animal models. Also, studies have shown that increased levels of MMPs correlates with disease activity in direct proportion. Among the MMPs, MMP-3 and MMP-9 expression was predominant in most studies on colitis induced in laboratory animals, sodium dextran sulfate, trinitrobenzenesulfonic acid and Salmonella typhimurium. MMP-3 and MMP-9 have been associated with lesions of the colon and mucosal complications, fistulae in patients with CD.

Chapter V - "Phenotypic classification of inflammatory bowel disease" Montreal presents phenotypic classification of the two clinicopathological entity of IBD.

II. PERSONAL CONTRIBUTIONS

Chapter VI - AIMS AND OBJECTIVES OF THE STUDY

The objective of this thesis is to *complex analysis* in terms of epidemiological and serological phenotype in patients with IBD (CD and UC), in the Referral Center of Craiova, Dolj County. Research project focuses on the following *specific objectives*:

- Analysis of key epidemiological parameters (incidence, prevalence) in IBD and their change trends in Dolj County;
- Evaluation of phenotypic characteristics of patients with IBD in terms of age of onset, location, severity of inflammatory lesions and intestinal and extra intestinal complications;
- Serological profiling of patients with identification of association between dynamics serum concentrations of serological markers and different clinical phenotypes of disease and establishing correlations between serum levels of serological markers and severity of IBD flare activity by :
  - The quantification of MMP-3 (stromelysin 1), MMP-9 (gelatinase B) ;
  - Measuring the levels of cytokines (pro-inflammatory cytokine IL-17 and anti-

inflammatory cytokine IL-13) ;

- Measurement of CRP by highly specific determination (hs-CRP), a marker important to quantify the inflammatory response.

- Evaluation of diagnostic accuracy of the determination of matrix metalloproteinases and interleukins compared with CRP determination using ROC curve analysis.

Chapter VII - MATERIAL AND METHOD

VII.1. Establishing lots and inclusion of patients in the study

In this study included 67 patients diagnosed with CD and UC, based on history, clinical examination, the lower gastrointestinal endoscopy and pathological examination of biopsy pieces. Patients included in this study were diagnosed in Gastroenterology Clinic of Emergency County Hospital Craiova, between October 2011 - May 2014.

For comparative analysis was constituted a control group (patients without a history of active IBD) consisting of 30 healthy subjects selected from patients investigated for other digestive disorders, also in Gastroenterology Clinic. Both groups were established on the basis of inclusion and exclusion. Both, IBD patients and control subjects signed an informed consent for inclusion in the study.

For each patient with IBD has developed a structured form which included contact information, demographic data, family history and personal pathology, clinical manifestations, laboratory analysis, phenotypic classification Montreal, intestinal and extraintestinal complications, classes of drugs used and the scheme current treatment.

VII.2. Sampling and biological material

Biological material for both groups (group with IBD and control group) was represented by blood (approximately 5 mL of venous blood) collected in tubes without anticoagulant. After harvesting, followed by centrifugation to separate clot (15 min at 1500 rpm) within 4 hours of collection, as standard procedure. Serum tubes were sealed

to prevent contamination and stored at + 2 ° C to + 8 ° C for 3 days when the samples were immediately worked or having been deep-frozen to at least -20 ° C and even -80 ° C when the samples were worked to a greater period of time. Samples were coded with letters and numbers in the order given crop.

VII.3. Determination of the presence and concentrations of serological markers

Serological profiling of patients with IBD and identification of association between dynamics serum concentrations of serological markers and different clinical phenotypes of disease and establishing correlations between serum levels of serological markers and severity of IBD flare activity, were performed in the *Laboratory of Immunology of UMF Craiova*. For the quantitative determination of IL-17, IL-13, MMP-3, MMP-9, CRP, we used *ELISA* technique.

VII.4. Statistical analysis

Information obtained were stored in Microsoft Excel files were then processed statistically to analyze the relationship between clinical and laboratory data of patients. The management of patient data and the processing of these data was performed using Microsoft Excel with XLSTAT suite for MS Excel and statistical analysis was performed using statistical software dedicated to scientific calculations, *GraphPad Prism 5* and *IBM SPSS Statistics 20.0*. We used ROC curve analysis to detect possible threshold values, cut-off, which use them in practice to make the separation of patients with inflammatory bowel disease in patients without such a pathology.

Chapter VIII - epidemiological aspects in inflammatory bowel diseases

Our research is the first epidemiological study conducted in our Referral Center and provides the first epidemiological data, characteristics of patients diagnosed with IBD:

1. The data are consistent with previous studies provided by the Referral Center of Bucharest and confirms the low average annual incidence of CD and UC in Dolj

County (0.82 /10^5 inhabitants, respectively 1.26 /10^5 inhabitants) (Figure 1) [2,3].

2. We found a higher incidence of UC compared to CD.
3. Crohn's disease started at a younger age (mean age 39.52 years) than the population of Western and Central Europe, with a balanced distribution, with a slight preponderance of females and a single peak incidence [4- 10].
4. Ulcerative colitis had an age of onset (mean age 44.80 years) similar to the western population [4-10], with a bimodal distribution (ranges 20-29 and 50-59 years) incidence in women and equal gender distribution.
5. The incidence of CD is slightly higher in rural areas than in urban areas.
6. In urban areas there is high incidence of UC statistically significant ($p = 0.013$) compared to rural areas.

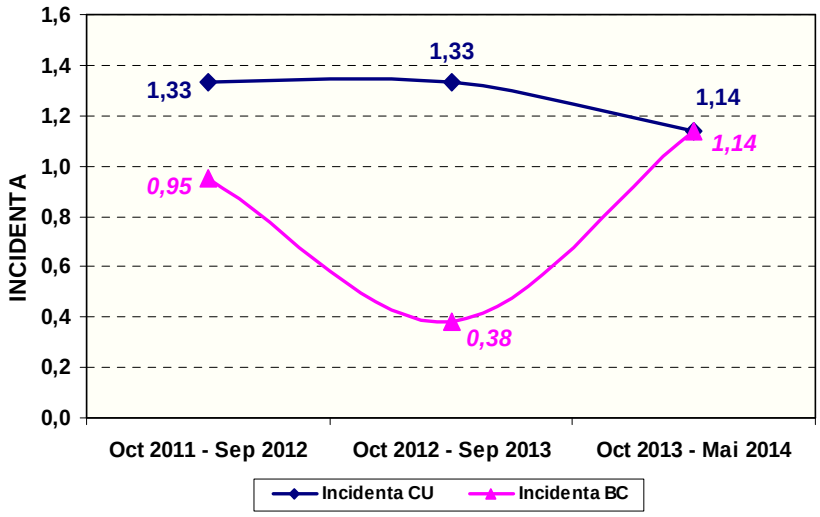

Figure 1. Evolution of the annual incidence of CU and BC

Chapter IX. PHENOTYPIC FEATURES OF PATIENTS WITH IBD

To analyze the phenotypic features of patients diagnosed with IBD, we studied two groups of patients:

- **Lot 1** - represented the 21 adult patients with *CD* (all patients known or newly diagnosed), hospitalized and investigated in Gastroenterology Clinic of Emergency County Hospital Craiova in the analyzed period October 2011 - May 2014. Data were retrieved and analyzed medical documents existing in the archives of the Clinic.

  Each patient was classified according to the *Montreal classification* according to: age at diagnosis (A1 <16, A2: 17- 40, A3:> 40 years), location of lesions (L1: ileal, L2: colon, L3: ileo -colonică, L4: upper digestive tube, p: perianal) and pattern (type) lesions (B1: non-stenosing, non-penetrating, B2: stenosing, B3: penetrating). For each case was calculated *Harvey-Bradshaw score* (*HBI*) useful in evaluating patients according to severity of flare of disease activity. Depending on the score obtained each patient was placed in the appropriate activity type, as follows: HBI score = 5-7, light activity; HBI score = 8-16, moderate activity; HBI score => 16, severe activity [11].

- **Lot 2** - accounted for 46 adult patients with *UC* (all patients known or newly diagnosed), hospitalized in Clinical Gastroenterology also investigated in the same period under review, October 2011 - May 2014.

  And for this group to do the same, each case was classified according to the *Montreal classification* according to the extent of the lesion: E1 - proctitis, E2 - left colitis, E3 - extensive colitis. To classify cases according to severity of disease we used *Truelove-Witts scale* (*TWI*). Depending on the score obtained each patient was placed in the appropriate activity type, as follows: TWI score = <5, easy activity; TWI score = 5-9 moderate activity; TWI = score> 9 severe activity [12].

❖ Results

1. We see that the age of onset for the two clinico-pathological entity is 5 years older than age in West and Central European countries. There is a tendency of developing UC at younger ages in recent years [4, 5, 14, 15].

2. More than half of newly diagnosed cases of CD had significant colonic affectation. Left colitis prevailed in patients with UC. We observe phenotypic progression of the disease over time in patients newly diagnosed with UC, weight loss colitis left, weight doubles extensive colitis and recorded cases of proctitis (Figures 2, 3 and 4)

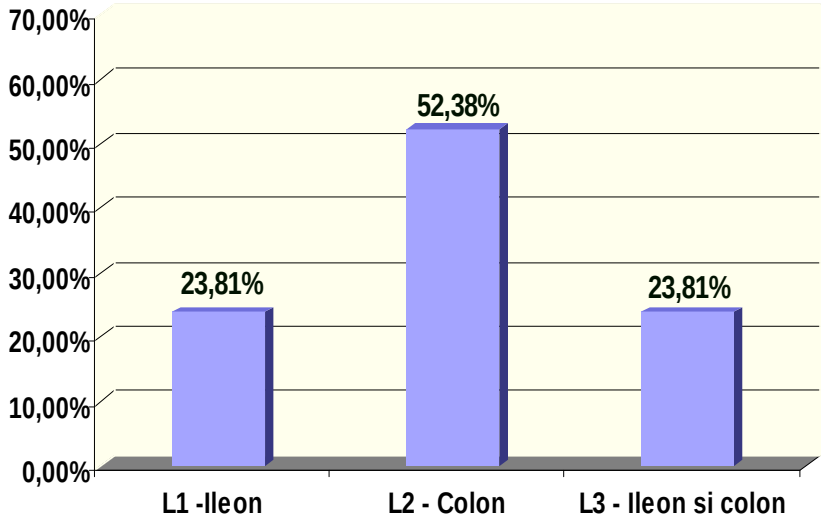

Figure 2. BC - Montreal L Classification - classification of patients according to location of lesions

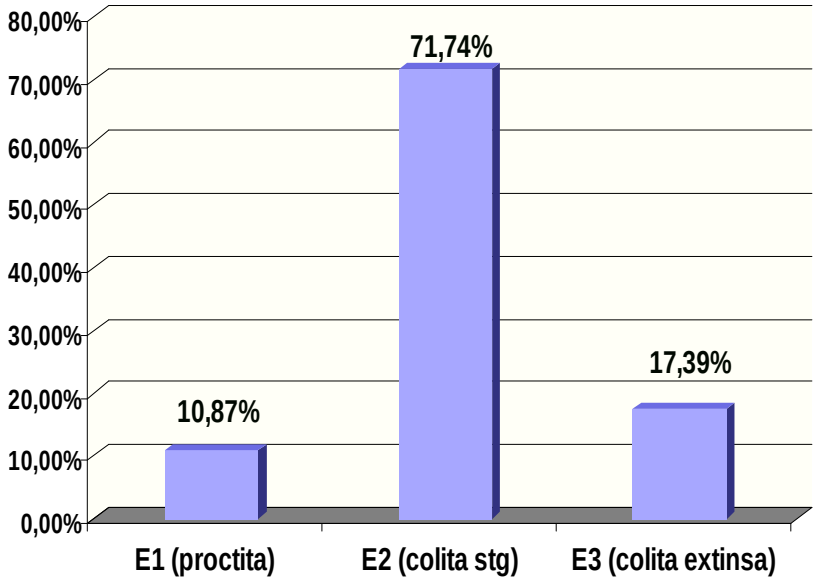

Figure 3. - Distribution of cases according to the location of lesions

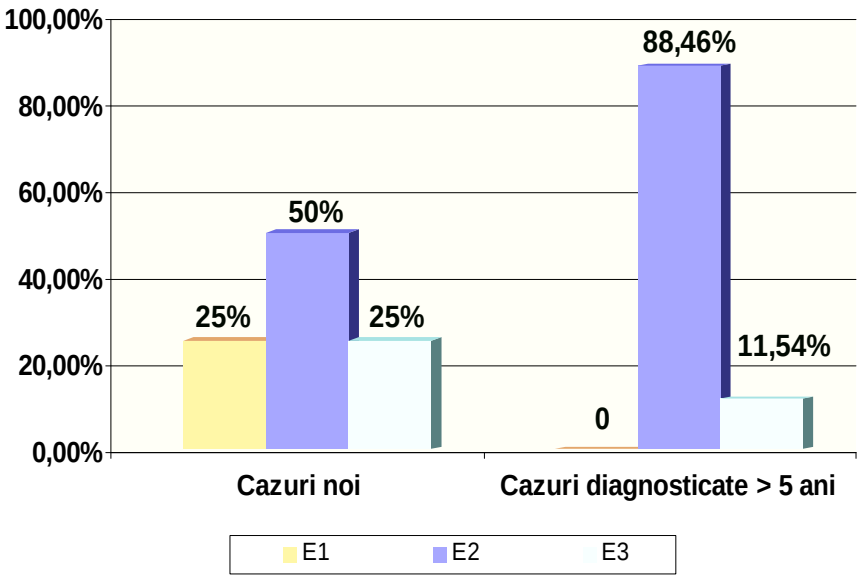

Figure 4. - Location of lesions in patients with diagnosis 5 years old *vs* newly diagnosed cases (E1: proctitis, E2: left colitis, E3: extensive colitis, *p = 0.0067*)

1. Non-stenosing, non-penetrating phenotype (inflammatory form) is most commonly found in CD (Figure 5)

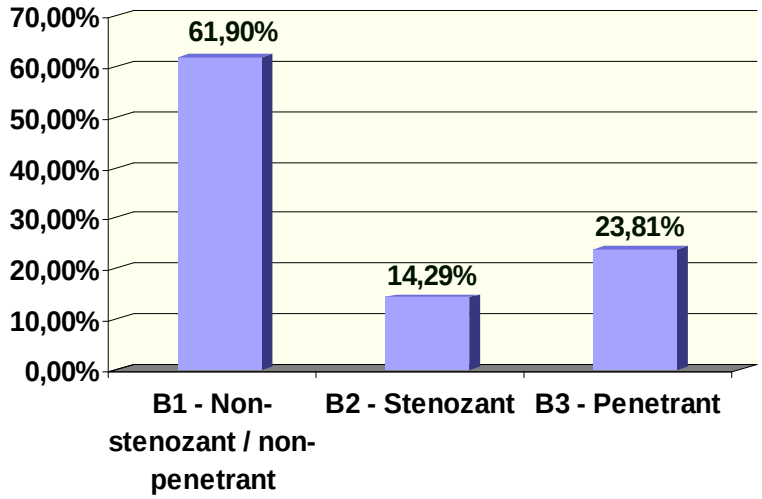

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Figure 5. BC - Montreal B Classification - Classification of patients depending on the pattern of injuries

2. Mild and moderate forms predominated in both entities.

3. No perianal manifestations, low frequency of intestinal and extra-intestinal complications in our study, suggesting a mild evolution of CD and UC in our Referral Center (Dolj county). Data are small compared with those reported in Western European literature.

Chapter X. SEROLOGICAL ASPECTS IN IBD

Quantitative dosage of IL-17, IL-13, MMP-3, MMP-9, CRP was performed using ELISA kits supplied by Invitrogen Corporation (Camarillo, CA, USA), following the protocol that, according to the manufacturer's recommendations. Values were expressed as *pg/ml* (IL-13, IL-17, MMP-9), *ng/mL* (MMP-3) and *mg/mL* (CRP). To identify the association between concentrations of these serological markers and different clinical phenotypes of disease, for both CD and UC, we used data provided by the Montreal classification.

For statistical analysis of the results we checked Gaussian distribution series values obtained with the *Kolmogorov-Smirnov* normality test or *D'Agostino & Pearson*. Thus serum levels of MMP-3 had a normal distribution after log transformation (due to statistical considerations and look, through logarithm is reduced values beach, keeping initial reports), while the concentrations of other serological markers, MMP-9, IL 13, IL-17 and CRP to analyze D'Agostino test for normality & Pearson had a Gaussian distribution. All values were expressed as arithmetic mean (mean), accompanied by 95% confidence interval (95% CI).

➢ Results

❖ Associations between serum concentrations of markers and different phenotypic profiles in IBD

1. The study conducted in our Center highlighted association between certain phenotypic profiles with serological markers analyzed in patients with IBD.
2. For the first time we have demonstrated the presence of significantly higher concentrations of MMP-3, -9, IL-13, -17 and CRP in the serum of patients with IBD compared to the control group.
3. Significantly higher concentrations of MMP-3 has been observed in patients over 40

years start (A3) of the CD, in patients with colonic damage (L2), in non-stenosing, non-penetrating (B1) and penetrating/fistulizing (B3) patterns and patients with extension to the left colon (E2) in UC.

4. Serum concentrations of MMP-9 were associated with early onset of CD (under 40 years) in patients with ileal involvement (L1), with stenosing pattern (B2) and to patients with left colitis location (E2).

5. Serum levels of cytokine IL-13 showed significantly higher in patients with onset over 40 years, with ileal involvement and to patients with left colitis.

6. Pro-inflammatory cytokine IL-17 was associated with onset under 40 years CD, with ileo-colonic and colonic location and proctitis.

7. The only significantly higher association of serum levels of CRP were with extensive colitis.

❖ Associations between serum concentrations of markers and clinical activity in IBD

Complex analysis of the two groups included in the study (group 1, patients diagnosed with CD and group 2 patients with UC) also included the investigation of the existence of various associations between concentrations of serological markers and severity of clinical activity flares for each clinical entity. The severity of clinical activity flares were estimated by addressing two scales / scores (HBI and TWI) used in clinical practice to assess patients and for monitoring progress and assessing the effectiveness of therapeutic regimens. Depending on the score obtained each patient was placed in the appropriate activity type.

The distribution of patients according to clinical and pathological entity and the severity of the clinical activity flare, on the score obtained is shown in Tables 2 and 3.

Lot 1 - Patients diagnosed with CD				
	Activity	**HBI**	Patients	***%***
Harvey-Bradshow score	Mild	6.00 (5.24-6.75)	7	33.33
(The severity of flare activity)	Moderate	12.56 (10.67-14.44)	9	42.86
	Severe	18 (16.76-19.24)	5	23.81

Table 2. CD - Distribution of patients according to severity of the clinical activity flare

Lot 2 – Patients diagnosed with UC				
	Activity	**TWI**	Patients	***%***
Truelove-Witts scale	Mild	3.20 (2.89-3.51)	15	32.61
(The severity of flare activity)	Moderate	7.23 (6.72-7.74)	22	47.83
	Severe	11.33 (10.18-12.49)	9	19.56

Table 3. UC - Distribution of patients according to severity of the clinical activity flare

Comparing the concentrations of markers analyzed, we found that their serum levels in subgroups of patients with mild, moderate and severe activity were significantly higher than in controls ($p < 0.0001$).

Serum levels of MMP-3, -9, IL-13, -17 and CRP for CD respectively UC are shown in Tables 4 and 5.

Parametru (mean ± SD)	**Activitate severă**	**Activitate moderată**	**Activitate uşoară**	***Lot control***
MMP-3 (ng/ml)	9.69 ± 1.40	9.88 ± 1.60	7.00 ± 2,27	*3.23 ±0.86*
MMP-9 (pg/ml)	544,42 ± 189,30	531.90 ± 109,80	505,60 ± 67,91	*112.20 ±21.10*
IL-13 (pg/ml)	65,78 ± 21,54	82,11 ± 19,42	64,83 ± 25,52	*26.16 ±4.64*
IL-17 (pg/ml)	53,99 ± 4,54	58,55 ± 34,42	45,74 ± 7,61	*34.08 ±10.33*
hs-CRP (mg/ml)	13,00 ± 4,80	9,00 ± 4,87	7,14 ± 2,12	*5.17 ±3.48*

Table 4. Concentrations of serological markers according to different stages of clinical activity of CD

Parametru (mean ± SD)	**Activitate severă**	**Activitate moderată**	**Activitate uşoară**	***Lot control***
MMP-3 (ng/ml)	7.08 ± 3.76	5.89 ± 3.34	3.60 ± 1,17	*3.23 ±0.83*
MMP-9 (pg/ml)	683.50 ± 93.61	646.13 ± 101.20	519.19 ± 17.39	*112.20 ±21.10*
IL-13 (pg/ml)	61.81 ± 22.48	64.62 ± 31.15	57.40 ± 33.29	*26.16 ±4.64*
IL-17 (pg/ml)	42.32 ± 5.32	47.76 ± 10.83	34.48 ± 15.42	*34.08 ±10.33*
hs-CRP (mg/ml)	16,11 ± 10.06	10.86 ± 6.47	7.17 ± 5.20	*5.17 ±3.48*

Table 5. Concentrations of serological markers according to different stages of clinical activity of UC

In our study, we identified significant association of MMP-3, MMP-9, IL-13, IL-17 and CRP with various stages of clinical activity of CD and UC.

- **Crohn's disease** - we noticed that MMP-3 was increasing up to the moderate stage of the disease activity, with a subsequent decline in the severe stage. Were statistically significant differences between the levels of MMP-3 in sera of patients with severe and moderate activity *vs* mild activity (Figure 6).

In contrast to MMP-3, in the case of MMP-9, serum concentrations increased proportionally with the activity of CD, the highest concentrations were in patients with severe activity disease. The only difference that reached statistical significance was observed between the concentrations of MMP-9 in serum of patients with severe *vs* mild activity (Figure 7). Our data are consistent with observations from the literature, as outlined above [16-20].

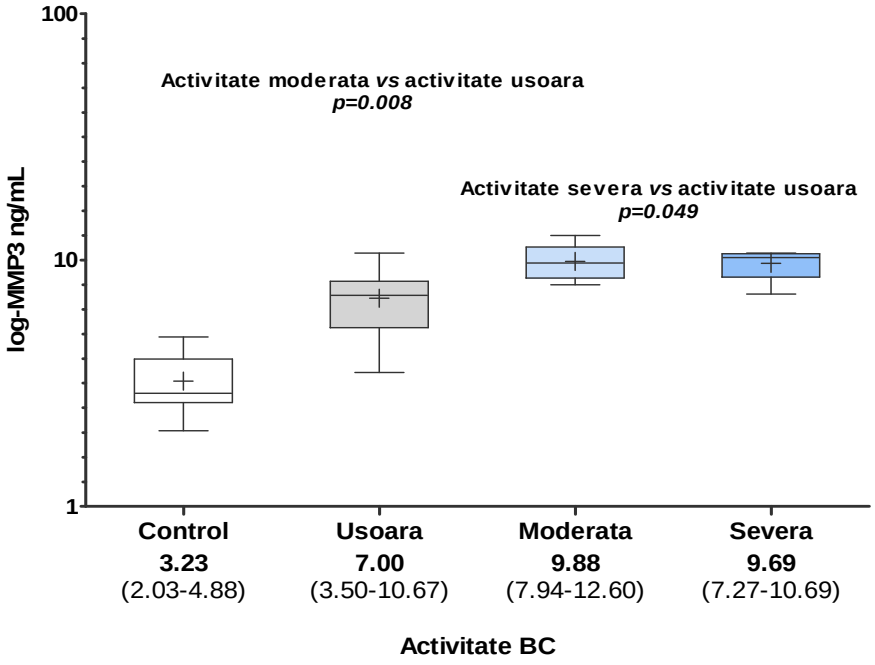

Figure 6. CD - The concentrations of MMP-3 in sera of patients with different stages of clinical activity *vs* control group

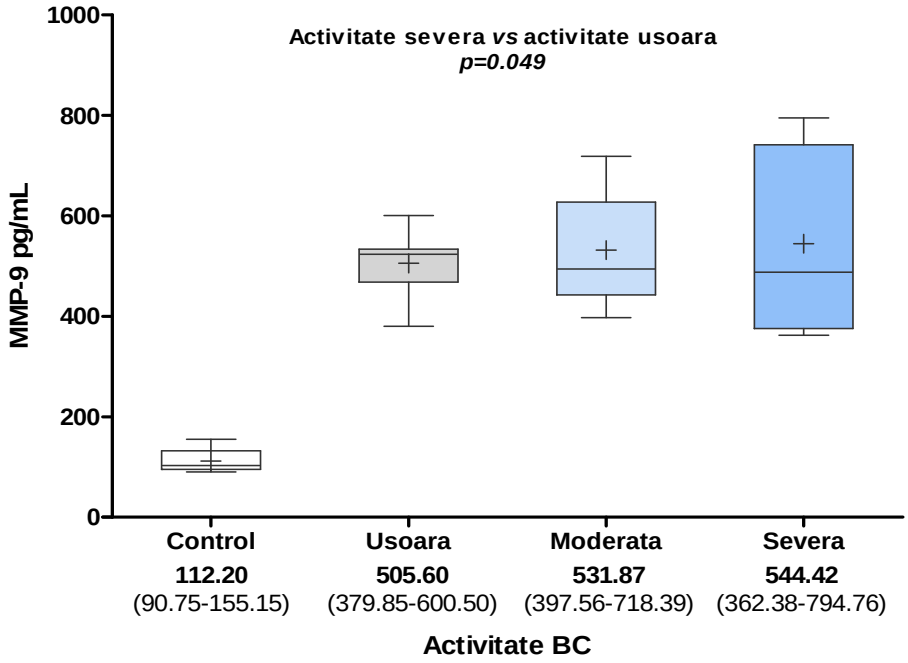

Figure 7. CD - Concentrations of MMP-9 in sera of patients with different stages of clinical activity *vs* control group

For the two cytokines, IL-13 and IL-17, we observed increasing concentrations with peak in the group of patients with moderate activity of CD, following a slight decrease in the group of patients with severe activity. Statistical significance levels are reached in groups with moderate *vs* severe activity and moderate *vs* mild activity (IL-13) and between concentrations in serum of patients with severe and moderate activity *vs* mild activity (IL-17). For the two cytokines have not found data in the literature to which we refer.

CRP - serum concentrations increased with the disease activity, the only statistically significant differences were between the group of patients with severe *vs* mild activity.

- **Ulcerative colitis** – for both proteinases, MMP-3 and MMP-9, serum concentrations increased direct proportionally with disease activity, with the highest concentrations in patients with severe activity disease. Both MMP-3 and MMP-9 showed statistically significant differences between concentrations in the serum of patients with severe and moderate *vs* mild activity. The data obtained in our study are consistent with recent data published in the literature [16-20].

  Concentrations of IL-13 and IL-17 in serum of patients with UC, showed a trend similar to those in the group with CD, with a maximum in the group of patients with moderate activity, following a slight decrease in group of patients with severe activity. Statistical significance was reached only for IL-17 serum levels between patients with severe and moderate *vs* mild activity (Figures 8, 9).

  CRP recorded in the serum of patients with an increase in direct proportion to the severity of activity flare, maximum levels were in the group of patients with severe activity. CRP was associated with significant levels in group of patients with severe and moderate *vs* mild activity. The data are consistent with previous observations in the literature [369].

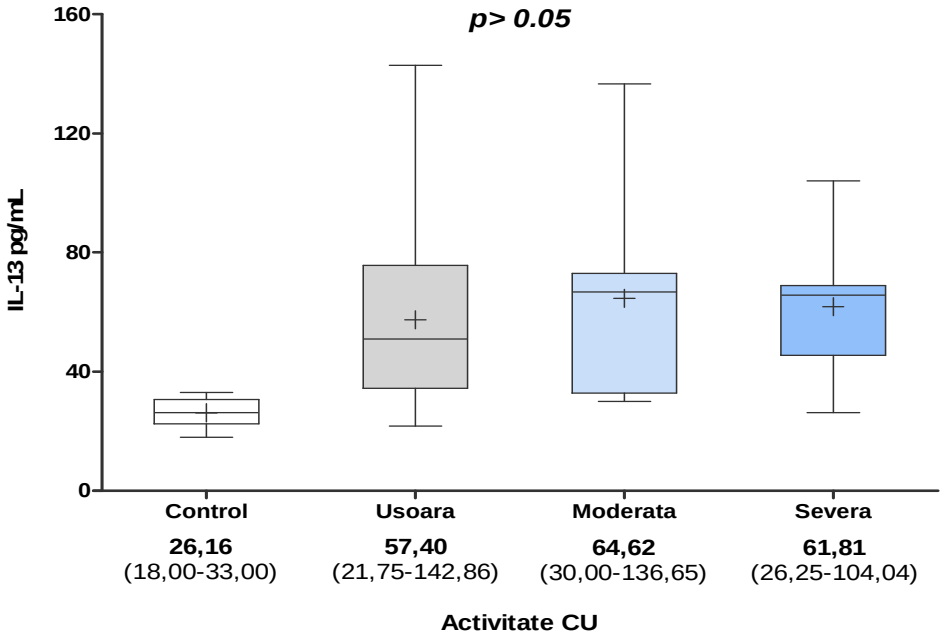

Figure 8. UC- Concentrations of IL-13 in sera of patients with different stages of clinical activity *vs* control group

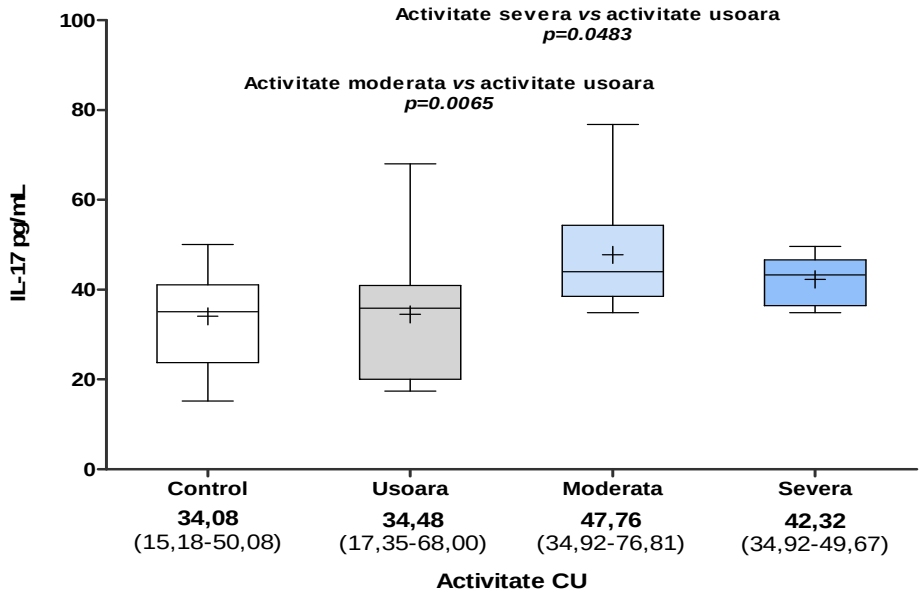

Figure 9. The concentrations of IL-17 in sera of patients with different stages of clinical activity *vs* control group

❖ Correlations in IBD

Study serological profile of patients diagnosed with IBD, continued with another specific objective to establish correlations between serological markers investigated (MMP, IL and CRP) and between serological markers and HBI, TWI severity assessment scores.

Using the software *GraphPad Prism 5* we calculated the Pearson correlation coefficient and demonstrated the presence of correlations between serum levels of MMP-3, MMP-9 and activity of IBD in adults (HBI, TWI score) and other indices evaluated to quantify the body's inflammatory response in CD (Table 6), respectively UC (Table 7)

Markeri	MMP-3	MMP-9	HBI	IL-13	IL-17	CRP
MMP-3		***r = 0.554****	***r = 0.276****	***r = - 0.341****	***r = 0.216****	*r* = 0.016
		p = 0.037	***p = 0.049***	***p = 0.030***	***p = 0.047***	*p* = 0.943
MMP-9			***r = 0.608****	***r = - 0.349****	*r* = -0.037	*r* = 0.246
			p = 0.039	***p = 0.029***	*p* = 0.874	*p* = 0.282
HBI				r = 0.063	***r = 0.300****	***r = 0.398****
				p = 0.785	***p = 0.046***	***p = 0.047***
IL-13					r = 0.365	r = -0.061
					p = 0.103	p = 0.791
IL-17						r = -0.226
						p = 0.322

r coeficientul de corelație Pearson, * Corelație semnificativă statistic

Table 6. Correlation between serological markers evaluated in CD

Markeri	MMP-3	MMP-9	IL-13	IL-17	CRP	TWI
MMP-3		r = 0.248	r = 0.036	r = 0.213	r = 0.209	***r = 0.344****
		p = 0.095	p = 0.810	p = 0.154	p = 0.162	***p = 0.029***
MMP-9			***r = 0.259****	***r = 0.563****	***r = 0.425****	***r = 0.308****
			p = 0.048	***p = 0.00004***	***p = 0.003***	***p = 0.037***
IL-13				***r = 0.354****	r = 0.108	r = 0.093
				p = 0.015	p = 0.473	p = 0.538
IL-17					***r = 0.588****	***r = 0.312****
					p= 0.00001	***p = 0.034***
CRP						r = 0.223
						p = 0.136

r coeficientul de corelație Pearson, * Corelație semnificativă statistic

Table 7. Correlation between serological markers evaluated in UC

- In the group of patients with Crohn's disease, serum levels of MMP-3 were correlated much better with hints of disease evaluated for this entity. Also the only cytokine IL-17 showed correlation with disease HBI score.
- In the group of patients with ulcerative colitis, serum levels of MMP-9 were correlated much better with indices evaluated. Among cytokines, we found that serum concentrations of IL-17 were better correlated with indices of disease.
- All the data obtained in our study, both for the group of patients with Crohn's disease and ulcerative colitis are consistent with previous observations in the literature.

❖ DIAGNOSTIC ACCURACY OF SEROLOGICAL MARKERS

A final objective of our study was to determine the performance / diagnostic accuracy of serological markers CRP comparative analysis using ROC curve analysis. We used ROC curve analysis to detect possible threshold values (cut-off), which use them in practice to make the separation of patients with inflammatory bowel disease (BC and CU) in patients without such a pathology. For various threshold values investigated at each marker, we calculated the sensitivity (Sn), specificity (Sp), their sum, and positive predictive values (PPV) and negative (NPV) and likelihood ratio positive (LR +) or false positive rate and likelihood negative ratio (LR) or false negative rate.

The performance was expressed as the area under the ROC curve (AUC, area under ROC curve) together with 95% confidence interval (95% CI) and *p* statistical difference between the calculated AUC and AUC = 0.05 (weak discriminative marker) .

- Comparing the ROC curves for the 5 parameters analyzed (Table 8), it appears that the best separation of patients with ***Crohn's disease*** can be done with the help of matrix metalloproteinases, the best detection being made by means of ***MMP-9*** (*accuracy of 100 %* of correctly diagnosing a sick person), followed by interleukins, ***IL-13*** (*97.90% accuracy*) with better performance than IL-17, but less than MMP, the less good detection determination being made by CRP (and thereby making a good separation of cases) (Figure 10).

- Also the comparison of ROC curves for the 5 parameters analyzed in ***ulcerative colitis*** (Table 9), we observed that the best separation of patients with CU can be done using ***MMP-9***, with the most advanced detection (*100% accuracy*) to diagnose, followed by ***IL-13*** (*92.80% accuracy*), the less quality detection being carried out by determination of IL-17. Performance/diagnostic accuracy of CRP was slightly better than detection by dosing MMP-3 (Figure 11).

Parametru	*AUC*	*Prag propus (cut-off)*	*Valoarea p AUC<>50%*	*Sensibilitate*	*Specificitate*
MMP-9	**1.000**	**155.15**	**< 0.0001**	**100.00%**	**100.00%**
MMP-3	**0.992**	**4.88**	**< 0.0001**	**95.24%**	**100.00%**
IL-13	**0.979**	**32.95**	**< 0.0001**	**95.24%**	**93.33%**
IL-17	**0.885**	**42.35**	**< 0.0001**	**85.71%**	**80.00%**
CRP	0.819	6.00	< 0.0001	80.95%	76.67%

Table 8. CD - Diagnostic performance of serological markers

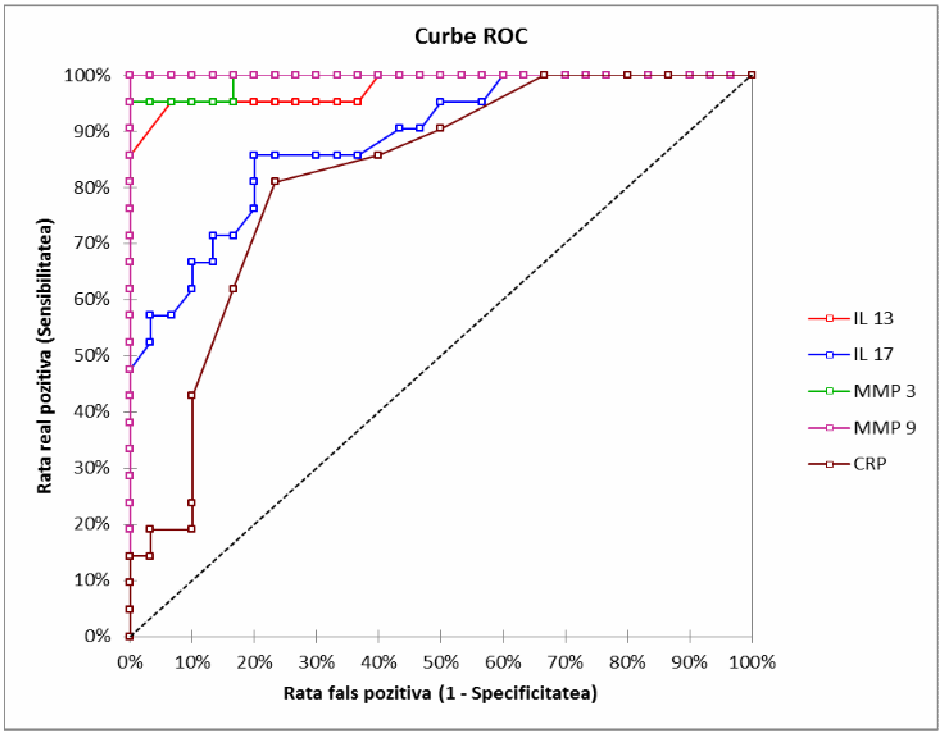

Figure 10. CD - ROC curves of serological markers analyzed

Parametru	*AUC*	*Prag propus (cut-off)*	*Valoarea p AUC<>50%*	*Sensibilitate*	*Specificitate*
MMP-9	**1.000**	**155 (- 410)**	**< 0.0001**	**100.00%**	**100.00%**
IL-13	**0.928**	**31.5 (30-33)**	**< 0.0001**	**86.96%**	**83.33%**
CRP	0.766	6.00	< 0.0001	73.91%	76.67%
MMP-3	0.764	3.55	< 0.0001	71.74%	70.00%
IL-17	0.690	37.50	0.00146	65.22%	63.33%

Table 9. UC - Diagnostic performance of serological markers

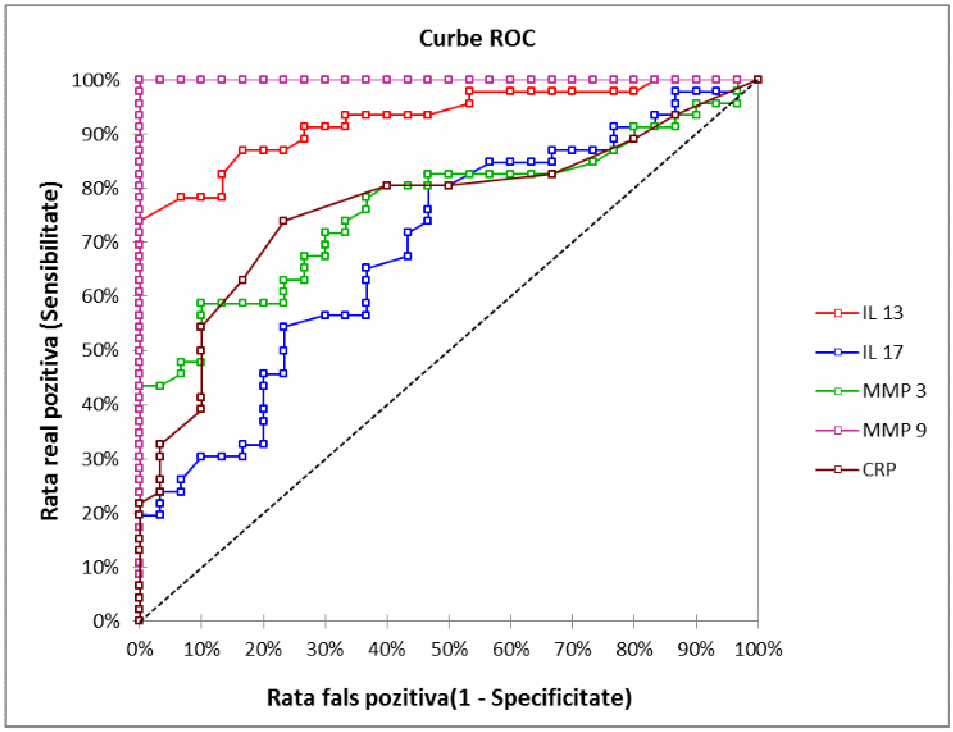

Figure 11. - ROC curves of serological markers analyzed

1. In our study we obtained high diagnostic accuracy for the determination of matrix metalloproteinases, MMP-9 and MMP-3 compared with CRP determination, in correct diagnosis of patients with or without IBD.

2. We also identified a better diagnostic performance for the determination of serum IL-13 compared with CRP determination in patients with CD and UC.

3. Interleukin IL-17 had a better diagnostic performance than serum CRP determination only in patients with Crohn's disease diagnosis.

4. Due to the high sensitivity and specificity, we can consider the matrix metalloproteinases and interleukins as an alternative or additional diagnostic for CRP, ESR and other inflammatory disease indices used in the diagnosis of IBD.

Chapter XI. CONCLUSIONS

1. The first epidemiological study performed in our Referral Center shows a reduced incidence of CD and UC in Dolj county (0.82/10^5 inhabitants per year respectively 1.26/10^5 ; we found a higher incidence of UC compared with CD). The data are consistent with previous observations provided for Referral Center Bucharest and confirms the low incidence of IBD in Romania.

2. Crohn's disease started at a younger age, with a balanced distribution with a slight preponderance of females and a single peak of incidence, while age of onset of ulcerative colitis was similar to that of the western population, with a bimodal incidence in women and equal gender distribution.

3. In the Dolj county, notice that the age of onset for the two clinicopathological entity was with 5 years older than age population of Central and West European countries. There is a tendency of developing UC at younger ages in recent years.

4. In terms of localization of lesions we observed that more than half of newly-diagnosed with CD had significant colonic affectation. Colitis left prevailed in patients with UC. We observe phenotypic progression of the disease over time in patients newly diagnosed with UC weight loss colitis left, weight doubles extensive colitis and recorded cases of proctitis

5. Non-stenosing, non-penetrating (inflammatory form) phenotype is most commonly found in CD.

6. Mild and moderate forms predominated in both entities. The absence of perianal manifestations, low frequency of complications of intestinal and extra-intestinal in our study, suggesting a mild evolution of CD and the UC in Dolj county.

7. Our study showed for the first time, certain phenotypic association with serological markers analyzed in serum of patients with IBD. We have achieved significantly

higher concentrations of MMP-3, -9, IL-13, -17 and CRP in the serum of patients with IBD compared to the control group. Significantly higher levels of MMP-3 were observed in patients with onset over 40 years (A3) of CD, in those with impaired colonic (L2) with non-stenosing, non-penetrating (B1) and penetrating/fistulizing (B3) pattern and patients with extension to the left colon (E2) of the UC. Serum levels of MMP-9 were associated with early onset of CD (under 40 years) in patients with ileal involvement (L1), with stenosing pattern (B2) and in patients with left colitis location (E2).

8. Cytokine IL-13 showed significantly higher in serum patients with onset over 40 years, with ileal involvement and to patients with left colitis. Cytokine IL-17 was associated with onset under 40 years CD, with ileo-colonic and colonic location and proctitis. The only significantly higher serum CRP were associated with extensive colitis.

9. CD and UC have obtained significantly higher serum concentrations in subgroups of patients with mild, moderate and severe activity than in controls, and we demonstrated that serum levels of MMP-3, -9, IL-13, -17 and CRP were significantly associated with various stages of clinical activity in two entities. In the serum of patients with CD and smokers have identified significant higher levels of MMP-9 and IL-13 *vs* ex-smokers and nonsmokers groups.

10. We have demonstrated in our study, the presence of correlations between IBD activity in adults and serum levels of MMP-3 and MMP-9. In the group of patients with CD, serum levels of MMP-3 were correlated much better with hints of disease evaluated for this entity, while for the group of patients with UC serum levels of MMP-9 were correlated much better with indices evaluated for this entity. Also the only cytokine IL-17 showed correlations with disease HBI score for CD and that concentrations correlated well with hints of disease in UC.

11. Using ROC curve analysis we obtain a higher diagnostic accuracy for determination of serum MMP-9 and MMP-3 compared to the dosage of CRP, in the accurate diagnosis of patients with IBD. The determination of serum IL-13 showed a better diagnostic performance compared with serum CRP determination in patients with CD and UC. Determination of serum IL-17 had a better diagnostic performance than that of CRP, only in the diagnosis of patients with CD.

12. Due to the high sensitivity and specificity, we can consider the matrix metalloproteinases and interleukins considered as an alternative or additional diagnostic for CRP, ESR and other inflammatory disease indices used in the diagnosis of IBD.

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