

The Role of Anti- Lactoferrin and Anti- Lysozyme Autoantibodies in Ulcerative Colitis: Serological Study

Sinan Bahjat Alrifai*, Khalid Abdullah Al-Khazraji**,
Mohammed Marouf Al-Ani***, Leen Khalook Mustafa****

* Dept. of Medical Microbiology, College of Medicine, Tikrit University.

** Dept. of Internal Medicine, College of Medicine, Baghdad University.

*** Dept. of Medical Microbiology, College of Medicine, Baghdad University.

**** Dept. of Immunology, Directorate of Teaching Laboratories, Ministry of Health.

Summary: Background: Autoantibodies, including anti- neutrophil antibodies, have been detected in the serum of UC patients. Serological antibodies have a role in primary diagnosis of UC. Preoperative measurement of serological antibodies can help to predict the likelihood of complications among patients undergoing surgery. They also appear to be of value in predicting disease progression.

Subjects, Materials and Methods: Current prospective study was conducted at Medical City in Baghdad from Dec. 2011 to the end of May 2012, where 26 known patients with UC aging from 18 to 77 years (diagnosed by histopathology slide sections) and 27 apparently healthy subjects have enrolled in this study as patient group and control group, respectively. Anti-lactoferrin and anti-lysozyme autoantibodies in both groups sera were assessed quantitatively by ELISA technique. Chi- square test and T- test were used for statistical analysis.

Results: Data showed that the prevalence for anti- lactoferrin autoantibodies was (42.3%), while that for anti-lysozyme autoantibodies was (15.4%) in UC patients. All control group subjects showed seronegative results regarding both autoantibodies assayed. Quantitative assessment showed a significant correlation between UC patients and control group in regard to both autoantibodies. All cases of Proctocolitis- a type of UC, were associated with seronegative autoantibodies results.

Conclusion: Anti-lactoferrin and anti-lysozyme autoantibodies tests can be used as a primary diagnostic tool in UC, and also to differentiate pancolitis and distal colitis types from proctocolitis type.

I. Introduction:

Ulcerative colitis (UC) is one of the two major types of inflammatory bowel disease (IBD), the other one is Crohn's disease. It is an idiopathic and chronic intestinal inflammation¹. Ulcerative colitis affects the colon and rectum and typically involves only the innermost lining or mucosa, manifesting as continuous areas of inflammation and ulceration, with no segments of normal tissue. The peak age of onset of UC is between 15 and 30 years. A second peak occurs between 60 and 80 years².

Although IBD has been known as a clinical entity for over one hundred years, its etiology and pathogenesis has not yet defined, and many risk factors have been implicated¹. The pathogenesis of inflammatory bowel disease (IBD) is multifactorial and results from an interaction between genetic, immunologic, microbial, and environmental factors³. A consensus hypothesis is that in genetically predisposed individuals, both exogenous factors (e.g., infectious agents, diet, normal luminal flora) and host factors (e.g., intestinal epithelial cell barrier function, vascular supply, neuronal activity) cause a chronic state of deregulating mucosal immune function that is further modified by specific environmental factors (e.g., smoking)⁴.

Differing cytokine and other inflammatory- mediator profiles have been identified for UC. Autoantibodies, including anti- neutrophil antibodies, have been detected in the serum of UC patients². Serological antibodies have a role in primary diagnosis and in differentiating between CD and UC. Preoperative measurement of serological antibodies can help to predict the likelihood of complications among patients undergoing surgery. They also appear to be of value in predicting disease progression^{2,5}.

Besides, studies suggest high prevalence of these autoantibodies in unaffected relatives of patients with UC, particularly in those relatives whose probands were having positive titers of autoantibodies, thus, it can be used as a tool for early diagnosis of the disease in those relatives. Antineutrophil- cytoplasmic autoantibodies (ANCA) are directed against components of neutrophil granules and monocyte lysosomes. They are considered as serological marker for various diseases characterized by vascular inflammation^{6,7}.

In inflammatory bowel disease, ANCA are directed against a wide variety of antigens, including lactoferrin, bactericidal/permeability-increasing protein (BPI), proteinase III, lysozyme, elastase, cathepsin G and the cytosolic antigens catalase and α -enolase⁸⁻¹⁰.

Different patterns for cytoplasmic constituents are recognized; that with a perinuclear pattern (i.e. p-ANCA) have been described in IBD and shown as a sensitive and specific marker for UC. The p-ANCA is strongly associated with UC and may represent an indicator of genetic susceptibility to it ⁷.

The p-ANCA in UC in contrast to CD is more prevalent, while the anti-Saccharomyces cerevisiae antibody (ASCA) is more prevalent in CD than in UC, which provides a possibility to distinguish between them by serological testing. Moreover, Th2-related subclasses of IgG (IgG1 and IgG4) are associated with UC, as opposed to Th1-related subclasses (IgG2), which are more associated with CD. Together, these support distinct T-helper cell responses in CD and UC ^{11,12}.

Lactoferrin is an iron-storing protein from the specific granules of the neutrophils. It binds the free iron ions that bacteria need for their growth and thus has an antimicrobial effect. Anti lactoferrin antibodies stimulates the increase in adherence to fibronectin and laminin similar in degree to that induced by TNF- α (a known neutrophil agonist). Humoral immune responses to lactoferrin, likely expressed on the neutrophil surface, can activate neutrophils in proinflammatory responses that may be pathogenic ¹³.

Lysozyme is a glucosidase localized in the specific granules of neutrophils. Its antimicrobial activity protects the body from invading bacteria and it is expressed in normal human intestine. Chronic inflammation in colon results in induction of α and β defensins and increased lysozyme expression. This altered antimicrobial activity of epithelial cells in chronic colitis may be a consequence of changes in the epithelial lining, permitting adherence of both pathogenic bacteria and commensals directly to the epithelial cell surface ¹⁴.

Antibodies in ulcerative colitis were commonly directed against lysozyme, cathepsin G, elastase, and lactoferrin, while it is directed against lysozyme in Crohn's disease ¹⁵.

II. Materials and Methods:

This study was conducted at the endoscopy department outpatient clinic of Baghdad Teaching Hospital/ Medical City in Baghdad from December 2011 to the end of May 2012, where 26 known patients aging from 18 to 77 years with Ulcerative Colitis (UC) (diagnosed by histopathology slide sections) have been submitted to a prospective study. Other 27 subjects have enrolled in this study as a control group, selected blindly to their medical condition (apart from IBD).

Base line data about subjects were obtained from their history and clinical examination, a previously arranged questionnaire was used for this purpose.

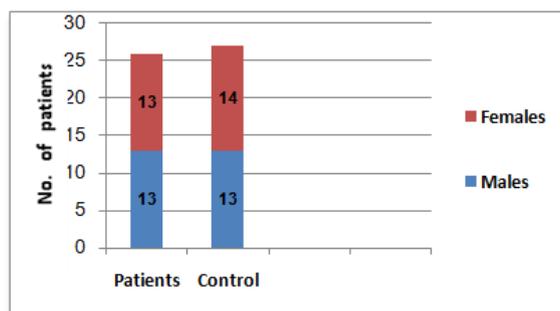
Venous blood samples of about 3-5 ml were collected by using disposable syringes, placed in plain tubes and allowed to clot (for minimum of 30 minutes) at room temperature (20-25°C), then sera are separated by centrifugation (3000 rpm for 5 minutes) and dispensed into sterile tightly closed Eppendorf tubes in 0.1 ml aliquots and stored at -20°C until assayed.

A commercial ELISA kits for the quantitative determination of IgG autoantibodies against both human lactoferrin and human lysozyme (IMMUCHEM TM, France) were used. This test was performed following the procedure protocol included within the kit packing as issued from the manufacturer company. The normal ranges that have been established for the anti- lysozyme and anti- lactoferrin test was < 10 U/ml. If the test value = 10 U/ml or more, the result is considered elevated.

Statistical analysis was done using statistical package for social sciences version 18 (SPSS V.18, Chicago, IL, USA). Chi square test was used to test the association between discrete variables. Two samples unpaired T- test was used to find the means of normally continuous samples of two sets of data. All P values used were asymptotic and two sided. Findings with P value less than 0.05 were considered significant.

III. Results:

This study enrolled two groups; a patient group that included 26 known Ulcerative Colitis patients whom already have been diagnosed by colonoscopy and histopathology slide sections, and control group of 27 apparently healthy individuals. The male and female distribution of both groups is shown in figure (1).



Fig(1): The distribution of patients and control groups according to their sex.

The age and sex distribution at the time of onset of UC symptoms is illustrated in table (1). This table shows that the mean age of UC patients at time of onset were about 32 ± 17.2 years old. Nearly an even distribution of sex among different age groups is also noticed.

Table (1): Age & sex distribution of IBD patients at time of onset.

Age (years)	No. of UC patients		No. of control subjects	
	male	female	male	female
10-19	3	3	3	2
20-29	4	4	2	3
30-39	4	3	3	2
40-49	1	-	2	3
50-59	-	-	3	2
60-69	2	1	-	2
70-79	-	1	-	-
Total	26		27	
C.I. Mean*	31.8		36.7	
S.D. **	17.2		15.8	

* C.I. = Class Interval,

** S.D.= Standard Deviation

Among the 26 UC patients, the assessments of anti- lactoferrin autoantibodies and anti- lysozyme autoantibodies using ELISA technique showed that 11 (42.3%) and 4 (15.4%) patients gave positive results, respectively. This is shown in figure (2).

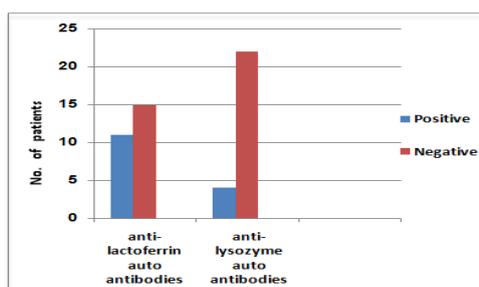


Figure (2): The distribution of positive cases among UC patients in regard to both anti- lactoferrin and anti- lysozyme autoantibodies.

Seronegative results for both autoantibodies were founded in all subjects of the control group.

Using unpaired T- test, table (2) shows the statistical analysis for the UC group compared with control group in regard to both anti- lactoferrin and anti- lysozyme autoantibodies. Significant correlation was found between both groups (P value < 0.05) regarding anti- lactoferrin and anti- lysozyme autoantibodies data.

Table (2): Statistical comparison between UC group data and control group data for anti- lactoferrin and anti- lysozyme autoantibodies.

Categories	Mean U/ml	SD*	T- Test	Df**	P- value
Anti- lactoferrin values in UC cases	7.56***	7.41	3.07	52	0.003
Control group	2.95	2.28			
Anti- lysozyme values in UC cases	6.21****	6.87	2.22	52	0.03
Control group	3.07	2.5			

* SD= Standard Deviation, **df= degree of freedom

*** Positive cases mean= 14.9 U/ml , negative cases mean= 3.6 U/ml

**** Positive cases mean = 17.9 U/ml , negative cases mean= 4.3 U/ml

Concerning the histopathological types of UC in current study, 6 cases were having pancolitis, 9 cases were of the distal colitis type, and another 11 cases were of the proctocolitis type, as seen in figure (3).

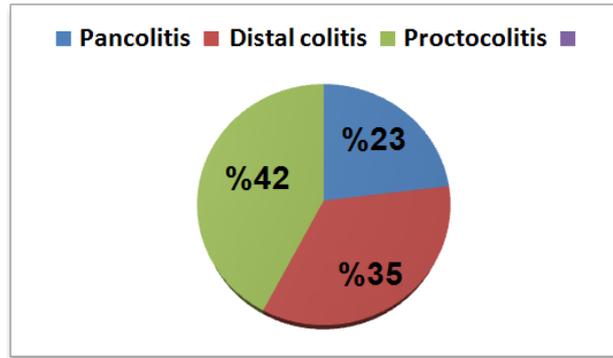


Figure (3): The distribution of UC patients according to histopathology.

Figure (4) shows the distribution of the positive cases of anti- lactoferrin and anti- lysozyme autoantibodies among the histopathological entities of UC patients. All the pancolitis cases show positive anti- lactoferrin assessment, four of them were also having positive results concerning the anti- lysozyme autoantibodies. The rest five positive cases for anti- lactoferrin assessment were shown to have the distal colitis entity of the disease. No positive cases for the autoantibodies assessment were noticed in proctocolitis patients.

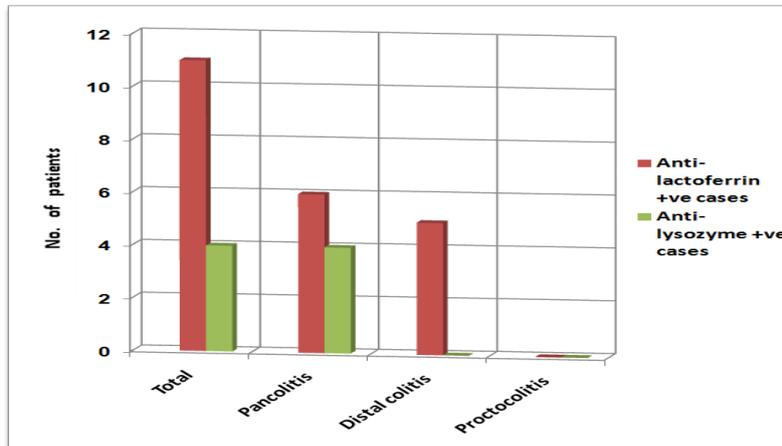


Figure (4): The distribution of the positive autoantibodies cases among different histopathological entities.

Only 17 of the 26 UC patients perform endoscopy at time of serological analysis, the other 9 cases have had non- recent histopathological study that, although confirming their condition, but not yield a recent activity of the disease at time of sampling. Only one UC case which is seropositive for both autoantibodies did not perform a recent histopathological study. The distribution of the seropositive autoantibodies cases according to the histopathological grading of the 17 UC patients is shown in figure (5), non- significant correlation was found ($P > 0.05$).

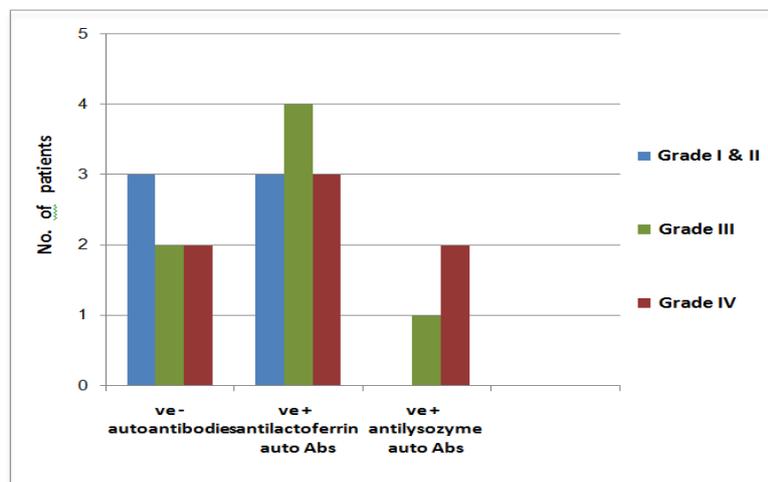


Figure (5): The distribution of the seropositive and seronegative cases in regard to the histopathological grading in some of UC patients.

IV. Discussion:

Inflammatory bowel disease (IBD) is traditionally considered to be common in the Western world, and its incidence has sharply increased since the early 1950s. In contrast, until the last decade, many cases have been reported from other parts of the world. These changes may represent differences in diagnostic practices and increasing awareness of the disease¹⁶.

In current study, nearly similar distribution of UC cases between box sexes were noticed. This is in agree with what was found in many areas of the world regarding UC¹⁷⁻¹⁹. Inflammatory bowel disease is diagnosed at a slightly older age in Asia than in the West¹⁹. In current study data, the mean age group for UC incidence was about 32 ± 17.2 years. In many areas of the Western countries, the peak incidence for UC patients is 15-30 years^{1,20,21}. Prideaux et al¹⁹ also found that a second incidence peak is less common in Asia compared to that of Western countries. This is also in agree with current study data as only 4 patients (15.4 %) out of twenty six with UC were above 60 years of age.

The worldwide prevalence of pANCA in UC is 23-81%⁸⁸. Current study data showed a prevalence for anti- lactoferrin autoantibodies in UC is 42.3%, which is in agree with formerly mentioned worldwide percentages and other studies^{22,23}. In contrast, anti- lysozyme auto antibodies showed lower prevalence rates (15.4%) in UC, this is in agree with Hauschild et al 1993, (15%) and Schmitt et al 1993, (20%) and disagree with Kossa et al 1995, who gave higher results (53%). This lower rate regarding anti-lysozyme autoantibodies in UC may be due to the fact that, in normal colon, no lysozyme was detected in surface epithelial cells or in mucous crypt cells, nor was lysozyme present in the lamina propria except in a few granulocytes. Also, normal rectum contained no lysozyme-positive cells²⁴, and since nearly 42% of current UC cases were of the proctocolitis type, this rate should be lower than expected²⁴⁻²⁷.

Quantitative analysis using T-test shows a significant correlation between both UC patients and control groups (P value < 0.05) regarding anti- lactoferrin and anti- lysozyme autoantibodies data. This positive correlation of anti- lactoferrin autoantibodies are in agree with chauveau et al 2009. Also, Bianca et al 2009, used multiple techniques to confirm that lactoferrin is the major target for anti-neutrophil perinuclear cytoplasmic antibodies in UC^{28,29}.

All positive autoantibodies titer cases were of either pancolitis or distal colitis. This is in agree with Nisihara et al 2010, who stated that positivity was correlated with pancolitis or left colitis. Senka et al 2011, also found higher concentrations of multi-specific ANCA in long-lasting, left-sided UC, and suggested an influence of bacterial stimulation on the break of tolerance. Also, Toader and Durnea 2011, found that more than half of their positive cases (60%) were of pancolitis type. This goes with current study data which showed that 54.5% of patients with positive autoantibodies were of pancolitis type³⁰⁻³². Regarding histopathological grading of UC and its relation to seropositivity for autoantibodies, non-significant correlation was found between these parameters ($p > 0.05$). This is in agree with Chlebowczyk et al³³ who found no link between p-ANCA antibodies and the clinical picture of UC.

V. Conclusion:

Anti-lactoferrin and anti-lysozyme autoantibodies tests can be used as a primary diagnostic tool in UC, and also to differentiate pancolitis and distal colitis types from proctocolitis type.

References:

- [1]. Friedman S, and Blumberg RS. Inflammatory bowel disease. In: Kasper DL, Braunwald E, Fauci AS, et al. Harrison's principles of internal medicine. 16th ed., McGraw- Hill, New York. 2005; (276): 1776- 1789.
- [2]. Head KA, and Jurenka JS. Inflammatory bowel disease part I: Ulcerative colitis- pathophysiology and conventional and alternative treatment options. *Alternative medicine review* 2003; 8(3): 247- 283.
- [3]. Dubeau MF, Iacucci M, Beck PL, et al. Drug-induced inflammatory bowel disease and IBD-like conditions. *Inflammatory Bowel Diseases*, 2012. Online ed.; Available at URL: <http://onlinelibrary.wiley.com/doi/10.1002/ibd.22990/abstract>
- [4]. Xavier RJ, Podolsky DK. Unraveling the pathogenesis of inflammatory bowel diseases. *Nature* 2007; 448: 427-434.
- [5]. Prideaux L, De Cruz P, Ng SC, et al. Serological antibodies in inflammatory bowel disease: A systematic review. *Inflammatory Bowel Diseases* 2012; 18: 1340-1355.
- [6]. Miettinen DA. Clinical implications and utility of Antineutrophil cytoplasmic antibodies in rheumatoid arthritis, spondylarthropathy, and ulcerative colitis. Academic dissertation submitted to the University of Tampere, Medical School-Dept. of clinical microbiology, Finland 2000.
- [7]. Monteleone G, Marasco R, Parrello T, et al. Low Prevalence of p-ANCA in Unaffected Relatives of Patients with Ulcerative Colitis from a Mediterranean Area. *Inflammatory bowel diseases* 1996; 2: 11-15.
- [8]. Peen E, Almer S, Bodemar G, et al. Anti-lactoferrin antibodies and other types of ANCA in ulcerative colitis, primary sclerosing cholangiitis and Crohn's disease. *Gut* 1993; 34:56-62.
- [9]. Stoffel MP, Csernok E, Herzberg C, Johnston et al. Anti-neutrophil cytoplasmic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated disorders. *Clinical & Experimental Immunology* 1996; 104:54-9.
- [10]. Hagen EC, Andrassy K, Csernok E, et al. Development and standardization of solid phase assays for the detection of anti-neutrophil cytoplasmic antibodies (ANCA). A report on the second phase of an international cooperative study on the standardization of ANCA assays. *Journal of Immunology Methods* 1996; 196: 1-15.

- [11]. Kerick M. Development of an HLA microarray and its application in Inflammatory Bowel Disease. Dissertation for the academic degree of Doctor of Natural Sciences filed in the Department of Biology, Chemistry, Pharmacy- The Free University of Berlin, Berlin 2008.
- [12]. Gupta A, Derbes C, and Sellen J. Cytoplasmic Antibodies and Anti-Saccharomyces cerevisiae Antibodies in the Evaluation of Inflammatory Bowel Disease at an Academic Medical Center. *Inflammatory Bowel Diseases* 2005; 11: 898–902.
- [13]. Zweiman B, von Allmen C. Comparative Effects of Antilactoferrin Antibodies and Tumor Necrosis Factor on Neutrophil Adherence to Matrix Proteins. *Clinical & vaccine immunology* 1999; 6(3): 364- 368.
- [14]. Fahlgren A, Hammarstrom S, Danniellsson A, et al. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clinical and experimental immunology* 2003; 131(1): 90-101.
- [15]. Kossa K, Colthart A, Ives CT, et al. Antigen specificity of circulating anti-neutrophil cytoplasmic antibodies in inflammatory bowel disease. *European journal of gastroe nterology and Hepatology* 1995; 7(8): 783-789.
- [16]. Lakatos PL. Recent trends in the epidemiology of inflammatory bowel diseases: up or down?. *World Journal of Gastroenterology*. 2006. 12(38):6102-6108.
- [17]. Cottone M, Cipolla C, Orlando A, et al. Epidemiology of Crohn's disease in Sicily: A hospital incidence study from 1987 to 1989. *European Journal of Epidemiology* 1991; 7(6): 636-640. Cited by: www.ivsl.org
- [18]. Camps RM, Van de Kruijs HM, Schouten LJ, et al. Inflammatory bowel disease in South Limburg (The Netherlands) 1991-2002: Incidence, diagnostic delay, and seasonal variations in onset of symptoms. *Journal of Crohn's and Colitis* 2009; 3(2): 115-124. Cited by: www.ivsl.org
- [19]. Prideaux L, Kamm MA, De Cruz PP, et al. Inflammatory bowel disease in Asia: A systematic Review. *Journal of gastroenterology and Hepatology* 2012; 27(8): 1266-1280. Cited by: www.ivsl.org
- [20]. Abraham C and Cho JH. Inflammatory bowel disease: mechanisms of disease. *New England Journal of Medicine* 2009; 361: 2066-78.
- [21]. Loftus EV Jr, Sandborn WJ. Epidemiology of inflammatory bowel disease. *Gastroenterology Clinics of North America* 2002; 31: 1–20.
- [22]. Peen E, Almer S, Bodemar E, et al. Anti-lactoferrin antibodies and other types of ANCA in ulcerative colitis, primary sclerosing cholangitis, and Crohn's disease. *Gut* 1993; 34(1): 56-62.
- [23]. Roozendaal C, Kallenberg CG. Are anti-neutrophil cytoplasmic antibodies (ANCA) clinically useful in inflammatory bowel disease (IBD)? Clinical and experimental immunology 1999; 116(2): 206-213.
- [24]. Klockars M, Reitamo S, Reitamo JJ, et al. Immunohistochemical identification of lysozyme in intestinal lesions in ulcerative colitis and Crohn's disease. *Gut* 1977; 18: 377-381.
- [25]. Hauschild S, Schmitt WH, Csernok E, et al. ANCA in systemic vasculitides, collagen vascular diseases, rheumatic disorders and inflammatory bowel diseases. *Advanced Experimental Medical Biology* 1993; 336: 245-251.
- [26]. Schmitt WH, Csernok E, Flesch BF, et al. Autoantibodies directed against lysozyme: a new target antigen for anti-neutrophil cytoplasmic antibodies. In: Gross WL. *ANCA-associated vasculitides: immunological and clinical aspects*. Plenum Press, New York, 1993: 267-272. .
- [27]. Kossa K, Coulthart A, Ives CT, et al. Antigen specificity of circulating anti-neutrophil cytoplasmic antibodies in inflammatory bowel disease. *European Journal of Gastroenterology and Hepatology* 1995; 7:783-789.
- [28]. Chauveau A, Delaperriere N, Cholet F, et al. Chronic inflammatory bowel diseases: which choice for the autoantibodies? *Immunanalyse & Biologie Spécialisée* 2009; 24(1): 24-31. Cited by: www.ivsl.org
- [29]. Bianca T, Stephanie N, Christian P, et al. DNA- bound lactoferrin is the major target for antineutrophil perinuclear cytoplasmic antibodies in ulcerative colitis. *Annals of the New York Academy of Science* 2009; 1173: 161-165. Cited by: www.ivsl.org
- [30]. Nishihara RM, De Carvalho WB, Utiyama SR, et al. Diagnostic role and clinical association of ASCA and ANCA in Brazilian patients with inflammatory bowel disease. *Digestive diseases and sciences* 2010; 55(8): 2309-2315.
- [31]. Senka D, Dragan P, Milos N, et al. Anti-neutrophil cytoplasmic antibodies (ANCA) specific for one or several antigens: useful markers for subtypes of ulcerative colitis and associated primary sclerosing cholangitis. *Clinical Chemistry and Laboratory Medicine* 2011; 50(3): 503–509.
- [32]. Toader E, Durnea C. P-anca prevalence in ulcerative colitis patients and their first degree relatives. *Revista medico-chirurgicala a Societatii de Medici si Naturalisti din Iasi's* 2011; 114(3): 687- 693.
- [33]. Chlebowczyk GU, Wos H, Aleksander L, et al. Serologic investigations in children with inflammatory bowel disease and food allergy. *Mediators of inflammation* 2009; Article ID 512695, 6 pages. doi:10.1155/2009/512695. Online publication.