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Ascitic Fluid Markers Hepcidin, Calprotectin, and Lactoferrin in Early Diagnosis and Follow-up of Spontaneous Bacterial Peritonitis

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Abstract

Background: Spontaneous Bacterial Peritonitis (SBP) is the most serious complication in patients with decompensated liver cirrhosis and ascites. Its diagnosis is based on Polymorph Nuclear Leucocyte (PMNL) count in ascitic fluid >250 cell/mm^3, however; SBP occurs at even lower levels, hence the need for early sensitive and specific markers for early detection and prognosis of SBP.

Aim of Study: The aim of the study was to assess ascitic fluid Calprotectin, Lactoferrin and Hepcidin as reliable markers for diagnosis and follow-up of SBP.

Patients and Methods: Ascitic fluid samples were collected from 88 patients with ascites; 37 with SBP (patients group) and 51 without SBP (control group). Calprotectin, Lactoferrin and Hepcidin levels were measured using Enzyme Linked Immunosorbent assay (ELISA).

Results: Calprotectin, Lactoferrin and Hepcidin were significantly higher in patients with SBP than those without SBP (p=0.002, 0.001, 0.029 respectively). The areas under the receiver operator characteristic curve for Calprotectin, Lactoferrin and Hepcidin were (0.695, 0.860 & 0.661 respectively) with sensitivity 91.9% for the three markers. Hepcidin showed higher specificity (64.7%) than the other two markers. Calprotectin and Hepcidin decreased significantly after proper antibiotic therapy than before therapy in SBP group (p=0.002 & 0.004 respectively).

Conclusion: Ascitic fluid Calprotectin, Lactoferrin and Hepcidin can be used as diagnostic markers of SBP. Calprotectin and Hepcidin can be used as markers for follow-up of SBP. Hepcidin was the most specific among them.

Key Words: *SBP – Calprotectin – Lactoferrin – Hepcidin – Ascites.*

Introduction

SPONTANEOUS Bacterial Peritonitis (SBP) is the most frequent life threatening infection of ascitic fluid in patients with advanced liver cirrhosis **[1]**. The incidence of SBP is higher in hospitalized patients with routine paracentesis (12%), while it develops in up to 3.5% of patients who are treated as outpatients **[2,3]**. Its mortality has reduced to approximately 20% with early diagnosis and treatment **[4]**. However; survivors of SBP have a poor prognosis. After an initial diagnosis of SBP, the 6-month mortality rate is 50% **[5]**.

The bad prognosis and outcome lead to the urgent need for rapid and early diagnosis of SBP. Diagnosis of SBP is made in patients with liver cirrhosis if the Polymorphonuclear (PMN) cell count in ascitic fluid exceeds 250cells/mm^3, if microbiological cultures show only one organism and other forms of peritonitis have been excluded **[6,7]**. Several markers have been developed and studied for early detection of SBP. These markers include Calprotectin, Lactoferrin, Hepcidin, lipopolysaccharide binding protein, and Complement 3 **[8]**.

Calprotectin is a protein belongs to the S 100 group of proteins. It is present in large amounts in neutrophils, and in smaller amounts in monocytes and activated macrophages. Its presence in body fluids is proportional to the neutrophils activation **[9,10]**. Ascitic fluid PMNs count more than 250 cell/mm^3 can be reliably predicted by measuring ascitic fluid Calprotectin. Subsequently, it can be a useful marker for early diagnosis of SBP **[11]**.

Lactoferrin is an iron binding glycoprotein that is present in human breast milk and bovine milk. Also, it is present in mucosal secretions as in gastrointestinal fluids, vaginal fluids, semen, tears **[12]**. It is synthesized and stored in the secondary granules during the transition of neutrophils from promyelocyte to myelocyte **[13]**. Lactoferrin is released on activation of PMNs, and its presence in body fluids is proportional to neutrophils influx

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[14]. Its measurement in ascitic fluid showed high sensitivity and specificity for diagnosis of SBP even after initiation of systemic antibiotics [15,16].

Hepcidin is an iron regulatory hormone that is mainly synthesized by the hepatocytes and macrophages [17]. Hepcidin has a dual role as an 'iron-hormone' and 'antimicrobial peptide. It maintains iron homeostasis by decreasing iron absorption from the duodenum and iron release from hepatocytes or macrophages. Serum Hepcidin has been identified as a useful marker of infection in the evaluation of late-onset sepsis in very low birth weight infants [18,19] and plays a role in innate immunity; where it can bind ferro-protein resulting in its degradation and promoting its protective role through decreasing the amount of the available iron in the peripheral circulation to limit the overgrowth of bacteria in acute infections [20,21]. Hepcidin can be used as a sensitive and specific marker for diagnosis of bacterial translocation that causes SBP [22].

This work aimed to assess the role of ascitic fluid Calprotectin, Hepcidin and Lactoferrin in diagnosis of SBP, and follow-up after 6 month of proper antibiotic treatment.

Patients and Methods

This is a prospective case-control study that was conducted from the period August 2016 to December 2017. The study enrolled 88 hepatitis C cirrhotic patients with decompensated liver cirrhosis. They were 52 males and 36 females with a mean age 52.75±7.97 years. They were admitted to Endemic Hepato-Gastroenterology Department, Faculty of Medicine, Cairo University, Egypt. The patients were subdivided to two groups. Group (1) included 37 patients (20 male and 17 females) with SBP and without other evidence of infection in other organs or other sites. SBP patients were originally diagnosed depending on ascitic fluid PMNL count higher than 250/mm^3 with or without having any signs or symptoms of peritoneal infection (abdominal pain, tenderness on palpation and fever) [23]. Group (2) included 51 ascitic patients (32 male and 19 females) without SBP or any other overt infection (ascitic PMNL less than 250/mm^3) as a control group. Inclusion criteria include: HCV patients with decompensated chronic liver disease.

Exclusion criteria include: Patients who received antibiotic treatment during the last two weeks before admission. Patients with secondary peritonitis, tuberculous peritonitis, malignant ascites or ascites due to other causes than cirrhosis caused by HCV infection as cardiac, renal diseases or Budd-Chiari syndrome, patients with ulcerative colitis, Crohn's, colorectal cancer, and major surgical operations were excluded. The study was approved by the Local Ethical Committee and informed consents were obtained from all participants. All patients were diagnosed and managed in compliance with ethics principles of the declaration of Helsinki for Good Clinical Practice guidelines.

All patients were subjected to full history taking, full clinical examination, abdominal ultrasonography for assessment of liver and spleen sizes, portal and splenic vein diameters, and the degree of ascites and presence of any focal lesion. Laboratory investigations included serum Albumin, total bilirubin, creatinine, Prothrombin time, concentration and INR, ascitic fluid analysis for PMNL, and bacterial culture and sensitivity. Ascitic fluid measurements for the three studied makers (Calprotectin, Lactoferrin and Hepcidin). All laboratory analyses were done on Clinical and Chemical Pathology Laboratories, Faculty of Medicine-Cairo University.

Sample collection and analysis:

Three mL of venous blood and 20mL of ascitic fluid were withdrawn and sent to the central laboratory of the Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University. Serum and plasma samples were analyzed for Albumin, total bilirubin, and creatinine, prothrombin time and concentration. Ascitic fluid samples (20mL) were obtained through Paracentesis performed using a 20-gauge sterile needle under local anesthesia with lidocaine under complete aseptic conditions in the right or left lower quadrant with the patient in the supine position, part of the specimen was directly sent to the laboratory for examination of differential leukocyte counts (PMNLs) and for bacterial culture and sensitivity.

The other part (about three mL) of the ascitic fluid was centrifuged for 15min, the supernatant was transferred to three sterile eppendorf tubes and stored at –20ºC until analysis by ELISA technique for measuring the levels of Lactoferrin, Hepcidin and Calprotectin.

Ascitic fluid samples were obtained twice from SBP group for Lactoferrin, Hepcidin and Calprotectin levels assay; the first time was at the time of admission before starting antibiotic therapy (intravenous third generation cephalosporin) and the second time was 6 months after finishing regimen of antibiotic therapy to detect their prognostic role in response to therapy.

Methodology:

All the chemical analyses for serum samples were analyzed on AU 680 blood chemistry auto-analyzer (Beckman Coulter Diagnostics, USA) according to manufacturer's instructions. Manual PMNLs count on hemocytometer and microscopic examination for differential leucocyte count were done. 5ml of the ascitic fluid were cultured.

Measuring of AF Calprotectin, Lactoferrin and Hepcidin by ELISA:

Calprotectin (DEH 325 Calprotectin human ELISA kit; Demeditech Diagnostics, GMbH, Kiel-Wellsee, Germany), Lactoferrin (Assay Max Human Lactoferrin ELISA Kit; AssayPro-USA) and Hepcidin (Human Hepcidin ELISA kit; Glory Science Co.Ltd. USA) Kits were used to measure their levels in ascitic fluid according to the manufacturer's instructions.

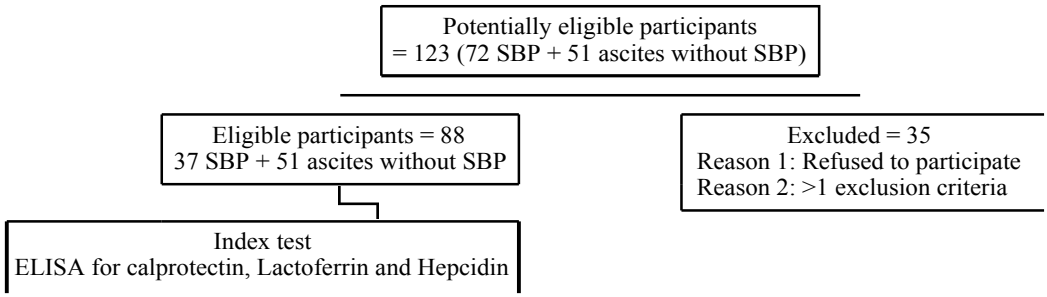

Fig. (1): Flow of selection of participants through the study.

Statistical analysis:

Quantitative data were expressed as mean ± SD and compared using *t*-test when normally distributed, and as median and range using Mann Whitney U-test when not normally distributed. *p*-value <0.05 is considered significant. Correlations between numerical data were determined with the Spearman's rank correlation coefficient. Receiver Operating Characteristic (ROC) curve analysis was used to identify optimal cut-off values of the studied markers with maximum sensitivity and specificity for differentiation of cirrhotic patients with SBP from those without.

Results

On comparing the two studied groups as regards to the demographic data, a statistically significant increase was observed among SBP group as regards abdominal pain, fever, mean serum albumin and ascitic fluid PMNLs levels (p=0.001, 0.001, 0.004 and 0.001 respectively) (Table 1).

The ascitic fluid markers (Hepcidin, Lactoferrin and Calprotectin) concentrations were significantly higher in SBP group than the control ascitic group (p=0.029, 0.001 and 0.002 respectively) (Table 2).

After 6 month of therapy, Calprotectin, Lactoferrin and Hepcidin were measured again in the SBP group and results were compared to the initial results. Both Hepcidin and Calprotectin showed statistically significant lower levels after therapy than before therapy (p= 0.004 & 0.002 respectively) (Table 3).

Table (1): Demographic, clinical and laboratory data of the studied groups.

	SBP group (n=37)	Control group (n=51)	*p*-value
Age (years)	52.49±6.866	52.75±7.975	0.871
Sex:			
Males	20/37 (54%)	32/51 (62.7%)	0.413
Females	17/37 (46%)	19/51 (37.2%+)	
Bleeding tendency	26/37 (70.2%)	33/51 (64.7%)	0.101
Abdominal pain	23/37 (62.1%)	2/51 (3.9%)	0.001
Fever	14/37 (37.8%)	0/51 (0%)	0.001
Encephalopathy	3/37 (8.1%)	9/51 (17%)	0.198
Jaundice	29/37 (78.4%)	26 (66.7%)	0.068
Child pugh score:			
Mean	10.43±1.9	10.04± 1.7	0.365
Classification:			
A	0 (0%)	1 (1.9%)	
B	11 (29.7%)	22 (43.1%)	0.275
C	26 (70.3%)	28 (55%)	
Meld score	22±8.6	17±7.3	0.109
Albumin (g/dl)	2.59±0.42	2.37±0.38	0.004
Bilirubin (mg/dl)	4.1 (0.54-16.4)	2.3 (0.5-18)	0.108*
Creatinine (mg/dl)	1.48±0.79	1.39±0.73	0.055
INR %	1.94±0.72	1.74±0.43	0.186
PMNLs in AF (cell/mm^3)	800 (250-7200)	180 (20-380)	0.001*

- Data is expressed as mean ± Standard Deviation (SD) or * median. Qualitative data are expressed as frequency and percentage.
AF : Ascitic Fluid.
PMNLs: Polymorphonuclear Leucocytes, p<0.05 is significant.

Table (2): Comparison of the levels of the three studied markers in SBP and control group (diagnostic value).

	SBP group (n=37)	Control group (n=51)	*p*-value
Hepcidin pg/mL	2205 (1766-12220)	2000 (0-3800)	0.029*
Lactoferrin ng/mL	282.8±68.1	178.4±63.5	0.001
Calprotectin ng/mL	72.1±21.9	56.18±24.58	0.002

- Results are represented as mean ± standard deviation, or *median with minimum and maximum. $p<0.05$ is significant.

Table (3): Comparison of the levels of the three studied markers at the onset of SBP and after six months (follow-up).

	At the onset of SBP	After 6 months	*p*-value
Hepcidin pg/mL	2205 (1766-12220)	2000 (1518- 12350)	0.004*
Lactoferrin ng/mL	282.5±68.1	262.8±77.5	0.343
Calprotectin ng/mL	72.1± 21.9	64.9±18.9	0.002

- Results are represented as mean ± standard deviation or *median minimum and maximum. $p<0.05$ is significant.

Correlation between the three measured ascitic fluid markers and other studied parameters showed that positive correlation was found between Calprotectin and PMNLs among SBP group before therapy (r=0.387, p=0.018) Fig. (2).

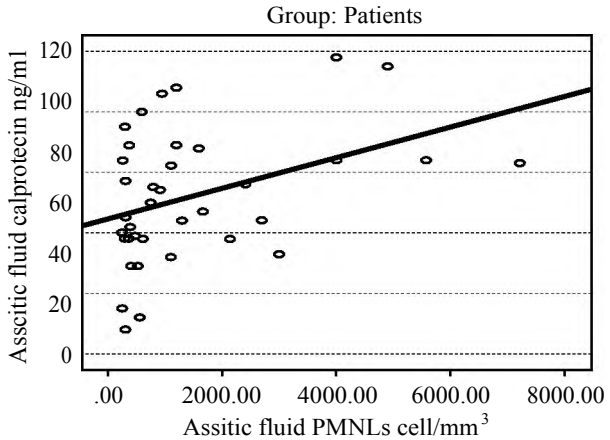

Fig. (2): Positive correlation between ascitic fluid Calprotectin and Ascitic Fluid PMNLs among SBP group before therapy (r=0.387 & p=0.018).

With the use of a cutoff value of 48ng/mL, Calprotectin gave sensitivity (91.9%) and specificity (62.7%) for detection of SBP (AUC=0.695), while Lactoferrin showed sensitivity (91.9%) and specificity (60.8%) with a cutoff value 189.9ng/ml (AUC=0.860). Hepcidin gave 91.9% sensitivity and 64.7% specificity for detection of SPB with the use of a cutoff value of 1952pg/ml (AUC= 0.661) Fig. (3A-C).

To detect whether each marker is more useful separtley or it is better to do combination of them, testing comination of the three markers considering that the combination is positive when all the three markers are above the cutoff value of each, and negative if any one of them is below the cutoff value revealed that their combination gives nearly the same sensitivity and specificity.

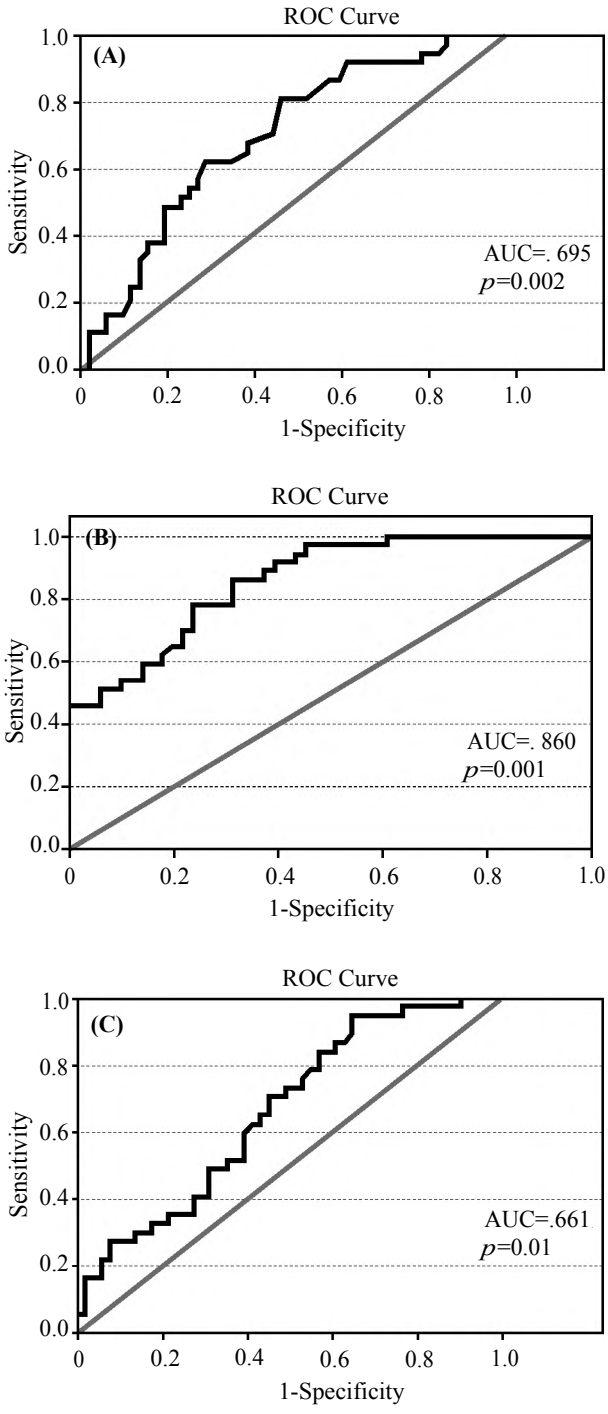

Fig. (3): Receiver Operating Characteristics (ROC) analysis for A: Calprotectin, B: Lactoferrin and C: Hepcidin.

Discussion

Spontaneous bacterial peritonitis is the most serious complication in patients with decompensated liver cirrhosis and ascites [24]. The gold standard method for diagnosis of SBP is diagnostic paracentesis and PMNL in ascitic fluid analysis [25]. Many clinical trials for early diagnostic markers were performed and mostly focused on Calprotectin and Lactoferrin. They were studied individually to detect their role in diagnosis of SBP [11,12,26].

In this study, we assessed the role of Calprotectin, Lactoferrin and Hepcidin in early diagnosis and prognosis of SBP. The results of our study showed that the levels of ascitic fluid Calprotectin, Lactoferrin and Hepcidin were significantly higher in patients with SBP group than the ascitic control group (p=0.002, 0.001 & 0.029 respectively).

These results agreed with Elbanna et al., and Ghweil et al., [27,28] who reported higher AF Calprotectin levels among Egyptian patients with SBP than in Ascitic patients without SBP. Also, Lutz et al., [11] reported higher levels of AF Calprotectin in German patients with SBP than ascitic non-SBP patients. Also, Khalifa et al., [15] reported higher Lactoferrin levels among SBP group than Ascitic group in a group of Egyptian patients, Wu et al., [16] and Lee et al., [29] reported higher Lactoferrin levels in SBP patients than non SBP ascitic patients in Taiwanese population.

To detect their role in follow-up cases with SBP, we compared the levels of ascitic fluid Calprotectin, Lactoferrin and Hepcidin before and after six months of antibiotic therapy in SBP group. Calprotectin and Hepcidin levels decreased significantly after long duration (6 months) therapy. Lactoferrin levels showed no significant difference before and after therapy. These results are in agreement with Wu et al., [16] who reported that Lactoferrin levels didn't significantly change after antibiotic therapy when compared to before therapy levels. As regards to AF Calprotectin, Fernandes et al., [26] reported that Calprotectin levels increased significantly in patients with SPB and decreased after antibiotic therapy.

There are several functions of Calprotectin; it is increased significantly during inflammation in serum and body fluids so it may play a regulatory role in inflammatory reactions. It induces apoptosis in cell lines, and it has an antimicrobial effect [30]. Calprotectin is mainly produced by activated neutrophils [10], so its concentration in body fluid is linked to neutrophil counts, in our study Calprotectin levels are positively correlated with PMNLs counts; this may explain the decrease in its levels in Ascitic group than SBP group, and in SBP after therapy. The clinical utility of serum, ascitic fluid and fecal Calprotectin in liver diseases has been evaluated in several studies. High plasma Calprotectin levels identified group of cirrhotic patients with recurring bacterial infections and was found to be a good prognostic marker of survival independent of severity of liver disease [11,31].

The high Lactoferrin levels in patients with SBP than control ascitic patients in our study can be explained by the theory that increased number of ascitic bacterial colonies that produce more inflammation lead to higher Lactoferrin levels in SBP group than in ascitic control group [12,16,32]. Lactoferrin major function resides in its bactericidal effects, either by sequestering free iron [33] or by the effects of lactoferricin, an antibacterial peptide generated by proteolytic cleavage of Lactoferrin [34].

Very few studies studied the role of Hepcidin in chronic liver disease [35] but to our knowledge; our study is the first one to study Hepcidin as marker for early diagnosis and follow up of SBP. Hepcidin showed high levels in SBP group that decreased significantly after therapy; also it gave a very high sensitivity and specificity more than Calprotectin and Hepcidin. This can be explained by the role that Hepcidin plays in host defense as it acts as a bridge between iron metabolism and immunity where it is markedly increased in cases of infections and inflammations [36,37]. Also, Hepcidin has antimicrobial activity against several micro-organisms as E. coli, Staphylococcus epidermidis and Staphylococcus aureus [17].

In conclusion, we found that ascitic fluid Calprotectin, Lactoferrin and Hepcidin can be used in diagnosis and follow-up of SBP. The three markers have the same sensitivity but Hepcidin showed higher specificity than the other two markers. Being reliable highly sensitive markers with levels correlating with disease progression, they can be used as good markers for diagnosis and follow-up of SBP specially that Calprotectin is available now as a rapid point of care test when other diagnostic tools are unavailable or can't be obtained rapidly. However, a large sample size from different centers in whole Egypt should be performed to confirm these results.

Declaration of interest statement:

The authors declare that they have no conflict of interest concerning this article.

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تقييم مستوى الكالبروتكتين واللاكتوفيريين والهيبسيدين فى السائل البريتونى وإستخدامهم كدلائل متابعة لحدوث الإلتهاب البريتونى التلقائى فى مرضى تليف الكبد

يعتبر الإلتهاب البريتونى التلقائى الحدوث من أخطر مضاعافات مرضى تليف الكبد فى وجود مضاعافات والإستسقاء. ويتم التشخيص إعتمادا على وجود خلايا الدم البيضاء فى سائل البطن بنسبة أكبر من ٢٥٠خلية/مم٣. وعلى الرغم من ذلك يتم حدوث المرض فى وجود عدد قليل من الخلايا البيضاء لهذا لزم البحث عن دلائل نو حساسية عالية ونوعية عالية للكشف المبكر عن المرض. تهدف الدراسة إلى تعيين مستوى Hepcidin, Calprotectin, Lactoferrin فى السائل البريتونى كدلائل متاحة للكشف المبكر ومتابعة المرض. وشملت الدراسة ٨٨ مريضا تم تقسيمهم إلى مجموعتين: المجموعة الآولى شملت ٣٧ مريضا بتليف الكبد مع وجود الإلتهاب البريتونى التلقائى والمجموعة الثانية شملت ٥١ مريضا بتليف الكبد فقط دون وجود الإلتهاب البريتونى التلقائى كمجموعة حاكمة. وتم دراسة مستوى الثلاث دلائل، Calprotectin, Lactoferrin و Hepcidin فى السائل البريتونى بطريقة الإليزا. وقد وجدت الدراسة آن مستوى الثلاث دلائل أعلى فى المجموعة الآولى عن المجموعة الحاكمة (p=٠.٠٠٠٢, ٠.٠٠٠١, ٠.٠٢٩) وقد وجد آن Hepcidin أعلى فى درجة الحساسية والنوعية عن باقى الدلائل. ونستخلص من الدراسة إمكانية إستخدام هذه الدلائل للكشف المبكر عن الإلتهاب البريتونى التلقائى وإمكانية إستخدام Hepcidin فى المتابعة لهذه الحالات.