The Egyptian Journal of Hospital Medicine (October 2022) Vol. 89, Page 5941- 5948

Ascitic Calprotectin, as a Potential Diagnostic and Prognostic Marker of Spontaneous Bacterial Peritonitis

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ABSTRACT

Background: The presence of an elevated absolute polymorphonuclear leukocyte count in the ascitic fluid (>250 cells/mm3) in combination with a positive ascitic fluid bacterial culture is diagnostic of spontaneous bacterial peritonitis. Among the families of calcium-binding proteins known as S100, calprotectin belongs to the subfamily known as calgranulins. **Objective:** The aim of the current work was to examine the value of ascitic fluid calprotectin for the diagnosis and prognosis of spontaneous bacterial peritonitis in people with liver cirrhosis.

Patients and Methods: Between April 2018 and May 2019, 50 Egyptians with liver cirrhosis and ascites were recruited. Forty individuals with spontaneous bacterial peritonitis (SBP) and 10 patients with ascites without SBP were studied. Biochemical, bacterial, and ascitic calprotectin level, as well as diagnostic paracentesis, were performed to all patients.

Results: When comparing individuals with cirrhosis and SBP to those without SBP, the level of calprotectin in the ascitic fluid was significantly higher in SBP patients (P= 0.000). Its mean values were statistically significantly reduced after treatment of SBP in comparison to its level before treatment [reduced from 626.75 ± 188.05 to 251.25 ± 223.13 ug/l] with p value 0.000. At a cutoff value of >320 ug/l, ascitic calprotectin may be a possible marker of development of SBP among cirrhotic patients with ascites with 95% sensitivity and 90% specificity.

Conclusion: It could be concluded that ascitic fluid calprotectin may be useful in the diagnosis and prognosis of patients with liver cirrhosis and spontaneous bacterial peritonitis.

Keywords: Liver cirrhosis, Spontaneous bacterial peritonitis, Calprotectin.

INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is the most prevalent bacterial illness in people with liver cirrhosis, and it accounts for ten to thirty percent of all bacterial infections diagnosed in hospitals **(1).**

SBP is diagnosed in cirrhotic patients with an ascitic polymorph nuclear leucocytic count (PMNL) of ≥ 250 cell/mm^3, regardless of whether bacteria are isolated from the ascitic fluid **(2)**. Abdominal pain, fever, as well as worsening of pre-existing ascites are the most common symptoms, though up to one-third of cases are asymptomatic **(3).** Some people with SBP don't have any symptoms, therefore finding a good marker is crucial.

Severe side effects of SBP include hepatorenal syndrome (HRS), hypovolemia, fluid and electrolyte changes that can lead to shock or abrupt renal failure, and peritoneal abscess. Early detection of SBP requires a paracentesis, however this isn't always feasible and may take too much time **(4).** It is therefore desirable to discover new and relevant biomarkers for the early detection of SBP. Also desirable are laboratory tests that can anticipate how a patient will respond to the first treatment as failure to respond to the initial treatment is associated with an increased risk of death and/or severe bleeding **(5).**

A noninvasive biomarker for gastrointestinal inflammation, calprotectin, has emerged in the last two decades **(6).** In neutrophils, the antibacterial and anti-proliferative protein calprotectin is found nearly exclusively; the number of neutrophils present is inversely proportional to the amount of this substance found in body fluids **(7).** Calprotectin titers in bodily fluids are known to rise dramatically in many forms of inflammation. Excessive levels of calprotectin have been seen in extracellular fluid in inflammatory diseases such as: Cystic fibrosis, temporal arthritis, rheumatoid arthritis and abscesses **(8).**

The ascitic fluid levels of calprotectin in individuals with liver cirrhosis who have SBP are much higher than those of patients without SBP **(7). Lutz *et al.* (6)** stated that the ascetic fluid calprotectin to total protein ratio was an independent predictive predictor of 30-day mortality and showed better diagnostic value for SBP than calprotectin alone. Insufficient study has made it difficult to determine how effective calprotectin levels are in distinguishing between SBP and sterile ascites **(9).**

In this study we aimed for examining the value of ascitic fluid calprotectin for the diagnosis and prognosis of spontaneous bacterial peritonitis in people with liver cirrhosis.

PATIENTS AND METHODS

This study included a total of fifty Egyptian patients with liver cirrhosis and ascites admitted to Ain Shams University Hospitals during the period from April 2018 to May 2019.

They were divided into two groups, one comprising 40 patients who had ascites and SBP detected by the existence of ≥ 250 PMNL/mm3 in ascitic fluid and/or positive culture with a single causative organism, and the other comprising 10 patients with ascites without SBP.

Received: 27/06/2022
Accepted: 03/09/2022

Ethical consent:

An approval of the study was obtained from Ain Shams University Academic and Ethical Committee. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Exclusion Criteria: Patients having secondary causes of peritonitis (T.B peritonitis, malignancy), surgical causes of peritonitis (abscess, appendicitis, cholecystitis, pancreatitis), recent abdominal surgery (<3 months), patients receiving antibiotics within 1 month before presentation, bilharziasis, hepatocellular carcinoma, history suggestive of inflammatory bowel disease, diabetes mellitus, hypertension, ischemic heart disease, renal impairment, and any infections other than SBP.

Laboratory testing comprising complete blood count (CBC), C-reactive protein (CRP), ESR, full liver profile, serum creatinine, HCV Ab, HBsAg, and HIV Ab and were performed for all patients in addition to a thorough history taking and physical examination.

Abdominal ultrasonography was performed with special emphasis on liver size, echotexture, splenic size, presence of ascites and HCC.

Diagnostic paracentesis was done under complete aseptic conditions to estimate PMNL, Albumin, total proteins, and glucose in the ascitic fluid.

Brain-heart infusion broth infused with 10 ml of ascitic fluid was cultured for 48 hours at 37°C in standard blood culture bottles for aerobic and anaerobic bacteria. Identification of the isolated organisms was done by gram stain, biochemical reactions, clonal morphology, and agglutination with specific antisera

Ascitic fluid Calprotectin using ELISA: Normal assay range: 3 ng/ml - 320 ng/ml, stored at 2-8°C, quantitative detection of calprotectin in serum, plasma, or cell culture supernatant of human subjects.

Statistical methods

IBM SPSS statistics was used to conduct a statistical analysis of the data (Statistical Package for Social Sciences). For qualitative variables, independent t-tests were used when there were two separate groups with parametric data to do inferential analysis. Chi square test ($\chi 2$) to calculate difference between two or more groups of qualitative variables. Quantitative data were expressed as mean ± SD (Standard deviation). Independent samples t-test was used to compare between two independent groups of normally distributed variables (parametric data). For numerical parametric data, we used Pearson correlation; for numerical nonparametric and qualitative data, we used the Spearman test. We used ROC curve and DeLong test to analyze how well different tests discriminated across various subgroups. SBP patients were studied using logistic regression analysis to identify potential determinants of their condition.

RESULTS

This study included fifty patients with liver cirrhosis and ascites: forty patients with SBP, (26 males (65%) and 14 females (35%)) with mean age 56.40±7.37, and ten patients without SBP (8 males (80%) and 2 females (20%)) with mean age 54.20 ± 6.86,

Table 1 shows that the inflammatory markers [Total leucocytic count, CRP, ESR] were significantly higher in participants diagnosed with SBP compared to those without the condition. Also, a statistically significant higher kidney function tests, liver function tests, and lower platelet count among SBP patients were found.

Table (1): Comparison between ascites patients with SBP and without SBP as regard laboratory investigations among groups.

		Ascites without SBP	Ascites with SBP	Test value•	P-value	Sig
		No. = 10	No. = 40			
CRP mg/l	Mean ± SD	6.09 ± 1.41	55.13 ± 12.61	-6.249	0.000	HS
ESR mm/hr	Mean ± SD	39.90 ± 7.65	77.03 ± 6.00	-7.098	0.000	HS
Urea mg/l	Mean ± SD	25.60 ± 6.19	101.38 ± 15.44	-14.684	0.000	HS
Creatinine mg/dl	Mean ± SD	0.76 ± 0.17	2.76 ± 0.44	-9.508	0.000	HS
Hemoglobin g/dl	Mean ± SD	8.74 ± 1.27	9.28 ± 0.90	-1.562	0.125	NS
WBCs mm^3/l	Mean ± SD	6.20 ± 1.31	16.33 ± 3.21	-9.053	0.000	HS
Platelet x 10^9/l	Mean ± SD	116.50 ± 26.31	71.03 ± 7.42	4.735	0.000	HS
Liver profile comparison among groups:						
AST units/L	Mean ± SD	41.80 ± 10.31	55.15 ± 11.32	-2.340	0.024	S
ALT units/L	Mean ± SD	39.20 ± 6.22	32.45 ± 6.08	2.121	0.039	S
Total bilirubin mg/dl	Mean ± SD	3.00 ± 0.71	3.02 ± 0.39	-0.041	0.968	NS
Direct bilirubin mg/dl	Mean ± SD	1.43 ± 0.20	2.52 ± 0.41	-3.271	0.002	HS
Albumin g/dl	Mean ± SD	2.36 ± 0.41	2.23 ± 0.42	0.607	0.547	NS
PT sec	Mean ± SD	11.80 ± 0.65	21.63 ± 5.10	-3.602	0.001	HS
PTT sec	Mean ± SD	38.50 ± 4.53	47.58 ± 11.48	-2.436	0.019	S
INR	Mean ± SD	0.88 ± 0.12	1.60 ± 0.31	-3.559	0.001	HS

The mean values of PMNL and LDH were substantially greater in patients with SBP compared to those without SBP statistically. The average levels of glucose and protein in ascitic fluid were considerably lower in SBP patients. Calprotectin levels in ascitic fluid were considerably greater in patients with SBP compared to those who don't have SBP (p0.0001) (Table 2).

Table (2): Comparison between the studied groups as regard ascitic fluid analysis and ascitic calprotectin

		Ascites without SBP	Ascites with SBP	Test value•	P-value	Sig.
		No. = 10	No. = 40			
Ascitic fluid PMN mm^3/l	Mean ± SD	177.30 ± 46.64	288.68 ± 22.30	-11.055	0.000	HS
Ascitic fluid LDH u/l	Mean ± SD	179.70 ± 43.19	368.10 ± 90.54	-6.027	0.000	HS
Ascitic fluid Glucose mg/dl	Mean ± SD	129.10 ± 31.30	44.48 ± 10.62	13.577	0.000	HS
Ascitic fluid Protein g/dl	Mean ± SD	3.95 ± 0.59	1.97 ± 0.43	6.707	0.000	HS
Ascitic calprotectin ng/mL	Mean ± SD	237.00± 56.41	626.75 ± 51.12	-6.171	0.000	HS

Distribution of different organisms in ascetic fluid cultures among 40 SBP patients: 18 (45%) positive, 17 (42.5%) negative, 5 (12.5%) Not performed. Among the positive culture patients, it included: 13 (72.2%) E coli, 2 (11.1%) Streptococcus pneumoniae, 1 (5.5%) pseudomonas species and 1 (5.5%) for each Staphylococcus species and Enterococcus species. A statistically significant reduction in inflammatory markers (WBCs, CRP, ESR) as well as Kidney function tests (creatinine & urea) after treatment of SBP as shown in (table 3). Also, a statistically significant improvement in full hepatic synthetic function after treatment of SBP were noted as shown in (table 4).

Table (3): Comparison between before and after treatment among SBP patients as regard Laboratory investigations:

		SBP before treatment	SBP after treatment	Test value•	P-value	Sig.
		No. = 40	No. = 40			
CRP mg/l	Mean ± SD	55.13 ± 11.42	6.62 ± 1.00	12.643	0.000	HS
ESR mm/hr	Mean ± SD	77.03 ± 6.00	40.15 ± 7.55	13.503	0.000	HS
Urea mg/l	Mean ± SD	101.38 ± 15.44	82.30 ± 10.17	5.757	0.000	HS
Creatinine mg/dl	Mean ± SD	2.76 ± 0.41	1.88 ± 0.26	7.926	0.000	HS
Platelet x $10^9/l$	Mean ± SD	71.03 ± 7.42	71.03 ± 7.42	0.000	1.000	NS
Hemoglobin g/dl	Mean ± SD	9.28 ± 0.90	9.37 ± 0.78	-0.645	0.523	NS
WBCs mm^3/l	Mean ± SD	16.33 ± 3.21	7.41 ± 1.80	16.342	0.000	HS

Table (4): Comparison between before and after treatment among SBP patients as regard Liver profile:

		SBP before treatment	SBP after treatment	Test value•	P-value	Sig.
		No. = 40	No. = 40			
AST units/L	Mean ± SD	55.15 ± 12.32	53.75 ± 12.31	0.521	0.605	NS
ALT unit/ L	Mean ± SD	32.45 ± 5.08	31.73 ± 5.46	0.560	0.579	NS
Total bilirubin mg/dl	Mean ± SD	3.02 ± 0.42	1.92 ± 0.33	4.925	0.000	HS
Direct bilirubin mg/dl	Mean ± SD	2.52 ± 0.31	0.75 ± 0.23	10.425	0.000	HS
Albumin g/dl	Mean ± SD	2.23 ± 0.51	3.13 ± 0.38	-8.468	0.000	HS
PT sec	Mean ± SD	21.63 ± 5.12	17.03 ± 2.15	3.621	0.001	HS
PTT sec	Mean ± SD	47.58 ± 11.48	40.30 ± 4.19	3.866	0.000	HS
INR	Mean ± SD	1.60 ± 0.31	1.26 ± 0.16	3.621	0.001	HS

Regarding ascitic fluid analysis, a statistically significant reduction of ascitic fluid PMN & LDH with significant elevation of ascetic fluid glucose & protein after treatment of SBP patients were detected. On measuring calprotectin in the ascitic fluid, its mean values were statistically significantly reduced after treatment of SBP in comparison to its level before treatment with p value 0.000 (Table 5)./

Table (5): Comparison between before and after treatment among SBP patients as regard ascitic fluid and Ascitic calprotectin

		SBP before treatment No. = 40	SBP after treatment No. = 40	Test value•	P-value	Sig.
Ascitic fluid PMN mm^3/l	Mean ± SD	288.68 ± 22.30	226.63 ± 15.32	14.329	0.000	HS
Ascitic fluid DH u/l	Mean ± SD	368.10 ± 95.54	213.73 ± 57.06	8.915	0.000	HS
Ascitic fluid Glucose mg/dl	Mean ± SD	44.48 ± 2.51	152.23 ± 29.41	-22.827	0.000	HS
Ascitic fluid Protein g/dl	Mean ± SD	1.97 ± 0.28	5.33 ± 1.48	-11.263	0.000	HS
Ascitic calprotectin ng/mL	Mean ± SD	626.75 ± 18.05	251.25 ± 23.13	10.693	0.000	HS

Correlations of ascitic fluid calprotectin and different parameters are shown in (Table 6).

Table (6): Correlation between ascitic calprotectin & other laboratory investigation before treatment

Ascites with SBP before treatment	Ascitic calprotectin ng/mL	
	R	P-value
Age (years)	0.028	0.863
AST (units/l)	-0.089	0.584
ALT (units/l)	-0.140	0.390
Total bilirubin (mg/dl)	.440**	0.005
Albumin (g/dl)	0.036	0.824
CRP (mg/l)	.530**	0.000
ESR (mm/hr)	-0.032	0.845
Ascitic fluid PMN	.589**	0.000
Ascitic fluid LDH	.425**	0.006
Ascitic fluid Glucose	-.468-**	0.002
Ascitic fluid Protein	.354*	0.025
Platelet (x $10^9/l$)	0.025	0.878
Hemoglobin (g/dl)	0.018	0.914
WBCs (mm^3/l)	.329*	0.038
PT sec	0.105	0.520
PTT sec	-0.179	0.270
INR	.472**	0.002

At a cutoff value of >320 ug/l , ascitic calprotectin may be a possible marker of development of SBP among cirrhotic patients who had ascites with 95% sensitivity and 90% specificity (Table 7, Figure 1)

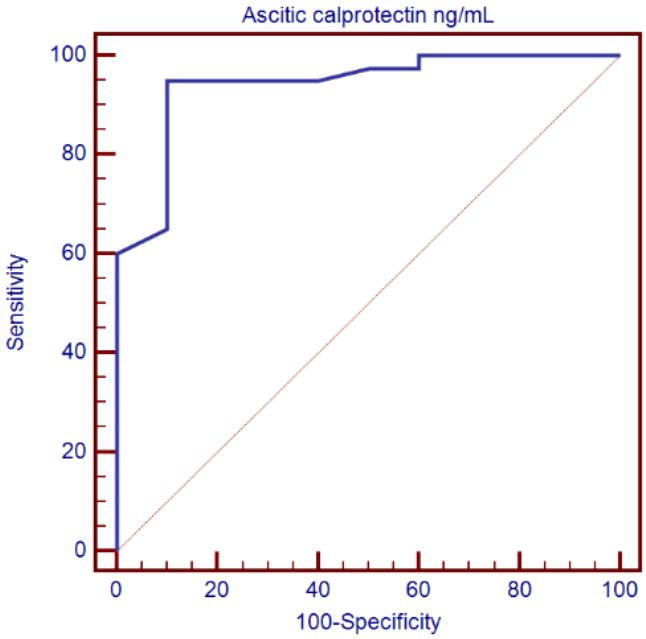

Figure (1): Ascitic calprotectin as a predictor of SBP

Table (7): Ascitic calprotectin as a predictor of SBP.

Parameter	AUC	Cut of Point	Sensitivity	Specificity	PPV	NPV	Accuracy
Ascitic calprotectin	**0.941**	**>320**	**95.0**	**90.0**	**97.4**	**81.8**	**94.1%**

While at a cutoff value of ≤780 ug/l, ascitic calprotectin may be a possible marker for prognosis of SBP after treatment [response to treatment] with 82,86% sensitivity and 100 % specificity (Table 8, Figure 2).

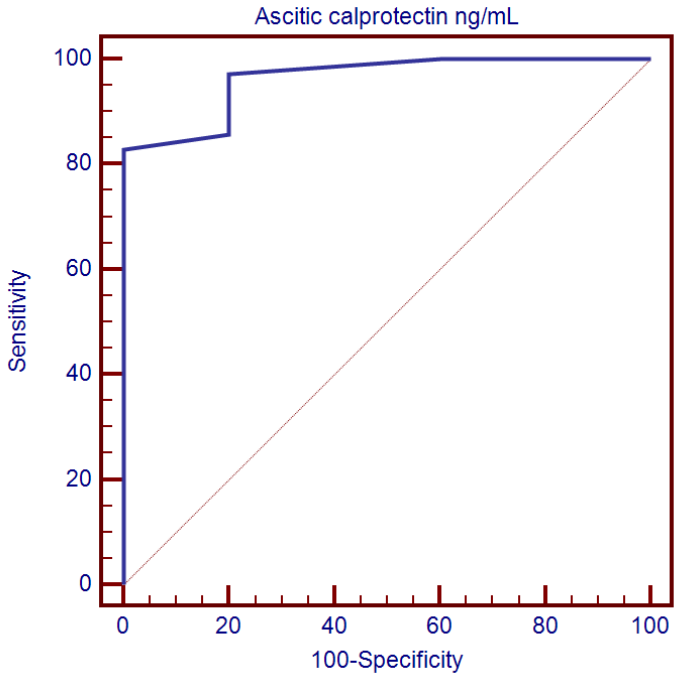

Figure (2): Ascitic calprotectin as a predictor for prognosis of SBP among cirrhotic patients after treatment.

Table (8): Ascitic calprotectin as a predictor for prognosis of SBP among cirrhotic patients after treatment.

Parameter	AUC	Cut of Point	Sensitivity	Specificity	PPV	NPV	Accuracy
Ascitic calprotectin	**0.963**	**≤780**	**82.86**	**100.0**	**100.0**	**45.5**	**96.3%**

DISCUSSION

Ten percent to thirty percent of all bacterial infections diagnosed in hospitalized patients are due to spontaneous bacterial peritonitis, and the vast majority of these cases occur in cirrhotic patients [10, 11, 12].

All patients with ascites should have paracentesis performed to rule out an infection in the abdomen, according to current guidelines [11]. Ascitic fluid samples with a PMN concentration of more than 250/mic/l are used to diagnose SBP patients with liver cirrhosis. For the detection of SBP, other criteria (PMN > 250 mic/l, white blood cell count > 500 mic/l) are considered more sensitive [13].

Up to 60% of patients with an elevated PMN count are reported as culture negative, making a diagnosis of SBP based purely on bacterial culture inaccurate [14]. It has been proposed that automated PMN counting replace the time-consuming and error-prone process of manually counting cells in order to speed up diagnosis and reduce patient wait times [15].

Several inflammatory situations raise the neutrophil turnover surrogate marker calprotectin. Plasma, urine, faces, cerebrospinal fluid, saliva, synovial fluid, colonic biopsies and empyema supernatant are all good indicators of its presence [16].

Calprotectin is a non-specific protein that is enhanced in a variety of organic gastroenterological disorders, despite the fact that it is not specific. A variety of regularly encountered organic disorders can be diagnosed and prognosticated using measurements of this protein. Calprotectin has been studied as a potential diagnostic and prognostic marker of SBP in patients with liver cirrhosis.

Consistent with the findings of Moodithaya's study (in which 79% of the SBP group were males and SBP were not impacted by age), the present study found no significant difference in age or gender between the SBP and non-SBP groups [17].

On comparing different laboratory parameters of patients with and without SBP, there was a statistically significant higher AST among SBP patients than those without SBP attributed to the toxic liver injury by bacterial infection [18], and this agreed with **Metwally *et al.*** [19], who detected higher AST in SBP patients than in non SBP patients with a p value of 0.006. However, in comparing the two groups, **Moravej *et al.*** [20] observed no statistically significant differences (ALT, AST).

Also, cirrhotic patients with SBP had higher bilirubin level. It could be attributed to intrahepatic shunts and significant relationship between the fasting serum bile acids and the intrinsic clearance of indocyanine green (ICG) [21], and this result agreed with **Umgelter *et al.*** [22] who stated that individuals with SBP had an abnormally high rate of serum bilirubin abnormalities.

At the present study, there was significant higher values in the coagulation profile (INR, PT, PTT) among SBP than non SBP group because blood clotting is maintained by the liver synthesizing several procoagulant and anticoagulant proteins. In addition, the liver disease is frequently linked to an increased risk of bleeding [23].

Tsung *et al.* [24] stated that mean levels of creatinine and urea in this research were much higher in patients with SBP than in those without SBP, corroborating the hypothesis that renal impairment occurs in SBP patients and is an independent predictor of mortality. Possible additional causes include anomalies in systemic and renal hemodynamics, which can lead to acute kidney injury.

Furthermore, acute phase reactants (total leucocytic count, CRP, and ESR) were significantly greater in cirrhotic patients with SBP compared to those without SBP. These markers are utilized as independent predictors of SBP [25]. This agreed with **Jayachandran *et al.*** [26] who reported that CRP is a reliable predictor of SBP and an index of therapeutic effectiveness in adults.

Regarding ascitic fluid analysis in the present study, patients with SBP had substantially greater ascitic PMNL values than those without SBP; these findings were consistent with those of **Elsadek *et al.*** [27] who found the same results with a p value of 0.001.

SBP patients, on the other hand, had considerably lower mean values of ascitic protein than non-SBP patients. We were in agreement with **Paul *et al.*** [28], who first reported that protein synthesis is decreased in patients with severe hepatic impairment.

SBP patients also had considerably lower mean levels of glucose in their ascitic fluid compared to those without SBP. These results are in conformity with **Tsung *et al.*** [24], who found that cirrhotic patients with SBP had lower glucose levels in their ascitic fluid, and who concluded that this was an independent predictor of overall survival. The ascitic glucose concentration drops when bacteria, white blood cells, or cancer cells consume it, as occurs in tuberculous peritonitis, severe bacterial peritonitis, and malignancy [29].

On measuring calprotectin in the ascitic fluid, its mean values were statistically significantly higher in patients with SBP in comparison to those without SBP with p value 0.000. At cut off value 320 ng/ml could predict the presence of SBP. This is also close to **Abdel-Razik *et al.*** [7] who reported that ascetic calprotectin was higher in patients who had SBP with cut off value 470 ng/ml.

Also, **Burri and colleagues** [30] stated that ascitic calprotectin as assessed by enzyme-linked immunosorbent assay was shown to have the highest sensitivity (94.8), specificity (89.2), positive predictive value (60%), and accuracy (90%) when used to diagnose SBP.

Also, these findings supported by the present study on using Pearson correlation coefficient test revealed that Ascitic calprotectin level was positively correlated with (total leucocytic count, C-reactive

protein, acetic fluid PMNL, lactate dehydrogenase, proteins). These results were also close to those of **Abdel-Razik *et al.* [7]** who found that elevated ascitic calprotectin was linked to increased total leucocytic count in a cohort of individuals with SBP brought on by inflammation.

These findings could be explained by the fact that inflammatory response proteins like calprotectin have antibacterial and antiproliferative properties **[31].**

Moreover, a substantial negative association was identified between ascitic calprotectin level and ascetic fluid glucose among SBP group of patients and these findings agreed with **Fayrouz *et al.* [32],** who found a positive link between them.

On treatment of SBP cirrhotic patients, the percentage of improved cases was 87.5% on regimen of 3rd generation cephalosporines (cefotaxime), while the morbidity rate was 10% and mortality rate was 2.5% after treatment of 10 days duration. This is close to the result of **Shamseya *et al.* [33]** who showed that cefotaxime has successful effect in treating SBP in 77 to 98% of cases. But showed the overall in-hospital mortality was 17.6% in 30 days duration.

Regarding ascitic fluid analysis, it showed improvement after SBP treatment in the cirrhotic patients as following: rise in (Ascitic fluid Glucose & Protein) with decrease in (Ascitic fluid. Polymorphs & LDH). These results were close to results of **Fernandez *et al.* [34]**, who stated decrease in ascitic polymorph cell count of more than 25% of the starting value and it was really proposed as the primary criterion for determining the antibiotic's success and the necessity of switching the medication.

Lastly, on measuring calprotectin in the ascitic fluid, its mean value was statistically significantly reduced after treatment of SBP in comparison to its baseline level and at cut off value ≤ 780 ng/ml it had sensitivity 82.86%, specificity 100%, PPV 100%, NPV 45.5%, accuracy 96.3% in prediction of better prognosis in form of response to treatment.

CONCLUSION

It could be concluded that ascitic fluid calprotectin may be useful in the diagnosis and prognosis of patients with liver cirrhosis and spontaneous bacterial peritonitis.

Financial support and sponsorship: Nil.
Conflict of interest: Nil.

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