Ascitic Fluid Calprotectin and Serum C-Reactive Protein as Diagnostic Markers for Spontaneous Bacterial Peritonitis

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Background and study aim: Spontaneous bacterial peritonitis (SBP) is an important cause of morbidity and mortality in cirrhotic patients with ascites. The diagnosis of SBP is based on PMN leukocyte cell count exceeding 250/µL in ascitic fluid. However, this procedure is time consuming as well as subjective. C-reactive protein (CRP) has been reported to be a reliable predictor of SBP and an index of therapeutic effectiveness in adults. Ascitic fluid calprotectin reliably predicts PMN count >250/µL, which may prove useful in the diagnosis of SBP. This work was planned aiming to evaluate both ascitic fluid calprotectin and serum CRP as accurate diagnostic laboratory markers for detecting SBP

Patients and Methods: From 140 patients; only 124 patients with ascites were included in this study. They were divided into SBP group including 70 patients (49 males and 21 females) and non-SBP group of 54 patients (25 males and 29 females). Serum CRP was determined by latex agglutination and ascitic fluid calprotectin was measured using an enzyme-linked immunosorbent assay.

Results: Ascitic fluid calprotectin and serum CRP were significantly higher in SBP patients in comparison with the non-SBP group (754.67 ±256.06 vs. 280.77 ±230.97 and 62.4 ±28.39 vs. 9.81 ±8.98) respectively. In addition, both were positively correlated with ascitic fluid proteins and PMN count as well as with each other. At a cutoff value of 270 mg/dl, ascitic fluid calprotectin had 86% specificity and 97.5% sensitivity for detecting SBP [Area under the receiver operating characteristics curve (AUC) = 0.924 with negative and positive predictive values (NPV, PPV) for ascitic calprotectin 96% and 69% respectively.

Conclusion: Ascitic fluid calprotectin and serum CRP may be used as accurate and reliable markers for the diagnosis of SBP.

INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is an important cause of morbidity and mortality in cirrhotic patients with ascites. SBP is estimated to affect 10-30% of cirrhotic patients hospitalized with ascites, and mortality in this group approaches 30% [1]. Many of these patients are asymptomatic, and it is therefore recommended that all patients with ascites undergo paracentesis at the time of admission to confirm the SBP status [2]. Although SBP is less prevalent in an outpatient setting, it is reasonable to also evaluate the ascitic fluid of outpatients because of the high mortality associated with SBP.

The diagnosis of SBP is based upon the polymorphonuclear (PMN) leukocyte cell count exceeding 250/µL in ascitic fluid [3]. However, this procedure is time consuming and subjective. Alternative methods using automated PMN counting [4], reagent strips (urine dipsticks) [5], or ascitic lactoferrin [6] have been developed; unfortunately, their diagnostic accuracies are limited and their use is dependent upon availability of laboratory personnel and reagents/components from the commercial source. Therefore, an accurate and convenient method of rapid diagnosis of SBP remains an unmet clinical need.

C-reactive protein (CRP) is an acute phase reactant which binds to different substrates. It activates the complements, takes part in cytokine secretion, and increases the phagocytosis of leucocytes. CRP has been reported to be a reliable predictor of SBP and an index of therapeutic effectiveness in adults [7].

Calprotectin, a calcium and zinc-binding protein, is detected almost exclusively in neutrophils [8], and its presence in body fluids is proportional to the influx of neutrophils [9].

Calprotectin is primarily expressed in neutrophils and macrophages, while it is not usually present in lymphocytes. It has been estimated to account for more than 40% and 5% of cytosolic and total proteins of neutrophils, respectively [10]. Ascitic fluid calprotectin reliably predicts PMN count >250/μL, which may prove useful in the diagnosis of SBP, especially with a readily available bedside testing device [11].

This work was planned aiming to evaluate ascitic fluid calprotectin as an accurate diagnostic laboratory marker for detecting spontaneous bacterial peritonitis (SBP).

PATIENTS AND METHODS

In this prospective observational study, we recruited 140 patients with ascites referred for paracentesis to Tropical Medicine Department-Mansoura University, from October 2012 to March 2013. All patients were subjected to the following evaluations: complete history taking and physical examination, abdominal ultrasound, laboratory assessment including full blood count, liver profile, creatinine, CRP, ESR, and ascitic fluid analysis (WBCs, protein, bacteriologic culture with sensitivity, pathological assessment and calprotectin level). Paracentesis of ascitic fluid was performed for every patient with cirrhosis and ascites that was admitted to our department, independently of the clinical suspicion of AFI, as a routine procedure. AFI diagnosis was based on the presence of ≥250 cells/mL PMN in the ascitic fluid, with or without positive ascitic fluid culture in the absence of a hemorrhagic ascites and secondary peritonitis (by WBCs count, bacteriological culture, LDH and clinical examination)

Exclusion criteria included patients who were immunocompromised and patients who had received antibiotic prior to hospital admission. Moreover, patients with heart failure, diabetes mellitus, hematological disorders and neoplastic disorders and patients with clinically overt hypo- or hyperthyroidism or with clinically and laboratory evident autoimmune diseases were also excluded from this study. None of the study participants had received anticoagulant medications, non-steroidal anti-inflammatory drugs (NSAID) or oral contraceptive drugs before hospital admission.

Sampling

1. Five ml blood was withdrawn by venipuncture, one ml in EDTA tube for CBC and four ml delivered into plastic tube and allowed to clot. Non-hemolyzed sera was separated by centrifugation and used for determination of creatinine, uric acid, CRP, and liver functions (ALT, AST, total bilirubin and albumin).
2. Ascitic fluid sample was taken by paracentesis performed under aseptic conditions from a puncture site in the left or right lower quadrant with the patient in the supine position. All samples for diagnostic testing were immediately collected at the bedside and processed by laboratory personnel without further delay.

Methodology

1- Blood glucose, liver profile, and creatinine concentrations were measured on a Dimension Xpand plus chemistry analyzer (Roche Diagnostics, Basel, Switzerland) using commercially available reagents and an enzyme-based kit.
2- Complete blood picture was measured using CELL-DYN Emerald cell counter (ABBOTT, Germany).
3- Serum CRP was determined using latex agglutination test kit (Omega diagnostics LTD AVITEX CRP Ref OD073/OD023/ OD023/E. Scotland, UK) **[12]**.
4- Ascitic fluid calprotectin was measured by an enzyme-linked immunosorbent assay using immune diagnostic AG ELISA kit ([MRP 8/14] Stubenwald-Allee 8a–D-64625, Bensheim) **[13]**.

This study was approved by the Ethical Committee of Mansoura University and all patients provided written informed consent prior to participation in any protocol-specific procedures. The study was conducted in accordance with the guidelines of the Helsinki Declaration.

Statistical analysis

All statistical analyses were performed using the SPSS version 17.0 software, Chicago, USA. Data were first tested by Kolmogorov–Smirnov test for

distribution of data. Parametric data was expressed in mean and standard deviation (SD). The mean and SD of the differences and the limits of agreement, defined as the mean ± 2 SD of the difference (95%CI), were calculated. Unpaired *t* test was used for intergroup comparisons. A P-value of less than 0.05 indicated statistical significance. Correlations between numerical data were determined with the Pearson's rank correlation coefficient. All hypothesis testing were two-tailed. Analysis of the receiver operator characteristics (ROC) and calculation of the area under the curve (AUC) were used to evaluate the capability of calprotectin to identify a PMN count >250/μL.

RESULTS

***Patient characteristics*:**

From 140 patients, only 124 patients with ascites were included in this study. After three weeks of admission of each case, the final diagnosis (SBP) and the aetiology of ascites were assessed. According to ascitic fluid analysis and clinical data, they were divided into a SBP group including 70 patients (49 males and 21 females) and non-SBP group of 54 patients (25 males and 29 females). A total of 16 patients had malignant ascites (which included 6 leaking hepatocellular carcinoma, four ovarian, two lymphomas, one breast, one stomach, one colorectal and one pancreatic cancer) were excluded from this study.

Patients suffered from liver cirrhosis with different aetiologies (Table 1). 97 chronic hepatitis C related cirrhosis (78.2%), 21chronic hepatitis B related cirrhosis (16.9%), 3 autoimmune-related cirrhosis (2.4%), 2 nonalcoholic steatohepatitis-related cirrhosis (1.6%) and one cryptogenic cirrhosis (0.8%).

Table (1) : Baseline characteristics of patients with liver cirrhosis

Parameters	No. of patients
Aetiology of liver cirrhosis:	
Chronic hepatitis C (CHC)	97 (78.2%)
Chronic hepatitis B (CHB)	21 (16.9)
Autoimmune hepatitis (AIH)	3 (2. 4%)
Nonalcoholic steatohepatitis (NASH)	2 (1.6%)
Cryptogenic	1 (0. 8%)
Child-Turcotte-Pugh class:	
Child A	0
Child B	88 (71%)
Child C	36 (29%)
MELD score	11.3 (10.5-18)

MELD: Model for end-stage liver disease

Fever was the most common presentation found in 49 cases (70%), followed by abdominal pain in 39 patients (55.7%), abdominal tenderness in 34 cases (48.6%), altered mental status in 23 cases (32.9%) and upper GIT bleeding in 19 cases (27.1%), while 21 cases (30%) were asymptomatic (Table 2).

Table (2): Clinical presentation in patients with SBP

Parameters	No. of patients (n=70)
Fever	49 (70%)
Abdominal pain	39 (55.7%)
Abdominal tenderness	34 (48.6%)
Altered mental status	23 (32.9%)
Upper GIT bleeding	19 (27.1%)
Asymptomatic	21 (30%)

GIT: gastrointestinal tract

According to the Child-Turcotte-Pugh Score, 88 (71%) of the patients were classified as stage B and 36 (29%) of the patients were classified as stage C (Table 1).

Laboratory and ascitic fluid cell count:
There was significant increase in WBCs, platelets, CRP, AST and creatinine in the SBP group versus the non-SBP group **(**10.16±2.88 vs. 6.99 ±1.78; 107.52±19.13 vs. 130.9±29.73; 62.4 ±28.39 vs. 9.81 ±8.98; 43.15±13.75 vs. 51.95 ±10.34 and 1.77±0.44 vs. 1.19±0.42 respectively). There was no correlation between hemoglobin (Hb), ALT, serum bilirubin, serum albumin and random blood sugar (RBS) in both groups 10.09 ±0.69 vs. 9.74 ±0.81; 50.5±21.42 vs. 40.31 ±15.33; 1.82 ±0.81 vs. 1.87 ±0.48; 2.68 ±0.33 vs. 2.57 ±0.43 and 141.2 ±38.18 vs. 148.4 ±21.66 respectively) (Table 3).

Table (3): Biochemical parameters in the studied groups

	SBP group (n=70)	**Non-SBP group (n=54)**	**P-value**
Hb (gm/dl)			
Range	8.10 – 11.0	8.9 – 11.4	0.09
Mean ±SD	10.09 ±0.69	9.74 ±0.81	
WBCs (10^3/cmm)			
Range	3.5 – 16.0	4.3 – 11.0	0.001
Mean ±SD	10.16 ±2.88	6.99 ±1.78	
Platelets (10^3/cmm)			
Range	60 – 142	68 – 201	0.001
Mean ±SD	107.52 ±19.13	130.9 ±29.73	
CRP (mg/dl)			
Range	12 – 96	6 – 48	0.001
Mean ±SD	62.4 ±28.39	9.81 ±8.98	
ALT (U/L)			
Range	20 – 96	22 – 65	0.054
Mean ±SD	50.5 ±21.42	40.31 ±15.33	
AST (U/L)			
Range	22 – 75	29– 72	0.01
Mean ±SD	43.15 ±13.75	51.95 ±10.34	
Bilirubin (mg/dl)			
Range	0.8 – 3.3	1.3 – 2.8	0.79
Mean ±SD	1.82 ±0.81	1.87 ±0.48	
Albumin (gm/dl)			
Range	2.0 – 3.0	1.7 – 3.0	0.27
Mean ±SD	2.68 ±0.33	2.57 ±0.43	
Creatinine (mg/dl)			
Range	0.8 – 2.7	0.9 – 2.5	0.017
Mean ±SD	1.77 ±0.44	1.19 ±0.42	
RBS (mg/dl)			
Range	110 – 181	112 – 189	0.24
Mean ±SD	141.2 ±38.18	148.4 ±21.66	
Ascitic fluid analysis			
WBCs (cell/cmm)			
Range	250 – 400	5 – 200	
Mean ±SD	296 ±48.55	66.13 ±38.6	0.001
Protein (mg/dl)			
Range	112 - 288	71 – 122	
Mean ±SD	213±28.23	93.5±11.1	0.001
Calprotectin (ng/ml)			
Range	230 – 1080	120 – 920	
Mean ±SD	754.67 ±256.06	280.77 ±230.97	0.001

Hb: hemoglobin, WBC: white blood cell, CRP: C reactive protein. ALT: alanine aminotransaminase, AST: aspartate aminotransferase, RBS: Random blood sugar

In addition, there was a significant increase in ascitic fluid WBCs, protein, and calprotectin in the SBP group vs. the non-SBP group (296 ±48.55 vs. 66.13 ±38.6; 213±28.23 vs. 93.5±11.1 and 754.67±256.06 vs. 280.77±230.97 respectively) **(**Table 3).

There was positive correlation between serum CRP and ascitic fluid proteins and WBCs (r= 0.686, p= 0.001 and r= 0.793, p= 0.001 respectively) (Figure 1).

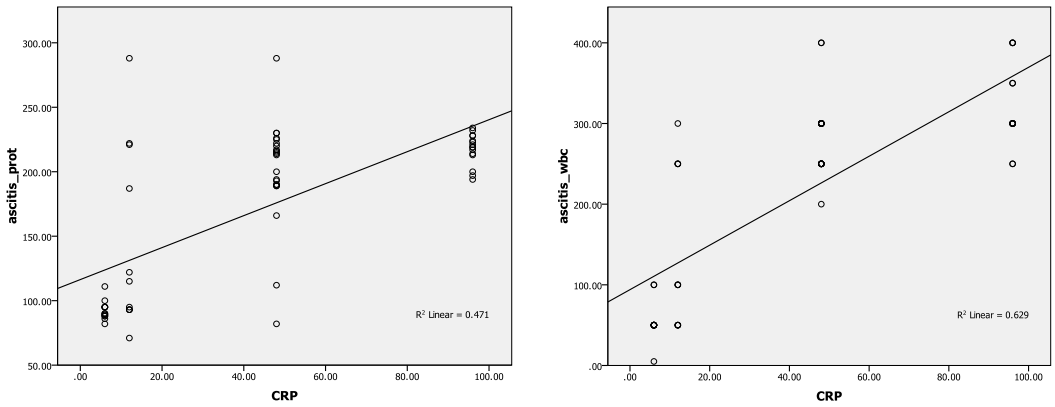

Figure (1): Correlation between serum CRP and ascitic fluid protein and WBCs

Diagnostic value of CRP:

Receiver operating characteristics (ROC) curve for sensitivity and specificity of CRP. At a cutoff value of 30 mg/dl, CRP was shown to have 96% specificity and 90% sensitivity for detecting SBP [Area under the receiver operating characteristics curve (AUC)= 0.91 with negative and positive predictive values (NPV, PPV) for CRP 95% and 70% respectively].

Diagnostic value of ascitic calprotectin:

Receiver operating characteristics (ROC) curve for sensitivity and specificity of calprotectin. At a cutoff value of 270 mg/dl, ascitic fluid calprotectin had 86% specificity and 97.5% sensitivity for detecting SBP [Area under the receiver operating characteristics curve (AUC)= 0.924 with negative and positive predictive values (NPV, PPV) for ascitic calprotectin 96% and 69% respectively].

There was positive correlation between ascitic fluid calprotectin and ascitic fluid proteins and WBCs (r= 0.524, p= 0.001 and r= 0.692, p= 0.001 respectively) (Figure 2).

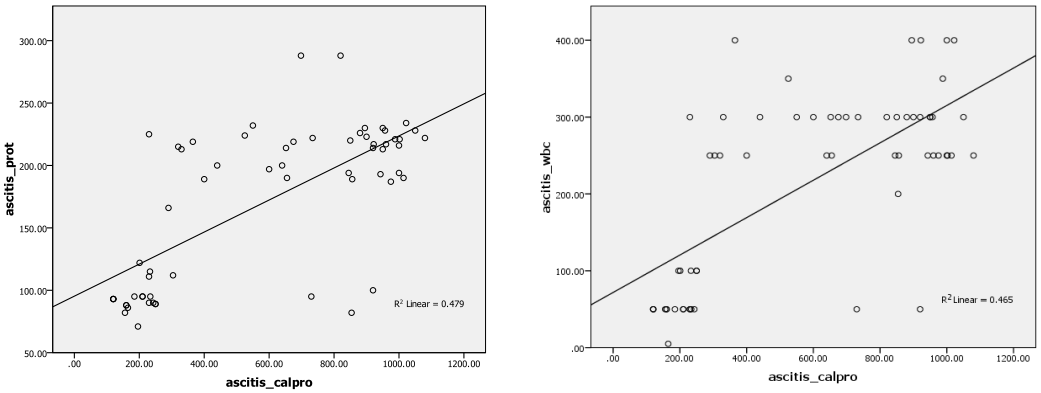

Figure (2): Correlation between ascitic fluid calprotectin and ascitic fluid proteins and WBCs

There was positive correlation between ascitic fluid calprotectin and serum CRP (r =0.793, p= 0.001) (Figure 3).

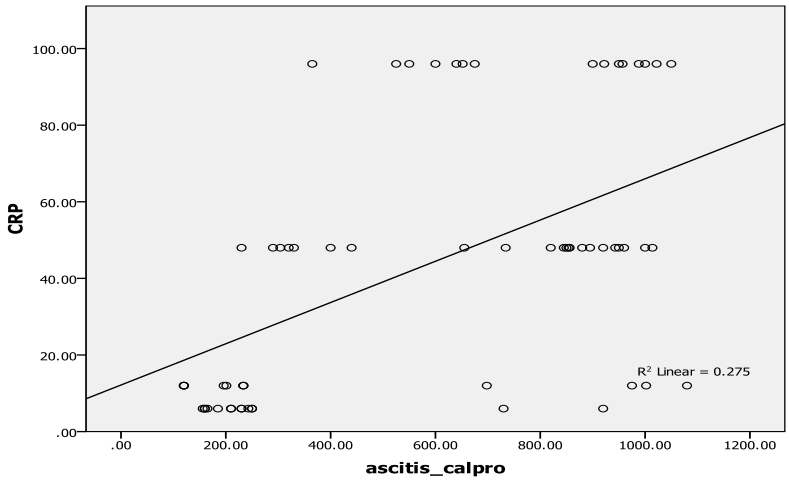

Figure (3): Correlation between ascitic fluid calprotectin and serum CRP

DISCUSSION

Ascites is commonly found in patients with liver cirrhosis and may promote bacterial translocation, enhancing the risk of SBP [1]. SBP in outpatients is rare, but when it occurs it often requires hospitalization to manage to disease course [2].

Spontaneous bacterial peritonitis (SBP) is an important cause of morbidity and mortality in cirrhotic patients with ascites. The diagnosis of SBP is based upon the PMN leukocyte cell count exceeding 250/µL in ascitic fluid, however this procedure is time consuming and subjective. Ascitic calprotectin reliably predicts PMN count >250/µL, which may prove useful in the diagnosis of SBP.

In this prospective observational study which conducted on 124 patients with ascites, most of the patients were in their fourth to sixth decades of life which was consistent with the mean age (49.09±11.3 years) reported by Ajitpal et al. [14]. SBP was found to be more common in males than in females (70% vs. 30%, respectively), a result which is in agreement with Reiberger et al. [15] who similarly reported a 68% male incidence of SBP. The male predominance in our study may be due to higher incidence of bilhariziasis and HCV in our locality.

The most common clinical presentation was fever (70%), followed by abdominal pain (55.7%), abdominal tenderness (48.6%), altered mental status (32.9%), upper GIT bleeding (27.1%) while 30% of patients were asymptomatic (Table 2). These results were consistent with the study conducted by Runyon et al. [16] in which fever was the most common feature (67%), followed by abdominal pain (60%), abdominal tenderness (42%) and encephalopathy (57%). Bandy and Tuttle [17] reported that as many as 30% of patients with paracentesis-proven SBP may be completely asymptomatic.

A significant decrease in hemoglobin levels were found in patients with SBP in comparison with non-SBP patients (9.74±0.81 vs. 10.09±0.69) (Table 3). Syed et al. [18] reported a mean hemoglobin level of 9.6 gm/dl in SBP patients. In the current study, an increase in WBC count was seen in SBP versus non-SBP (10.1±2.8 vs. 6.99±1.7), which is also in agreement with the results reported by Syed et al. [18]. A significant decrease in platelets in the SBP group versus the non-SBP group (107.5±19.1 vs. 130.±29.7) (Table 3) is consistent with findings of the study by Ajitpal et al. [14], who found that platelet count decreased in SBP patients than those without SBP.

The significant increase in creatinine concentration in the SBP group versus the non-SBP group (1.77±0.44 vs. 1.19±0.42, p 0.017). This is in agreement with results of the study conducted by Ajitpal et al. [14] in which the levels of serum creatinine were significantly higher in patients with SBP compared to those without (2.44 ± 0.84 vs. 1.8 ± 1.35, p <0.05).

A significant increase in WBCs and protein in ascitic fluid was reported between the two patient groups (296 ±48.55 vs. 66.13 ±38.6 for WBCs and 213±28.23 vs. 93.5±11.1 for protein) (Table 3). Subhas et al. [19] reported that the highest concentration of protein in ascitic fluid was 1.9gm/dl and the lowest was 0.40gm/dl. Mean ascitic fluid concentration was 0.93 ± 0.44gm/dl. Ascitic fluid analysis at admission by Syed et al. [18] showed mean TLC, polymorphonuclear (PMN) and protein as 903.34±3342/mm3, 411.62 ±1109/mm3 and 1.18±0.746gm/dl respectively. However, our results were much lower than the results reported by the previous studies. This may possibly be due to the difference in immune status as well as etiology of cirrhosis in patients in our study (due to HCV infection), compared to other studies (alcoholic cirrhosis). Runyon [20] demonstrated that cirrhotic patients with ascitic protein concentrations below 1 g/dl were 10 times more likely to develop SBP than individuals with higher concentration.

There was a significant increase in CRP in the SBP group versus the non-SBP group (62.4 ±28.39 vs. 9.81±8.98, p 0.001), with a significant positive correlation being observed between serum CRP and ascitic fluid protein and WBCs count (Figure 1). It was reported that at a cutoff value of 30 mg/dl, CRP had 96% specificity and 90% sensitivity for detecting SBP (Figure 2). Preto-Zamperlini et al [7] reported that the SBP group demonstrated significantly elevated CRP levels, leading to the conclusion that CRP was an independent variable in the prediction of SBP. Being an acute phase reactant, CRP binds to different substrates and stimulates the complements system, has a crucial role in cytokine secretion and increases the phagocytosis of leukocytes. In study by Yildirim, et al. [21] it was founded that CRP was increased in the serum and ascitic fluid of SBP patients.

In this study, ascitic fluid calprotectin was found to be significantly elevated in patients with SBP compared to non-SBP patients (754.67 ±256.06 vs. 280.77 ±230.97, P= 0.001) (Table 3), a result which is consistent with those demonstrated in the studies of Elbanna et al. [22] and Ali et al. [23].

A significant positive correlation was observed between ascitic fluid calprotectin and ascitic fluid protein and WBC count (Figure 3). A study conducted by Ali et al. [23] similarly reported a significant positive correlation between ascitic fluid calprotectin and PMN cell count. Burri et al. [11] reported that ascitic calprotectin levels correlated well and reliably with PMN count. Samples with PMN >250/μL also had higher ascitic calprotectin levels than the samples with PMN ≤250/μL.

The present study demonstrated that at a cutoff value of 270 mg/dl, ascitic fluid calprotectin had 86% specificity and 97.5% sensitivity for detecting SBP (Figure 4). Burri et al. [11] reported that at a cut-off value of 0.63 μg/mL, ascitic calprotectin yielded a sensitivity of 95% and a specificity of 89.2%.

This prospective study evaluated the diagnostic utility of measuring calprotectin in ascites to identify ascitic PMN counts >250/μL in patients referred for paracentesis, and provides the following new information: Patients with an elevated PMN count (>250/μL) had higher ascitic calprotectin levels than those with normal cell counts; this finding indicates that ascitic calprotectin levels correlate well and reliably with PMN count. It is clinically significant that calprotectin levels in ascitic patients can identify elevated PMN counts using ELISA methods. Indeed, ascitic calprotectin may serve as a surrogate marker for PMN count and would be amenable to routine SBP screening, especially when measured by a bedside test.

There are several limitations to the current study that merit consideration. First, we included all patients with ascites, irrespective of the aetiology, and it may be that our results cannot be generalized to all patients with liver cirrhosis. Second, our sample size was small and larger studies are needed to evaluate this test in different clinical settings and to establish a reliable cut-off for ascitic calprotectin for optimal identification of PMN counts >250/μL.

In conclusion, both ascitic fluid calprotectin and serum CRP are significantly elevated in SBP patients in comparison with non-SBP patients. In addition, they also correlate well with the PMN count and protein levels in ascitic fluid and reliably diagnose SBP.

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