**Diagnostic Value of Ascitic Fluid Homocysteine and Calprotectin in**

 **Cirrhotic Patients with Spontaneous Bacterial Peritonitis**

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ABSTRACT

**Background and study aim:** Spontaneous bacterial peritonitis (SBP) is mainly diagnosed by ascitic polymorphonuclear (PMNL) leukocyte greater than 250/mm3. We intended to assess ascitic homocysteine and calprotectin for SBP diagnosis.

**Materials and methods:** In our study, we collected ascitic fluids from 70 patients with liver cirrhosis (46 SBP plus 24 non-SBP according to PMNL>250 cells/mm3). Complete blood count, alanine aminotransferase, aspartate aminotransferase, serum albumin, total bilirubin, prothrombin time, INR, and serum creatinine were measured. Ascitic fluid sample was taken for chemical analysis, homocysteine was calculated in ascites by human homocysteine enzyme-linked immunosorbent assays (ELISA) kits and calprotectin was measured in the ascitic fluids using available human calprotectin enzyme-linked immunosorbent assays (ELISA) kits.

**Results:** SBP patients had considerably greater ascitic homocysteine levels than non-SBP (5.66± 7.15 vs. 2.97±.61 μmol/l) P=0.001. Homocysteine at a cut-off of 3.6 μmol/l had 91.7% specificity, 69.9% sensitivity, PPV 94.1% and NPV 61.1% for SBP diagnosis (area under the curve: 0. 754). SBP patients had considerably greater ascitic calprotectin than the non-SBP (182.98± 76.27 vs. 118.1± 27 ng/mL) P=0.000. Using a cut-off 142 ng/mL, calprotectin had 91.7% specificity, 71.7% sensitivity, PPV 94.2% and NPV 62.9% for SBP diagnosis (area under the curve: 0.768).

**Conclusion:** We found that ascitic homocysteine and calprotectin can be suitable diagnostic markers for SBP diagnosis.

**Keywords:** Calprotectin, Homocysteine, Liver cirrhosis, SBP.

 **INTRODUCTION**

Spontaneous bacterial peritonitis (SBP) is a common consequence in cirrhotic patients. It is about 10 to 30% in cirrhotic patients with ascites at hospitalization and nearly 50% develop along the stay, with a rate of mortality of around 20-30% according to many circumstances [***1***].

SBP means infection of ascites without surgical cause as visceral perforation or intra-abdominal inflammation such as abscess, acute pancreatitis [2].

Patients with cirrhotic ascites must have a diagnostic paracentesis when admitted for the first time and/or if there are signs of infection, encephalopathy, hypotension, GIT hemorrhage, and deterioration of liver or kidney function [3].

It was established that late SBP diagnosis is connected to a higher mortality rate. So, a precise marker for the early SBP diagnosis would be of great help. SBP is diagnosed if ascitic fluid neutrophils are > 250/mm3 [4].

PMNs lysis during transfer to the laboratory can cause false-negative results. The operator's ability

to manually count PMN in ascitic fluid affects the diagnosis [5]. So, finding novel biomarkers may help in SBP diagnosis and treatment [6].

Homocysteine is an amino acid which present in small amounts in human cells. Homocysteine may present as disulfide proteins or freely. Free form is about 1–2%

from the overall homocysteine, while protein-bound homocysteine is about 80%, mainly to albumin [7].

Calprotectin.is.an.acute.phase.inflammatory protein, which has regulatory and antimicrobial functions and related to the neutrophils influx [8]. Ascitic calprotectin can predict polymorphonuclear leukocytes (PMNs) count more than 250/mm3, that can help in SBP diagnosis [9].

All markers for SBP diagnosis present dissimilar sensitivities and specificities and are not routinely applied in all laboratories. So, we have to find a new, dependable, and accessible indicator for SBP diagnosis.

We tried to assess ascitic homocysteine and calprotectin in diagnosis of SBP.

**MATERIALS AND METHODS**

We enrolled 70 cirrhotic ascitic patients in the Tropical Medicine and Gastroenterology Department at Sohag University Hospital from July 2019 to January 2020. We divided them into 2 groups:

 1-Group A: 46 patients diagnosed as SBP with ascitic PMN count equal or more than 250 cells/mm³ [8].

 2- Group B: 24 patients with ascitic PMN count less than 250 cells/mm³ as a control.

* **Inclusion criteria:**

- Cirrhotic patients with ascites.

- Presence of ascites.

* **Exclusion criteria:**

-Other causes of ascites, as tuberculosis, malignancy, and pancreatitis.

* Patients with secondary peritonitis like tuberculosis (TB) or tumors; surgical causes of peritonitis such as intra-abdominal abscess, appendicitis, or pancreatitis; history of abdominal surgery in the previous 3 months
* Other etiologies with high homocysteine as thrombosis, neuropsychiatric diseases, and cancer mainly hepatocellular carcinoma.
* Other etiologies with high calprotectin as inflammatory bowel diseases, intestinal cystic fibrosis, and colorectal cancer.

**Methodology:**

The following was done:

1. Complete history taking and clinical examination.

2. Investigations:

a) Complete blood count (CBC).

b) Liver profile: alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin, total bilirubin, prothrombin time, and INR.

c) Renal profile: creatinine.

3. Abdominal ultrasonography.

4. Paracentesis was done under a strict aseptic condition. We divided the sample into 2 parts; the first for ascitic fluid analysis, and the second was stored at -80⁰ C for homocysteine and calprotectin measurement.

Differential cell count was done by a conventional optical microscope by experienced physicians in Clinical Pathology Department.

SBP diagnosis was if PMN equal or more than 250 cells/m3 in the ascitic fluid without secondary peritonitis [10]***.***

**Laboratory-based quantitative homocysteine and calprotectin measurement**

Ascitic homocysteine was tested by Human Homocysteine enzyme-linked immunosorbent assays (ELISA) kits (SinoGeneClon Biotech Co., Ltd, China).

Ascitic calprotectin was tested using commercially available Human Calprotectin (CALP) ELISA kits (SinoGeneClon Biotech Co., Ltd, China). That was done using a Mindray (MR-96A) machine.

**Ethical consideration:**

**The study was approved by the Ethics Board of Sohag. Acceptance of the trial was contingent on each patient signing an informed written permission form. This research was carried out in conformity with the World Medical Association's Code of Ethics (Declaration of Helsinki) for human studies.**

***Statistical analysis***

Statistical analysis was by the Statistical Package for the Social Sciences (SPSS 17; SPSS Inc., Chicago, IL, USA) software. Quantitative data were represented as the mean, standard error of the mean (SE). The Student T-test was used to compare quantitative data. The Chi-square test was used to compare qualitative data provided as numbers and percentages. The sensitivity, specificity, positive, and negative predictive values produced from the receiver operating characteristic (ROC) curve were used to examine the data. The area under the ROC curve (AUC) was used to measure the diagnostic accuracy of homocysteine and calprotectin in predicting SBP. Graphs were done by Excel. P-value was significant if < 0.05.

**RESULTS**

**Patient characteristics:** 70 patients were enrolled in the study: 46 (65.7%) of them had SBP. 9 patients (12.9%) died within admission. Most patients were female (52.9%) with a mean age of (57.3 ± 11.72) years **(Tables 1 and 2).**

**Table (1)** Clinical characteristics of cirrhotic patients with ascites included in the study

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameters** |  | **N=70 (100%)** | **Parameters** | **N=70 (100%)** |
| **Age (year)** | **Mean ±SE** | 57.3± 11.7 | **SBP rate** | 46 (65.7) |
| **Gender** | **Male**  | 33 (47.1) | **Abdominal pain** | 29 (41.4) |
| **Female** | 37 (52.9) | **Fever** | 21 (30) |
| **Hepatic encephalopathy** | **No** | 20 (28.6) | **Abdominal tenderness** | 13 (18.6) |
| **1** | 35 (50) | **Refractory ascites** | 53 (75.7) |
| **2** | 10 (14.3) | **Hepatorenal syndrome** | 4 (5.7) |
| **3** | 4 (5.7) | **Ascites turbidity** | 19 (27.1) |
| **4** | 1 (1.4) | **Mortality rate**  | 9 (12.9) |
| **Amount of ascites**  | **Mild** | 5 (7.1) |  |  |
| **Moderate** | 49 (70) |  |  |
| **Marked** | 16 (22.9) |  |  |
| **Child score** | **A** | 0 (0) |  |  |
| **B** | 16 (22.9) |  |  |
| **C** | 54 (77.1) |  |  |

**Table (2)** Laboratory characteristics of cirrhotic patients with ascites included in the study

|  |  |  |  |
| --- | --- | --- | --- |
| **Parameters** | **Mean ±SE** | **Parameters** | **Mean ±SE** |
| **WBCs (10^3 cells/ mm3)** | 9.34± 2.86 | **MELD score** | 17.77± 4.5 |
| **Platelets (10^3 cells/ mm3)** | 137.2± 27.8 | **Child-Pugh score** | 10.88± 1.73 |
| **RBCs (10^6 cells/ µL)** | 3.35± 0.82 | **Ascitic WBCs count (cells/ mm3)** | 1506.5± 215.1 |
| **Hb (g/dL)** | 9.63± 2.08 | **Ascitic PMNL count (cells/ mm3)** | 1193.1± 237.7 |
| **Serum Na (mmol/L)** | 126.3± 9.96 | **Ascitic protein (g/dL)** | 1.27± 0.31 |
| **Serum K (mmol/L)** | 4.03± 0.95 | **Ascitic albumin (g/dL)** | 0.44± 0.1 |
| **Serum RBS (mg/dL)** | 121± 30.05 | **Ascitic homocysteine (μmol/l)** | 4.74± 0.92 |
| **Serum total bilirubin (mg/dL)** | 4.37± 0.91 | **Ascitic calprotectin (ng/mL)** | 160.74± 30.69 |
| **Serum protein (g/dL)** | 5.06± 1.34 |  |  |
| **Serum albumin (g/dL)** | 2.28± 0.52 |  |  |
| **Serum ALT (U/L)** | 37.4± 3.3 |  |  |
| **Serum AST (U/L)** | 74.97± 4.2 |  |  |
| **Serum PT (seconds)** | 17.3± 2.94 |  |  |
| **Serum INR (ratio)** | 1.41± 0.22 |  |  |
| **Basal serum creatinine (mg/dL)** | 1.70± 0.05 |  |  |
| **Serum creatinine before discharge (mg/dL)** | 1.52± 0.3 |  |  |
| **Maximum serum creatinine (mg/dL)** | 1.80± 0.09 |  |  |
| **Renal impairment N (70)** | 28 (40%) |  |  |

SE: standard error, WBCs: white blood cells, RBCs: red blood cells, Hb: Haemoglobin, RBS: random blood sugar, ALT: alanine aminotransferase, AST: aspartate aminotransferase, PT: prothrombin time, INR: international normalized ratio, PMNL: polymorphnuclear cells

**Diagnosis of SBP:**

Patients were divided into two groups depending on ascitic fluid analysis: a SBP group of 46 patients (65.7%) (20 males and 26 females) with a mean age of 57.33± 12.43 years and a non-SBP group of 24 patients (34.3%) (13 males and 11 females) with a mean age of 57.25 ± 10.47 years.

The age and gender differences between the SBP and non-SBP groups were not significant. Also, difference in the clinical picture: fever, abdominal pain, tenderness, or encephalopathy or amount of ascites wasn’t significant (**Table 3**).

**Table (3)** Clinical characteristics of SBP group versus non-SBP

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Parameters** |  | **SBP (n=46)** | **Non-SBP (n=24)** | **P value** | **Parameters** | **SBP (N=46)** | **Non-SBP (N=24)** | **P value** |
| **Age (year)** |  | 57±12.4 | 57.±10.5 | 0.980 | **Abdominal pain** | 18 (39.1) | 11 (45.8)  | 0.589 |
| **Gender** | **Male**  | 20 (43.5) | 13 (54.2) | 0.395 | **Fever** | 16 (34.8) | 5 (20.8) | 0.227 |
| **Female** | 26 (56.5) | 11 (45.8) | **Abdominal tenderness** | 10 (21.7) | 3 (12.5) | 0.345 |
| **Hepatic encephalopathy** | **No** | 14 (30.4) | 6 (25) | 0.748 | **Refractory ascites** | 33 (71.7) | 20 (83.3) | 0.283 |
| **1** | 23 (50) | 12 (50) | **Hepatorenal syndrome** | 4 (8.7) | 0 (0) | 0.137 |
| **2** | 5 (10.9) | 5 (20.8) | **Ascites turbidity** | 18 (39.1) | 1 (4.2) | 0.002\* |
| **3** | 3 (6.5) | 1 (4.2) | **Mortality rate**  | 7 (15.2) | 2 (8.3) | 0.414 |
| **4** | 1 (2.2) | 0 (0) |  |  |  |  |
| **Amount of ascites**  | **Mild** | 4 (8.7) | 1 (4.2) | 0.767 |  |  |  |  |
| **Moderate** | 32 (69.6) | 17 (70.8) |  |  |  |  |
| **Marked** | 10 (21.7) | 6 (25) |  |  |  |  |
| **Child score** | **A** | 0 (0) | 0 (0) | 0.316 |  |  |  |  |
| **B** | 13 (28.3) | 3 (12.5) |  |  |  |  |
| **C** | 33 (71.7) | 21 (87.5 |  |  |  |  |

\*:P value is significant, Quantitative data are presented as mean and standard error

There was no significant difference in WBCs, Hb, platelets, liver enzymes, bilirubin, creatinine, Child score, and ascites protein between the 2 groups. But we had a considerable difference in ascites WBCs, Polymorph number, and ascites turbidity and a considerable rise in homocysteine and calprotectin (**Table 4**).

**Table (4)** Laboratory characteristics of SBP group versus non-SBP

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Parameters**Mean± SE | **SBP (n=46)**  | **Non-SBP (n=24)** | **P value** | **Parameters** | **SBP (N=46)**  | **Non-SBP (N=24)** | **P value** |
| **WBCs (10^3 cells/ mm3)** | 9.53± 2.06 | 8.97± 1.54 | 0.650 | **Renal impairment** | 15 (32.6) | 13 (54.2) | 0.081 |
| **Platelets (10^3/ mm3)** | 128± 5.3 | 154.5±31.9 | 0.233 | **MELD score** | 17.43± 4.6 | 18.4± 4.3 | 0.599 |
| **RBCs (10^6 cells/ mm3)** | 3.39±0.83 | 3.27± 0.83 | 0.566 | **Child-Pugh score** | 10.8± 1.84 | 11± 1.5 | 0.590 |
| **Hb (g/dL)** | 9.7± 2.28 | 9.5± 1.67 | 0.699 | **Ascitic WBCs count (cells/ mm3)** | 2215± 22 | 1487±15 | 0.001\* |
| **Serum Na (mmol/L)** | 126.8±8.9 | 126.4±11.9 | 0.869 | **Ascitic PMNL count (cells/ mm3)** | 17945±272 | 401.6± 42 | 0.002\* |
| **Serum K (mmol/L)** | 3.93±0.94 | 4.23±0.96 | 0.210 | **Ascitic protein (g/dL)** | 1.35± 0.04 | 1.13±0.03 | 0.302 |
| **Serum total bilirubin (mg/dL)** | 4.57± 0.6 | 3.98± 0.46 | 0.738 | **Ascitic albumin (g/dL)** | 0.45± 0.09 | 0.41±0.04 | 0.632 |
| **Serum protein (g/dL)** | 5.05± 1.42 | 5.1± 1.19 | 0.892 | **Ascitic Homocysteine (μmol/l)** | 5.66± 0.15 | 2.97±0.1 | 0.001\* |
| **Serum albumin (g/dl)** | 2.33±0.48 | 2.17±0.59 | 0.244 | **Ascitic Calprotectin (ng/mL)** | 182.9± 6.3 | 118.1± 207 | <0.001\* |
| **Serum ALT (U/L)** | 32.6± 6.2 | 46.7± 4 | 0.093 |  |  |  |  |
| **Serum AST (U/L)** | 68.3± 5.7 | 87.8±14.9 | 0.299 |  |  |  |  |
| **Serum PT (seconds)** | 16.9± 2.4 | 18.1± 3.72 | 0.128 |  |  |  |  |
| **Serum INR (ratio)** | 1.39±0.2 | 1.45±0.27 | 0.311 |  |  |  |  |
| **Basal serum creatinine (mg/dL)** | 1.68± 0.15 | 1.72±0.3 | 0.880 |  |  |  |  |
| **Serum creatinine before discharge (mg/dL)** | 1.5±0.04 | 1.55±0.09 | 0.834 |  |  |  |  |
| **Maximum serum creatinine (mg/dL)** | 1.78± 0.2 | 1.85±0.06 | 0.796 |  |  |  |  |

SE: standard error, WBCs: white blood cells, RBCs: red blood cells, Hb: Haemoglobin, ALT: alanine aminotransferase, AST: aspartate aminotransferase, PT: prothrombin time, INR: international normalized ratio, PMNL: polymorphnuclear cells. \*:P value is significant, Data are presented as mean and standard error

We found from **table (5)** that at a cut-off value of 3.6 μmol/l, the sensitivity, specificity, negative predictive values (NPV), and positive predictive values (PPV) of homocysteine were (69.9%, 91.7%, 61.1%, and 94.1.% respectively) in SBP diagnosis (AUC=0.754). On the other hand, with using cut-off 142 ng/ml for calprotectin, sensitivity, specificity, NPV, and PPV were (71.7%, 91.7%, 62.9%, and94.2% respectively) (AUC=0.768).

Using both homocysteine at a cut-off value of 3.6 μmol/l and calprotectin with a cut-off 142 ng/mL, the sensitivity, specificity, NPV, and PPV were (71.7%, 91.7%, 61.3%, and 87.2% respectively) (AUC=0.761) **(Table 5).**

**Table (5)** Sensitivity, specificity, NPV, PPV of homocysteine, calprotectin and homocysteine and calprotectin together for diagnosis of SBP

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Parameters** | **AUC** | **Sensitivity** | **Specificity** | **PPV** | **NPV** | **95% CI** | **P value** |
| **Homocysteine≥3.6 μmol/l** | 0.754 | 69.9% | 91.7% | 94.2%  | 61.1% | 0.639- 0.869 | 0.001 |
| **calprotectin≥ 142 ng/mL** | 0.768 | 71.7% | 91.7% | 94.2% | 62.9%  | 0.656- 0.880 | <0.001 |
| **Homocysteine and calprotectin** | 0.761 | 71.7% | 91.7% | 87.2% | 61.3% | 0.648- 0.874 | <0.001 |

AUC: Area under curve, PPV: Positive predictive values, NPV: Negative predictive values, CI: Confidence interval

**DISCUSSION**

Diagnosing SBP still depends on ascitic PMNL cells, which is not always accessible clinically [11]. So, there is a need for new diagnostic markers for SBP diagnosis [7]. In our study, we investigated ascitic homocysteine and calprotectin for SBP detection.

The difference in age and gender among SBP and non-SBP groups wasn’t significant, which was like **Abdel-Razik *et al.*** [7,12]**, Makhlouf *et al.*** [13], and **Nasereslami *et al.*** [14].

Abdominal discomfort was the most common presentation in SBP patients (39.1%) then fever (34.8%) and abdominal tenderness (21.7%). 15 (32.6%) of the patients had renal impairment. While **Makhlouf *et al.*** [13] found abdominal discomfort (89.8.7%), then fever (65.3%) and abdominal tenderness (55.1%) with (30.6%) renal impairment. **Sideris *et al.*** [15] also found that the most clinical presentation was abdominal pain (75.3%) followed by fever (6.3%).

We found no difference between the 2 groups in the clinical picture: fever, abdominal pain, tenderness in SBP prediction which was like what **Sideris *et al.*** [15] reported, but against what **Makhlouf *et al.*** [13] found. This could be due to the low specificity of the abdominal pain value for SBP prediction, as ascites buildup can generate stomach pain on its own. Also, fever could be due to other infections in cirrhotic patients [15]**.** Also, there was no significant difference in hepatic encephalopathy, amount of ascites, or mortality rate between the 2 groups which was like **Makhlouf *et al.*** [13] result.

There was no significant difference in WBCs, Hb, platelets, liver enzymes, bilirubin, creatinine, Child score, MELD, and ascitic protein between the 2 groups. This goes completely with the result of **Abdel-Razik *et al.*** [7] and was like **Abdel-Razik *et al.*** [12] result except that they found ALT and serum creatinine were significant.

There was a considerable difference in ascites turbidity which agreed with what **Makhlouf *et al.*** [13] reported (p=0.002). Also, ascitic WBCs and polymorph were considerable, which was like **Abdel-Razik *et al.*** [7,12]**, Makhlouf *et al.*** [13], and **Lutz *et al.*** [16].

Homocysteine (Hcy) was discovered to be a potential marker [7]. By using ascitic Hcy for diagnosing SBP, with a cut-off of 3.6 μmol/l, we got a sensitivity of 69.9%, a specificity of 91.7%, PPV 94.2%, and NPV 61.1%. We also have higher specificity and PPV but less sensitivity and NPV in comparison with **Abdel-Razik *et al.*** [7] the first to study Hcy, who got a sensitivity of 92.7%, a specificity of 84.4%, PPV 58.1%, and NPV 98.7% with a cut-off of 16.1 μmol/l.

The importance of calprotectin is not clear, but it has an anti-microbial function [17]. We found that ascitic calprotectin was considerably higher in SBP than non-SBP group. This goes with those established by **Heikl *et al.*** [8], **Burri *et al***. [9], **Abdel-Razik *et al.*** [12], **Makhlouf *et al.*** [13] **Elbanna *et al.*** [18], **Ghweil *et al.*** [19], **Fernandes *et al*.** [20], and **Selim *et al.*** [21].

We found that ascitic calprotectin at a cut-off of 142 ng/mL had 71.7% sensitivity, 91.7% specificity, PPV 94.2%, and NPV 62.9% for SBP diagnosis. Our cut-off value is near that of **Fernandes *et al.*** [20], which was 157 ng/mL with sensitivity, specificity, PPV, and NPV (87.8%, 97.9%, 97.3%, and 90.2% respectively). While **Abdel-Razik *et al.*** [12] reported that with using a cut-off 445 ng/mL ascitic calprotectin showed 95.4% specificity and a sensitivity of 85.2% with PPV 71% and NPV 93%. **Burri *et al.*** [9] stated that with a cut-off of 630 ng/mL, calprotectin showed 95% sensitivity, 70% specificity, 60% PPV, and 90% NPV. **Selim *et al.*** [21] showed that the sensitivity, specificity, PPV, and NPV of calprotectin were (90.91%, 95.45%, 95.2%, and 91.3%) with cut-off value of 620 ng/ml.

We tried in this study to assess using both homocysteine and calprotectin in SBP diagnosis. We found that using ascitic Hcy and calprotectin together for SBP diagnosis with a cut-off of (3.6 μmol/l and142 ng/mL respectively) showed 71.7% sensitivity, 91.7% specificity, 87.2% PPV, and 61.3% NPV. This is equal to sensitivity and specificity of calprotectin alone (71.7% and 91.7%) but with less PPV (87.2% vs 94.2%) and NPV (61.3%vs 62.9%). This is also equal to specificity of Hcy alone (91.7%), but with slightly higher sensitivity (71.7% vs 69.9%) and NPV (61.3% vs 61.1%) and less PPV (87.2% vs 94.2%). This means using both homocysteine and calprotectin doesn’t add much to using any of them alone.

But unfortunately, we had some limitations. First, we had a relatively small sample size. So, we need larger samples to evaluate homocysteine and calprotectin in different settings for SBP diagnosis. Second, we need to study a group of cirrhotic patients with other infections to judge if homocysteine and calprotectin are specific for SBP.

**CONCLUSION**

In conclusion, SBP patients had significantly greater levels of ascitic homocysteine and calprotectin than non-SBP patients with liver cirrhosis. So, it can be used as dependable markers to diagnose SBP.

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