Does ascitic fluid lactoferrin has a role in the diagnosis and follow up of spontaneous bacterial peritonitis in hepatitis C virus cirrhotic patients

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Background
Polymorphonuclear leukocyte (PMNL) count in the ascitic fluid (AF) is the gold standard method for the diagnosis of spontaneous bacterial peritonitis (SBP). Its measurement is routinely performed by traditional manual counting which is operator dependent and false-negative results may occur due to lysis of the leukocyte during transport.
Aims
The aims of the study were to assess the accuracy of AF lactoferrin and the best cutoff value for the diagnosis of SBP and also to compare its level before and after treatment to be used as a marker for follow up.
Patients and methods
The present study included 150 Egyptian patients with hepatitis C virus-related liver cirrhosis and ascites. The cases were divided into 100 patients with SBP and 50 patients with no SBP based on an elevated AF PMNL count of greater than or equal to 250 cells/mm^3, ascitic samples were examined for PMNL count, culture, chemistry, and lactoferrin concentration in non-SBP patients and in SBP patients before and after systemic antibiotic treatment.
Results
AF lactoferrin concentration is significantly higher in SBP patients than in non-SBP patients with a cutoff value of 75.55 ng/ml, significantly higher in culture positive than in culture-negative SBP patients and its concentration is decreased significantly in SBP patients after systemic antibiotic therapy.
Conclusion
Elevated AF lactoferrin levels in cirrhotic patients are reliable for the diagnosis and follow up of SBP after systemic antibiotic therapy. The level of AF lactoferrin level is higher in resistant cases of SBP than the cases that respond to systemic antibiotic treatment.

Keywords:
antibiotics, ascites, ascitic lactoferrin, spontaneous bacterial peritonitis

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Introduction

Spontaneous bacterial peritonitis (SBP) diagnosis is established when the ascitic fluid (AF) polymorphonuclear leukocyte (PMNL) count is greater than or equal to 250 cells/ml [1]. Lysis of the PMNL during transport to the laboratory may lead to false-negative results. Manual measurement of the AF PMNL is operator dependent, makes the quality control difficult, and can delay the diagnosis [2].

Lactoferrin is an iron-binding protein found in the specific granules of PMNL that is released on degranulation [3]. AF lactoferrin levels may be a reliable marker for the detection of SBP [4]. We aimed to evaluate the value of AF lactoferrin in the diagnosis of SBP and follow up after systemic antibiotic therapy.

Patients and methods

This study was an analytical cross-sectional study that included 150 Egyptian ascitic patients with liver cirrhosis due to the hepatitis C virus recruited from the inpatient ward and the outpatient clinic of Internal Medicine at Cairo University hospital during the period from July 2016 to February 2017. The protocol was approved by the ethics committee of Cairo University and informed consent was obtained from all participants.

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The patients were divided into two groups.

(1) Group A, which included 100 patients with SBP based on clinical picture and AF PMNL greater than or equal to 250/mm^3, not started systemic antibiotic treatment yet.
(2) Group B, which included 50 patients without SBP based on clinical picture and AF PMNL of less than 250/mm^3.

Exclusion criteria

(1) Ascitic patients due to any other cause (malignancy including HCC, cardiac, or tuberculosis) excluded by history, laboratory, and radiological findings.
(2) Patients with hemorrhagic ascites.
(3) History of system antibiotics in the previous 2 weeks.

All patients were subjected to the following.

Full detailed medical history including age, occupation and residence, history of drug intake including antibiotics, associated diseases, and clinical examination.

Abdominal ultrasonography for the assessment of liver and spleen, presence or absence of hepatic focal lesions, portal, hepatic and splenic veins diameter, and the degree of ascites and echogenicity.

The following laboratory investigations were carried out including urea, creatinine, aspartate aminotransferase, alanine aminotransferase, bilirubin, serum albumin, alkaline phosphatase, γ-glutamyltransferase (GGT), and lactic dehydrogenase (LDH) were measured using AU 480 (Beckman Coulter, California, USA) using its commercially available reagents, complete blood picture was carried out by Cell Dyn 3500 (Spectra Group, California, USA). Prothrombin time, concentration, and international normalized ratio were estimated.

Paracentesis was performed under strict sterile conditions in supine position guided by abdominal ultrasonography with the sample of AF being withdrawn and divided into:

(1) Sample for PMNL and total leukocyte count were collected in a heparin anticoagulant tube. Differential cell count and cytology were examined with a conventional optical microscope. A manual cell count with differential study was performed for all samples by experienced technicians.
(2) Sample for biochemical tests (glucose, protein, albumin, and LDH) and serum-ascites albumin gradient were calculated for each sample by calculating the difference between serum albumin and ascitic albumin.
(3) Culture samples were seeded at bedside with the inoculation of AF into aerobic and anaerobic media blood culture bottles (BACTEC, Gerresheimer Moulded Glass Gmbh, Germany).
(4) Lactoferrin levels in ascitic sample were quantified using a human lactoferrin enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Bethyl Laboratories Inc., Montgomery, Texas, USA). The lactoferrin concentrations in the test samples were then quantified by interpolating their absorbance from the standard curve generated in parallel with the samples. After factoring for sample dilutions, lactoferrin concentrations in the original sample were calculated. Lactoferrin levels were measured in SBP patients before and after systemic antibiotics for 2 weeks.

Statistical methods

Data were coded and entered using the SPSS (Statistical Package for the Social Sciences; SPSS Inc., Chicago, Illinois, USA) version 24. Data were summarized using mean, SD, median, minimum, and maximum in quantitative data and using frequency and percentage for categorical data. A *P* value of less than 0.05 indicated statistical significance. Differences between the groups were evaluated by an independent sample *t*-test and a χ^2-test. Receiver operating characteristic curve was constructed with area under curve analysis performed to detect the best cutoff value of AF lactoferrin for the detection of SBP.

Results

The present study included 150 Egyptian hepatitis C virus cirrhotic patients with ascites; 81 (54%) male patients and 69 (46%) female patients. Their ages ranged from 45 to 87 years with an average of 64.28 ±8.44 years divided into 100 patients with SBP (group A) and 50 patients with ascites but without SBP (group B) as a control group.

There was no statistically significant difference in age and sex distribution between the two groups (*P*=0.125, 0.728).

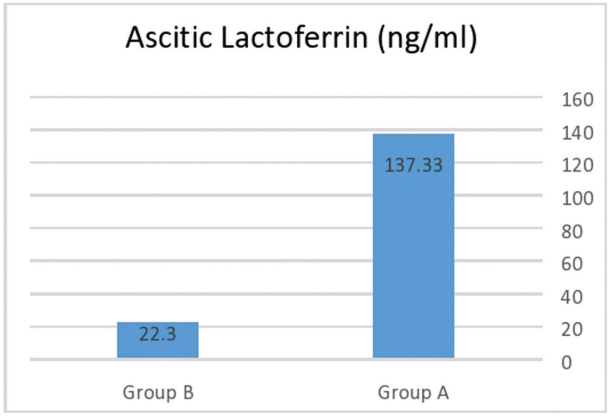
The main laboratory parameters included in our study are shown in Table 1. We found statistically significant higher levels of alanine aminotransferase (74.05±50.95), aspartate aminotransferase (56.37±43.65), total protein (4.82±0.53), and international normalized ratio (1.75 ±0.54) in group A than in group B with a *P* value of less

Table 1 Mean and SD of the main laboratory parameter among the groups included in our study

	Group A (n=100) (mean±SD)	Group B (n=50) (mean±SD)	P value
TLC (×1000 cells/ml)	8.63±4.92	6.80±1.95	0.249
Hemoglobin (g/dl)	7.98±1.33	7.92±1.43	0.915
Platelets (×1000/ml)	91.87±17.40	92.46±16.85	0.843
ALT (U/l)	74.05±50.95	31.88±18.02	<0.001
AST (U/l)	65.37±43.65	27.12±11.63	<0.001
Total bilirubin (mg/dl)	2.37±2.74	2.78±4.21	0.542
Direct bilirubin (mg/dl)	1.44±2.77	1.45±2.44	0.904
Serum albumin (g/dl)	2.43±0.32	2.42±0.35	0.805
Total protein (g/dl)	4.82±0.53	5.29±0.57	<0.001
INR	1.75±0.54	1.38±0.51	<0.001
Urea (mg/dl)	36.13±15.37	37.64±15.51	0.570
Creatinine (mg/dl)	0.92±0.47	0.92±0.48	0.980
Ascitic PMNL (cells/mm^3)	374.90±122.48	72.10±43.19	<0.001
Ascitic lactoferrin (ng/ml)	137.33±33.19	22.30±18.84	<0.001
Ascitic albumin (g/dl)	0.50±0.16	0.36±0.16	<0.001
Ascitic total protein (g/dl)	3.61±0.24	2.02±0.25	<0.001
Ascitic glucose (mg/dl)	48.67±31.92	107.66±65.46	<0.001
Ascitic LDH (mg/dl)	92.55±36.44	74.72±42.48	<0.001
SAAG	1.9±0.35	2.1±0.4	0.004

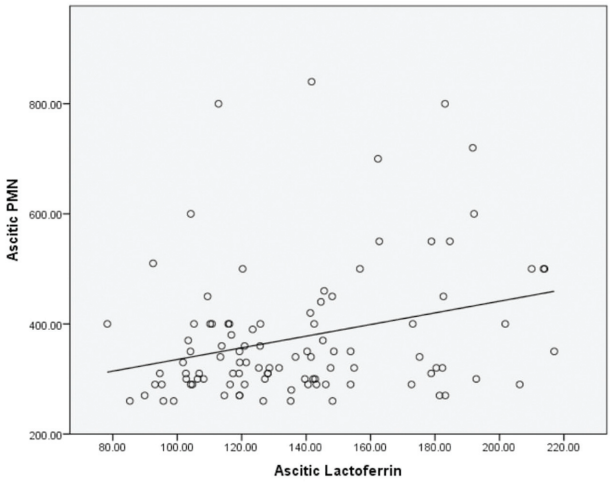
ALT, alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio; LDH, lactic dehydrogenase; PMNL, polymorphonuclear leukocyte; SAAG, serum-ascites albumin gradient; TLC, total leukocyte count.

Figure 1



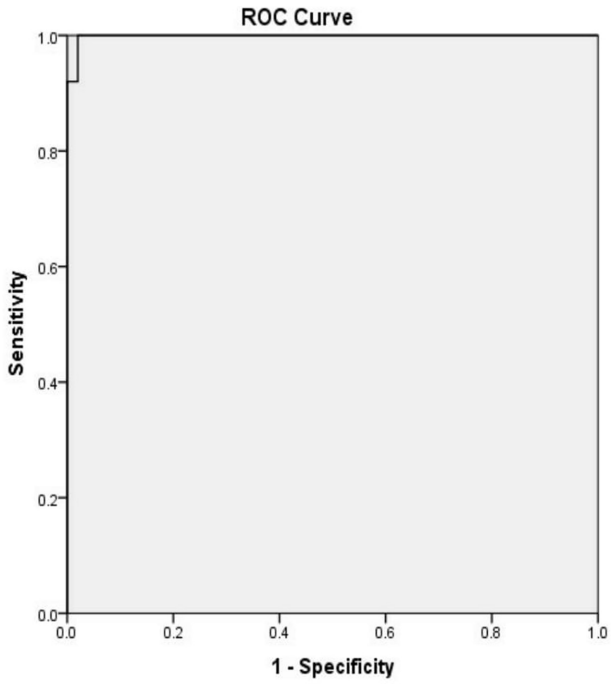
The mean ascitic lactoferrin concentration among SBP and non SBP groups.

Figure 2



Correlation between ascitic lactoferrin and ascitic PMNL.

Figure 3



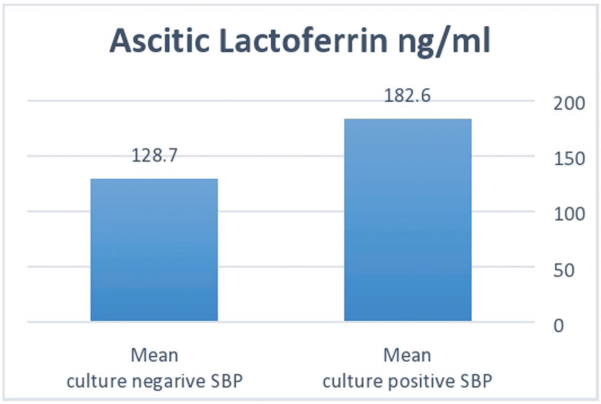
Roc curve for ascitic lactoferrin (AUC 0.998; CI 95%, 0.995-1.000).

than 0.001. Ascitic PMNL, ascitic albumin, ascitic total protein, ascitic glucose, and ascitic LDH were significantly higher in group A than in group B (P<0.001).

The mean AF lactoferrin concentration was significantly higher in group A (137.33±33.19 ng/ml) than in group B (22.30±18.84 ng/ml; P<0.001; Fig. 1).

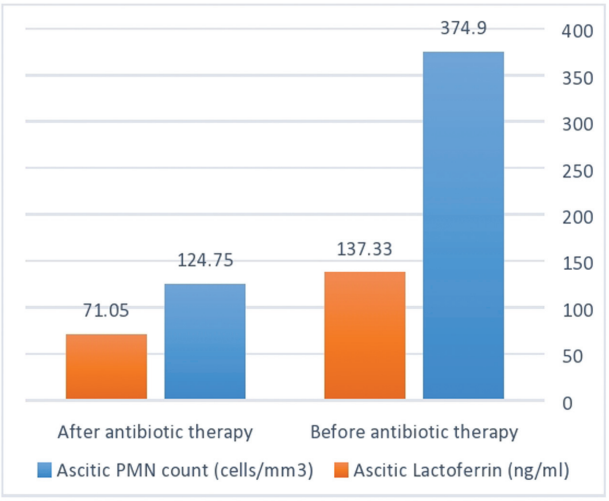
There was a significant positive correlation between AF lactoferrin levels and AF PMNL (P=0.010; r=0.255; Fig. 2). There was no statistically significant correlation between AF lactoferrin and ascitic albumin, ascitic total protein, ascitic glucose, ascitic LDH, or serum-ascites albumin gradient.

Figure 4



Mean of AF lactoferrin concentration in both culture positive and culture negative of SBP patients.

Figure 5



Ascitic lactoferrin and Ascitic PMNL before and after antibiotic treatment in SBP patients.

Table 2 Comparison between asciticfluid lactoferrin in spontaneous bacterial peritonitis patients (group A) before and after antibiotic therapy

	Before starting antibiotic (mean±SD)	After antibiotic therapy (mean±SD)	*P* value
Ascitic PMNs	374.90±122.48	124.75±78.31	<0.001
Ascitic lactoferrin	137.33±33.19	71.05±25.30	<0.001

PMNs, polymorphnuclear leukocyte.

The receiver operating characteristic curve showed that the cutoff value of AF lactoferrin for the diagnosis of SBP was 75.55 ng/ml with a sensitivity of 100% and specificity of 98% (Fig. 3).

According to the ascitic culture, group A (100 patients with SBP) were subdivided into two subgroups, culture-positive patients (16 patients, 16%) and culture-negative patients (84 patients, 84%). There was a statistically significant higher level of AF lactoferrin in culture-positive patients (182.6 ±32.70 ng/ml) than culture-negative patients (128.7 ±25.5 ng/ml; $P<0.001$; Fig. 4).

The patients of group A (100 SBP patients) started antibiotic therapy (third-generation cephalosporin); then reanalysis of AF was done as regards lactoferrin and PMNL count (Table 2).

AF lactoferrin after antibiotic therapy (71.05±25.3 ng/ml) was significantly lower than before antibiotic therapy (137.33±33.19 ng/ml; $P<0.001$).

Ascitic PMNL count after antibiotic therapy (71.05 ±25.3 cells/mm^3) was significantly lower than before antibiotic therapy (374.9±122.48 cells/mm^3; $P<0.001$; Fig. 5).

This means a significant effect of antibiotic therapy on the levels of AF lactoferrin and ascitic PMNL count. So, AF lactoferrin can be used for the follow up of SBP patients after antibiotic therapy.

Out of the 100 patients with SBP, there were only four (4%) patients whose ascitic PMNL count after antibiotic therapy was still greater than or equal to 250 cells/mm^3 (resistant cases), whereas in the other 96 (96%) patients, the ascitic PMN count decreased to less than 250 cells/mm^3 (responders). AF lactoferrin after antibiotic therapy was significantly higher in resistant cases (102.275±27.6 ng/ml) than in responders (69.45±24.5 ng/ml; $P=0.010$; Table 3).

We noticed also that AF lactoferrin in resistant cases after antibiotic therapy (102.275±27.6 ng/ml) was still higher than the cutoff value for the diagnosis of SBP (75.55 ng/ml). That result gives a higher diagnostic value for AF lactoferrin that it could be used to monitor the resistant cases of SBP.

Discussion

Ascites is the most common complication of cirrhosis in patients with liver disease and can become infected without any apparent intra-abdominal source of infection, a condition called SBP [5].

SBP is an important cause of morbidity and mortality. Diagnostic paracentesis is used commonly in cirrhotic patients with ascites to investigate the presence of SBP in symptomatic and asymptomatic patients. According

Table 3 Ascitic lactoferrin concentration in spontaneous bacterial peritonitis patients after systemic antibiotic therapy

	SBP patients (100 patients)		*P* value
	Resistant cases [4 (4%) patients] (mean±SD)	Responders [96 (96%) patients] (mean±SD)	
Ascitic lactoferrin After antibiotic therapy (ng/ml)	102.275±27.6	69.45±24.5	0.010

SBP, spontaneous bacterial peritonitis.

to the current guidelines, a diagnosis of SBP is established when the AF PMNL count is 250 cells/ml or greater [6].

Lysis of PMNs during transport to the laboratory may lead to false-negative results. Manual measurement of the AF PMNL count is operator dependent, makes quality control difficult, and can delay the diagnosis [2].

Lactoferrin is an iron-binding protein found in human mucosal secretions as well as in the specific granules of PMNL that is released on degranulation. Lactoferrin concentration is proportional to the degranulation of PMNL and can be used as a marker of inflammation [7]. Measurement of AF lactoferrin levels may be a reliable marker for the presence of PMNL and, so that, detection of SBP in patients with cirrhosis [4]. Patients with cirrhosis have an increased risk of developing bacterial infection, followed by sepsis and death. Infection either is present at admission or develops during hospitalization in ~25–35% of patients [7].

In this study, AF lactoferrin concentrations were assessed in cirrhotic patients with SBP and non-SBP and were assessed before and after systemic antibiotic treatment for SBP patients. We noted that AF lactoferrin was significantly higher in SBP patients (137.33±33.19 ng/ml) than non-SBP patients (22.30±18.84 ng/ml) with a *P* value less than 0.001. This result agrees with the results of previous studies which showed a higher level of ascetic lactoferrin in SBP patients than in non-SBP patients [8–10]. Our study showed that there was no statistically significant difference between male and female patients as regards ascitic lactoferrin levels in both SBP patients and non-SBP patients. This means that there is no effect of sex on ascitic lactoferrin level.

In our study the best cutoff value of AF lactoferrin for the diagnosis of SBP was 75.55 ng/ml with a sensitivity of 100% and specificity of 98%. Previous studies tried to reach to a standard cutoff value of AF lactoferrin for the diagnosis of SBP. The study by Mansour *et al.* [8] on 22 patients with SBP, the cutoff level was 242 ng/ml, the sensitivity and specificity of the assay for the diagnosis of SBP were 95.5 and 97%, respectively. Another study by Abulseoud *et al.* [9] who studied 60 patients with both SBP and non-SBP, showed that AF lactoferrin, at a cutoff level of 255 ng/ml, can distinguish patients with SBP from those without SBP with a sensitivity of 100% and specificity of 88.9%. A third study by Lee *et al.* [10] on 102 ascitic patients with 24 patients with SBP, 78 patients with no SBP, and 78 patients with HCC showed an AF lactoferrin level of 88 ng/ml as a cutoff can distinguish patients 'with' and 'without' SBP with sensitivity and specificity of 75 and 87.2%, respectively.

The differences in the cutoff value of the AF lactoferrin level between our study and other studies may be explained by the smaller sample sizes of SBP patients in other studies and, possibly, by the differences in the etiology of cirrhosis. HCC patients were excluded in our study, whereas other studies may have not excluded HCC patients. Thus, further multicenter studies are required to identify an optimal cutoff ascitic lactoferrin level for the diagnosis of SBP.

Our study showed a statistically significant positive correlation between ascitic lactoferrin and ascitic PMNL (P=0.010). It means that the higher the ascitic PMNL the higher the ascitic lactoferrin level. That result is in agreement with the study done by Lee *et al.* [10].

In this study, the ascitic culture was positive in 16 (16%) patients with SBP, whereas it was negative in the other 84 (84%) patients with SBP. Caldwell and Battle [11] reported that despite using good culture techniques, cultures were still negative in 30–50% of patients with an increased AF PMNL, whereas Friedman [12] detected positive AF bacterial cultures only in 41.7% of cases of SBP and negative in 58.3% of cases. Abulseoud *et al.* [9] found that AF bacterial cultures were positive in 43.4% of cases of SBP and negative in 56.6% of cases. Culture negativity was explained by low concentrations of bacteria in AF. So, larger samples are needed to give a higher percentage of positivity. Furthermore, AF culture is insensitive and leads to a delay in the diagnosis for several days. A delay in antibiotic therapy entails high mortality [1]. So, we do not depend on ascitic culture in the management of SBP. We found a statistically significant higher level of ascitic lactoferrin in culture-positive SBP patients (182.6 ±32.70 ng/ml) than culture-negative SBP patients (128.7±25.5 ng/ml; P<0.001), but still higher than

our cutoff value for the diagnosis of SBP. There was only one study that investigated ascitic lactoferrin in both culture-positive and culture-negative SBP patients conducted by Wu *et al.* [4], whose study included 22 cirrhotic patients with ascites, among them there were only four patients with SBP; three of them were culture positive and only one patient was culture negative with a statistically significant higher level of ascitic lactoferrin in culture-positive SBP patients (261.69±145.5 ng/ml) than the only one culture-negative SBP patient (6.057 ng/ml; P=0.002).

Ascitic lactoferrin level after antibiotic therapy (71.05 ±25.3 ng/ml) was significantly lower than before antibiotic therapy (137.33±33.19 ng/ml; P<0.001). This result means a significant effect of antibiotic therapy on the level of ascetic lactoferrin. So, ascitic lactoferrin can be used for the follow up of SBP patients after antibiotic therapy.

To our knowledge, no published study was done evaluating the effect of antibiotic therapy on the level of ascetic lactoferrin in SBP patients.

Also, we found that the ascitic lactoferrin level after antibiotic therapy was significantly higher in resistant cases with SBP (102.275±27.6 ng/ml) than in responding patients with SBP (69.45±24.5 ng/ml; P=0.010). Moreover, the level of ascitic lactoferrin in resistant cases with SBP was still higher than our cutoff value for the diagnosis of SBP.

This result gives a higher diagnostic value for ascitic lactoferrin that it could be used to monitor the resistant cases of SBP. To our knowledge, no published study was done comparing ascetic lactoferrin between resistant and responding cases of SBP after a course of antibiotic therapy.

Conclusion

AF lactoferrin concentration is higher in cirrhotic patients with SBP than in non-SBP and higher in culture-positive SBP patients than culture-negative SBP patients, so it can be used as a reliable marker for the detection of SBP. AF lactoferrin concentration is significantly decreased by the use of systemic antibiotic in SBP patients and its level remains high in resistant cases of SBP than the cases that respond to treatment, so it can be used for the follow up of these patients after antibiotics.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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