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RESEARCH ARTICLE

Amyloid A in Serum and Ascitic Fluid as a Novel Diagnostic Marker of Spontaneous Bacterial Peritonitis

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Abstract: ***Background*:** Diagnosis of Spontaneous Bacterial Peritonitis (SBP) depends mainly on ascetic fluid culture which may be negative in spite of the clinical suggestion of SBP and high ascetic fluid neutrophilic count.

***Aims*:** This study aimed to evaluate the biological importance of amyloid A biomarker in both serum and ascetic fluid to diagnose SBP as early as possible and to compare it to other markers (C-reactive protein (CRP), and the neutrophil-to-lymphocyte ratio (NLR)).

***Methods*:** This study included 37 patients with hepatic ascites; twenty-two of them had SBP, and 15 patients did not have SBP. Serum and ascetic fluid amyloid A, ascetic fluid neutrophil, C-reactive protein, and neutrophil-to-lymphocyte ratio were measured in all subjects before the start of antimicrobial chemotherapy to the infected ones.

***Results*:** Both the serum and ascetic fluid amyloid and also, CRP were significantly higher in patients infected with ascetic fluid than others. The cut-off point of serum amyloid A for early detection of SBP was 9.25ug/ml with the high sensitivity and specificity. For ascetic amyloid A, the sensitivity and specificity were 90.09% and 60% at cut-off point 2.85ug/ml, respectively.

***Conclusion*:** Amyloid A in serum and ascitic fluid can be considered as a good biomarker for early diagnosis of SBP.

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1. INTRODUCTION

Bacterial infections can be considered as serious complications in cirrhotic patients with hepatic decompensation [1]. One of them is spontaneous bacterial peritonitis which accounts for about 50% of cases. Others are pneumonia, urinary tract infections, and cellulitis [2].

SBP is a fatal ascetic fluid infection that typically occurs mainly in cirrhotic ascites. It was first demonstrated by Kerr and colleagues in 1963. It

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can be easily differentiated from secondary peritonitis by the absence of an intra-abdominal source of infection [3]. In 1964, Conn stated the term 'spontaneous bacterial peritonitis' to define a syndrome of peritonitis and bacteremia in Laennec's cirrhosis without an obvious source of infection [4].

It is important to stress that early diagnosis of SBP means good prognosis. However, it can be said that SBP diagnosis is too difficult to conduct earlier because of the lack of specific clinical manifestation and ascetic fluid biochemical markers [5]. According to current guidelines, the gold standard for the diagnosis of SBP is positive ascetic fluid culture. However, this culture is negative in 60% of patients in spite of the presence of clinical manifestations and high neutrophilic count in the ascetic fluid [6, 7].

The neutrophil-to-lymphocyte ratio (NLR) presents the junction between two different immune pathways. The neutrophil count gives an idea about inflammation while the lymphocyte count represents chronicity. So that their ratio can be considered as a marker of systemic inflammatory process [8].

Serum amyloid A (SAA) and C-reactive protein (CRP) are acute-phase proteins. They are secreted by hepatocytes, lymphocytes, monocytes, and macrophages. This is triggered by various cytokines, including interleukin 6 (IL-6) [9]. Causes of high levels of serum CRP and SAA are: bacterial infections, tissue injuries, neoplasm, and tissue rejection [10]. Be that as it may, the value of amyloid A in serum and ascetic fluid in early diagnosis of SBP, either alone or in combination with CRP and NLR, is still unclear. This study was conducted to determine the biological importance of amyloid A biomarker in both the serum and ascitic fluid to diagnose SBP as early as possible and to compare it to other markers (C-reactive protein (CRP), the neutrophil-to-lymphocyte ratio (NLR)).

2. PATIENTS AND METHODS

This case-control study was carried out on 22 patients with hepatic ascites who were suffering from SBP in comparison to 15 patients not affected by SBP. All the patients were from Tanta University, Tropical Medicine Department, Egypt.

All the selected patients were subjected to a bedside diagnostic paracentesis. A 22-G needle attached to a 20-cc syringe was used to administer the ascetic fluid after local anesthesia with lidocaine by a complete aseptic technique.

SBP was diagnosed according to the following criteria; (1) fever (temperature >37.5°C), and/or, abdominal pain with or without rebound tenderness (after exclusion of secondary peritonitis); and (2) ascitic fluid neutrophils $\geq 250/mm^3$ and/or positive ascetic fluid culture for bacteria [6, 11].

The patients were divided into two groups: Group I : 22 patients with SBP, and Group II: 15 patients without SBP. This classification was done according to neutrophilic count in ascitic fluid ≥, and $< 250/mm^3$ respectively.

Patients with liver cirrhosis (diagnosed on the basis of clinical, ultrasonographic, and laboratory findings) and ascites were included in the study. However, patients with hepatocellular carcinoma, extrahepatic malignancy, autoimmune diseases, organ transplant, any other infection due to parasites, fungi or bacteria, acute pancreatitis, recent abdominal surgery within three months before the study, and renal impairment were excluded from the study.

The aim of the research was clarified to all participants involved in the study. Informed consent was signed by every patient before enrolment in the study. The protocol was approved by the ethical committee of faculty of Medicine, Tanta University. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institution's human research committee. All the authors had access to the study data, and reviewed and approved the final manuscript.

Full medical history was taken and clinical examination was carried out of all subjects. Chest x-ray was performed to exclude chest infection. Furthermore, abdominal ultrasound was performed to assess the liver, spleen, kidneys, and portal vein diameter in addition to ultrasound guided paracentesis.

All the participants were subjected to laboratory investigations in the form of complete blood

count, ALT, AST, total and direct bilirubin, serum albumin, prothrombin time, and serum creatinine. Also biomarkers of SBP were analysed in the form of calculation of N/L ratio, CRP, and serum and ascitic fluid amyloid A.

2.1. Samples Collection and Laboratory Assays

Laboratory assays including prothrombin time, serum creatinine, albumin, bilirubin, liver enzymes, full ascitic fluid examination, serum and ascitic fluid Amyloid A levels (SAA), C-reactive protein (CRP) and complete blood count with the calculation of N/L ratio were all determined at Clinical Chemistry and Hematology units, Clinical Pathology Department, Tanta University Hospital, Egypt.

Ascitic fluid samples were collected by standard paracentesis protocol for a routine physical, chemical, microscopic and bacteriological examination in addition to amyloid A determination.

The aspirated AF was collected into ethylene diamine tetraacetic acid (EDTA) containing tubes. 5 ml was used for chemical and microscopic examination within 3 hours. A centrifuged aliquot for amyloid A measurement was stored at-20°C.

Amyloid A levels in serum or ascitic fluid were measured using abcam®Serum Amyloid A (SAA) Human ELISA Kit (ab100635, Cambridge, UK).

2.2. Statistical Analysis

The results were statistically analyzed using SPSS statistical package version 23 (SPSS Inc. Realesed 2015. Armnok, NY: IBM Corp.). Student's t-test and Mann Whitney's test were used to compare the variables of the two groups with normally and not normally distributed data respectively. Qualitative variables were compared by Chi-square test (χ^2), and Fischer's Exact test if one or more expected cells were <5. Receiver operator characteristic (ROC) with respective points of maximal accuracy for sensitivity and specificity was generated to determine the biomarker's performance. Area under the ROC curve (AUROC) measures the accuracy of the test. The significance of the test was considered at p value < 0.05.

3. RESULTS

Regarding demographic data, Group I included 16 males and 6 females; while in group II, there were 8 males and 7 females. The mean age was 47.27 ± 7.23 years and 50.40 ± 9.85 years in group I and group II, respectively. No significant difference was detected between the two groups regarding age, gender, and clinical signs (Tables **1** and **2**).

There was no significant difference in the results of most of the routine laboratory investigations except for the total leucocytic count, serum albumin, CRP, and serum amyloid and Ascitic amyolid which were significantly higher in group I (P value 0.001, 0.04, 0.004, <0.001 respectively) (Table **3**).

The areas under the curve regarding serum amyloid A and ascitic fluid amyloid for diagnosis of SBP were 1 and 0.91, respectively. The cut-off value of serum amyloid A for early detection of SBP was 9.25ug/ml with 100% sensitivity and specificity. For ascetic amyloid A, the sensitivity and specificity were 90.09% and 60% at a cut-off point 2.85ug/ml, respectively (Table **4**). The CRP showed 72.0% sensitivity and 80.0% as specificity, while N/L ratio showed 54.0% sensitivity and 40.0% as specificity at a cutoff point 3.53. ROC

Table 1. Demographic data of the included patients in both groups.

-	Group1 (n=22) Mean ±SD	Group2 (n=15) Mean SD	t- test	P value
Age	47.27 ± 7.23	50.40 ± 9.85	1.11	0.27
-	No. %	No. %	χ^2	P value
Gender				
Male	16 72.7	8 53.3	2.58	0.10
Female	6 27.3	7 46.7		

Table 2. Clinical manifestations of the included patients in both groups.

-	Group 1 (n=22) No. %	Group 2 (n=15) No. %	Fisher's Exact Test	P value
Jaundice				
Absent	16 72.7	9 60.0	0.21	0.64
Present	6 27.3	6 40.0		
LL edema				
Absent	6 72.7	3 20.0	0.01	0.90
Present	16 27.3	12 80.0		
Splenomegaly				
Absent	8 36.4	9 60.0	1.17	0.27
Present	14 63.6	6 40.0		

Table 3. Results of the routine laboratory investigations and biomarkers of the included patients.

-	Group1 (n=22) Mean ±SD	Group 2 (n=15) Mean ± SD	Test of Significance	P Value
Hb%	10.60 ± 1.05	10.68 ± 0.92	t=0.21	0.83
Platelets	130.18 ± 43.03	133.00 ± 60.32	U=0.37	0.70
WBCs	8.41 ± 2.99	5.82 ± 2.97	U=2.05	0.04
N/L ratio	5.96 ± 6.15	4.42 ± 2.37	U=0.18	0.85
TLC	6381.81 ± 7126.11	348.00 ± 224.44	U=4.37	<0.001
Neutrophils%	79.39 ± 15.32	69.60 ± 22.50	t=1.57	0.12
Bilirubin	2.43 ± 1.90	2.08 ± 1.40	U=0.65	0.51
Albumin	2.66 ± 0.59	2.36 ± 0.25	t=2.14	0.04
INR	1.60 ± 0.52	1.48 ± 0.17	t=0.98	0.33
ALT	44.54 ± 58.44	30.60 ± 11.36	U= 0.27	0.78
AST	112.36 ± 210.18	50.60 ± 13.10	U= 0.27	0.78
Serum amyloid A	149.34 ± 149.42	4.30 ± 0.95	U=5.12	<0.001
Ascitic amyloid	14.81 ± 23.30	2.80 ± 0.09	U=4.33	<0.001
CRP				
Absent	6 27.3	12 80.0	χ^2=7.93	0.004
Present	16 72.7	3 20.0		

*Hb (Hemoglobin); WBCs (White Blood Cells); N/L (Neutrophil/Lymphocyte ratio); INR (International Normalized Ratio); TLC (Total Leukocytic Count); ALT (Alanine Aminotransferase); AST (Aspartate Aminotransferase); CRP (C-Reactive Protein); SD (Standard Deviation).

curve analysis was performed and the best cutoff value was determined by maximizing the sensitivity and specificity to the left upper corner of the curve (Fig. **1**).

4. DISCUSSION

Liver cirrhosis is associated with the high risk of bacterial infections. Several mechanisms explain that including the deficiency of both humoral

Table 4. Areas under the curve and predictive values of serum and ascitic amyloid diagnosis of SBP patients.

Marker	AUC	Diagnostic Cut off point	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Serum amyloid A (ug/ml)	1.00	9.25	100.0	100.0	100.0	100.0	100.0
Ascitic amyloid (ug/ml)	0.91	2.85	90.09	60.0	77.0	82.0	78
CRP (+ve)	0.76	+ve	72.7	80.0	83.0	67.0	75.0
N/L ratio	0.48	3.53	54.5	40.0	57.0	38.0	49.0

*AUC (Area Under the Curve); PPV (Positive Predictive Value); NPV (Negative Predictive Value); CRP (C- Reactive Protein); N/L (Neutrophil/Lymphocyte ratio).

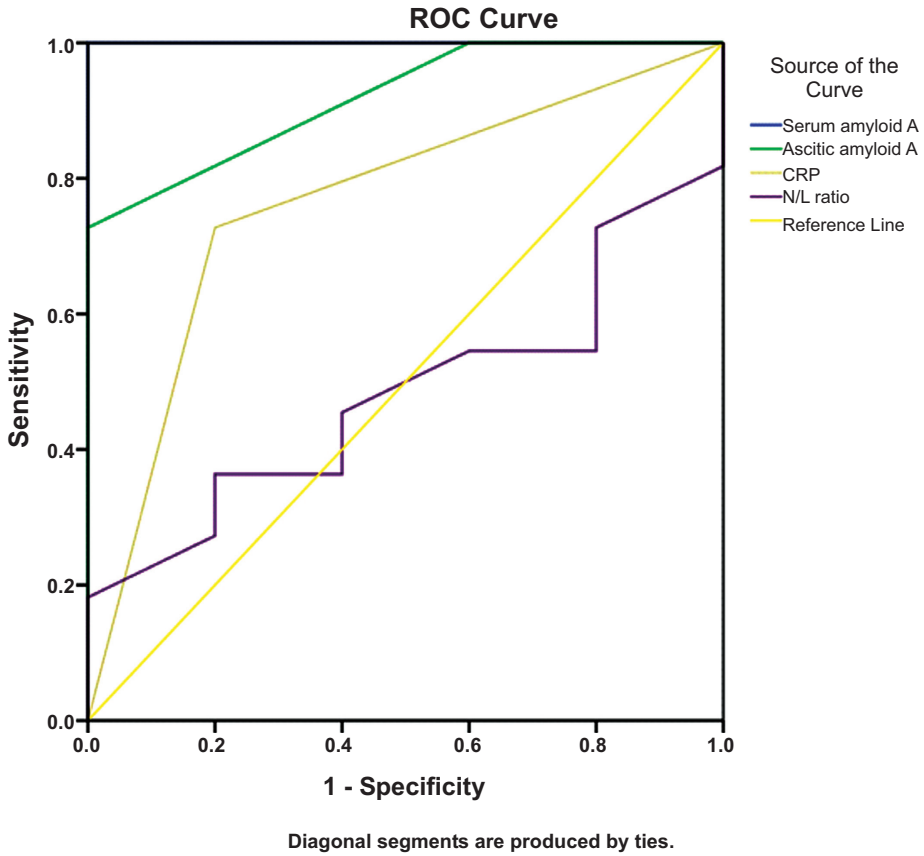

Fig. (1). ROC curve of sensitivity and specificity of serum amyloid, ascetic amyloid, CRP and N\L ratio.

and cell-mediated immune responses, structural and functional changes of intestinal mucosa increase intestinal permeability and the possibility of bacterial translocation. The risk of bacterial infection is increasing proportionally to the progression of liver disease [7].

Diagnosis of spontaneous bacterial peritonitis (SBP) is dependent mainly on ascitic fluid polymorph nuclear leukocytes (PMNs) and ascetic fluid culture. However, the neutrophil count is often manual and operator dependent; also, the lysis of PMNs can occur during transportation. Furthermore, culture has low sensitivity and is time consuming. This makes finding new diagnostic tools for SBP an interesting research area [12, 13].

Neutrophil-to-lymphocyte ratio (NLR) is a cheap, available test as a marker of a systemic inflammatory response [14]. In liver cirrhosis, pa-

tients often have leucopenia due to hypersplenism; so that, high NLR can predict poor prognosis in these patients [15]. In the present study, there was no significant difference observed between the two groups regarding N/L. This can be explained by the early hyperdynamic phase of infection (proinflammatory state), in which there is low neutrophil apoptosis [16], and high lymphocyte apoptosis in the thymus and spleen [17]. Neutrophil dysfunction was detected even in the compensated cirrhosis [18]. However, Kwon *et al*. 2015 stated that in the presence of infection, neutrophil count was higher and lymphocyte count was lower than in the absence of infection [15].

It has been suggested that C-reactive protein (CRP) is a predictor for SBP and its outcome after antibiotic therapy [19, 20]. In the present study, CRP and PMNLs levels were significantly higher in spontaneous bacterial peritonitis which was in agreement with Badawy, *et al.* (2013); Guler *et al*., (2009), and Lutz *et al*. (2015) [21-23]. CRP is known to be an inflammatory marker of bacterial infections, even in hepatic patients. Previous studies showed that CRP was also a good marker for bacterial infection in patients with chronic liver disease. However, it had diverse diagnostic accuracies and cut-off values [24, 25]. This variation may be affected by the nature of the liver disease. In 2017, Hamed *et al*. said that CRP with a cutoff value 10.5 mg/L provided the best sensitivity (91%) and specificity (97%) to diagnose SBP with AUC-ROC (0.975) [26]. This study was similar to the study by Preto-Zamperlini *et al*. (2014) who found that patients with SBP had a significantly higher CRP level than those without SBP [27]. In the study by Yuan *et al*. (2013), it was concluded that CRP was a better diagnostic marker for SBP in chronic hepatitis B patients than WBC count [28].

Wu *et al*. [29], in their study (2016) proposed a different point of view. They reported that CRP in ascitic fluid inflammation seems not to provide diagnosis with a higher level of accuracy. This may be explained by the higher baseline levels of CRP in liver cirrhosis patients than others. However, when infection occurs in advanced cirrhotic patients, there is a failure of a marked increase in CRP level due to functional hepatic impairment.

In our study, serum amyloid was observed to be a very good biomarker for early diagnosis of SBP with 100% sensitivity and specifity including NPV and PPV. However, ascitic amyloid A had lower sensitivity (90.%), lower specificity (60.0%), 77% PPV and 82% NPV.

This may be attributed to the leakage of amyloid A from serum through the serous membranes, and to some local synthesis. On the other hand, protein concentration may be decreased due to proteolysis and cell uptake. In the inflammatory process, the synthesis of SAA is expected. Certainly, activated monocytes express and release SAA [30, 31]. In spite of local SAA release, there is a serum contribution of SAA in the effusion due to a positive strong correlation between them [30-32].

The association between SAA and SBP may be attributed to SAA proteolysis by neutrophils lysosomal enzymes [33], and the presence of SAA specific receptor in macrophages [34] and neutrophils [35]. So, CRP and SAA are helpful markers for the diagnosis of SBP.

CONCLUSION

Although many research works showed the role of acute phase reactant (C-reactive protein (CRP) and Serum amyloid A (SAA)) in the inflammatory process and bacterial infections, this study focused on spontaneous bacterial peritonitis and found that SAA is a novel marker for its diagnosis.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol was approved by the ethical committee of the Faculty of Medicine, Tanta University, Egypt (approval no. 30776/02/16).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All human procedures were performed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Informed consent was signed by every patient before enrollment in the study.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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