

# Vitamin D and LL-37 in cirrhotic patients with culture-positive spontaneous bacterial peritonitis

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## Background and aims

Spontaneous bacterial peritonitis (SBP) is a common bacterial infection in patients with liver cirrhosis and ascites. Cirrhotic patients frequently have vitamin D (Vit-D) deficiency. Vit-D induces the production of LL-37, an important molecule of innate immunity, in macrophages. The aim of this study is to assess Vit-D and LL-37 levels in the ascitic fluid (AF) of cirrhotic patients with SBP in comparison to patients with simple ascites.

## Patients and methods

Forty male cirrhotic patients with ascites were included and were divided into two groups, 20 with culture-positive SBP (group I) and 20 with simple ascites (group II). AF Vit-D and LL-37 levels were measured.

## Results

Vit-D and LL-37 levels were significantly lower in patients with SBP than those with simple ascites. In univariate analysis, the risk factors for SBP were lower platelet count, higher leukocytic count, higher serum bilirubin, lower prothrombin activity, lower serum albumin, higher Child–Pugh and model for end-stage liver diseases scores, and lower AF Vit-D and LL-37 levels, while in multivariate analysis, a lower AF Vit-D level was the only risk factor for SBP.

## Conclusion

Patients with SBP have lower levels of AF Vit-D and LL-37 than simple ascites, where an inadequate expression of LL-37 in AF due to the low Vit-D level may increase the susceptibility to SBP.

## Keywords:

ascitic fluid, liver cirrhosis, LL-37, spontaneous bacterial peritonitis, vitamin D

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## Introduction

Spontaneous bacterial peritonitis (SBP) is a spontaneous infection of ascitic fluid (AF) in the absence of an intra-abdominal source of infection [1]. The prevalence of SBP in cirrhotic outpatients with ascites is 1.5–3.5% and about 10–30% in hospitalized patients [2]. The mortality rate of SBP is high ranging from 20 to 30% [3], and the 1-year survival rate is only 30–40% [4].

The pathophysiology of SBP is not completely understood. Translocation of bacteria from the gastrointestinal tract to the AF is believed to be a key mechanism behind the development of SBP, and is facilitated by impaired defensive mechanisms in cirrhotic patients [1]. SBP is diagnosed by polymorphonuclear (PMN) cell count in AF more than or equal to 250 cells/mm<sup>3</sup>, where the organism responsible for the infection is isolated in 60–70% of cases [5].

Cirrhotic patients frequently have vitamin D (Vit-D) deficiency. The liver is a vital organ for Vit-D

biotransformation, where it is the sole organ for 25-OH-D<sub>3</sub> production from Vit-D [6]. The decreased number of hepatocytes, reduced exposure to sunlight, decreased adipose tissue, malabsorption of Vit-D, and altered hydroxylation of Vit-D in the liver are all causes of low levels of Vit-D in patients with cirrhosis [7]. It was found that the low level of AF Vit-D is associated with an increased risk of AF bacterial infection in patients with cirrhosis and ascites [8].

Vit-D induces LL-37 production in human macrophages which is the only antibacterial peptide in humans that is regulated by Vit-D [6]. LL-37 is the only known cathelicidin in humans and considered as an important molecule of innate immunity with direct broad-spectrum antimicrobial, chemotactic, binding, and neutralizing lipopolysaccharide activities [9].

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LL-37 is released by cells of the immune system and is highly expressed at barrier sites, so it provides an important line of defense mechanism against pathogens that influence CYP27B1 and Vit-D receptor (VDR) upregulation in macrophages by enhancing toll-like receptors. Then, intracellular production of 1,25-hydroxyvitamin D<sub>3</sub> takes place to activate the endogenous LL-37, which is mediated by VDR [10,11].

Recently, it has been hypothesized that inadequate expression of LL-37 in the peritoneal cavity caused by Vit-D deficiency in cirrhotic patients with ascites might lead to increased risk of SBP with an increase in the mortality rates [12].

The aim of this work is to study the diagnostic role of Vit-D and LL-37 expression levels in the AF of cirrhotic patients with culture-positive SBP in comparison to patients with simple ascites.

## Patients and methods

This study included 40 male cirrhotic patients with ascites admitted to the Hepatobiliary Unit, Alexandria Main University Hospital. Patients were divided into two groups: 20 cirrhotic patients with ascites and culture-positive SBP (group I) and 20 cirrhotic patients with simple ascites (group II). Also, 20 age-matched and sex-matched healthy participants (group III) were included to obtain the normal range of biochemical assays.

Patients with renal disease, diabetes mellitus, collagen diseases, sepsis, or infection apart from SBP and patients who received Vit-D supplementation in the last 6 months were excluded from our study.

An informed consent was obtained from all participants included in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and Good Clinical Practice guidelines. It was approved by the Alexandria Faculty of Medicine Human Research Committee.

All participants underwent laboratory tests including: complete blood count [13], liver profile [alanine aminotransferase, aspartate aminotransferase, total serum bilirubin (TSB), alkaline phosphatase, prothrombin activity (PA), and serum albumin (ALB)] [14], viral markers [HBsAg; hepatitis C virus antibodies (HCV Abs), and confirmatory PCR in positive cases] [15]. The model for end-stage liver diseases (MELD) and Child-Pugh scoring systems for cirrhotic patients were calculated [16,17]. Also,

abdominal ultrasonography was done for grading of ascites [18].

All studied patients with cirrhosis and ascites underwent diagnostic paracentesis to obtain AF sample for PMN cell count, culture and sensitivity, Vit-D estimation using chemiluminescence [19] as well as quantification of LL-37 expression by real-time PCR where total RNA isolation from AF samples was carried out using PureLink RNA Mini Kit (Cat. No. 12183018A; Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol [20].

SBP diagnosis depended on the presence of more than or equal to 250 PMN cells/mm<sup>3</sup> in the AF and positive AF culture in the absence of an intra-abdominal source of infection.

## Statistical analysis

Data were fed to the computer and analyzed using the IBM SPSS software package, version 20.0 (IBM Corp., Armonk, New York, USA) [21]. Qualitative data were described using number and percent, while quantitative data were described using range (minimum–maximum), mean, SD, and median. Significance of the obtained results was judged at the 5% level.

The used tests were:  $\chi^2$  test, Fisher's exact test, Student's *t* test, *F* test (analysis of variance), Mann-Whitney *U* test, Kruskal-Wallis test, Pearson's coefficient, and Spearman's coefficient. Also, receiver operating characteristic (ROC) curve was used for AF Vit-D and LL-37 levels to predict SBP. Furthermore, univariate and multivariate analyses were done for detection of the parameters affecting SBP, where values of *P* less than or equal to 0.05 were considered statistically significant.

## Results

### Demographic data

Age showed close medians of 50, 55, and 51.5 years in groups I, II, and III, respectively, with no statistically significant difference between different studied groups (*P*=0.106). All cirrhotic patients with ascites and healthy participants who were enrolled in the study were men.

### Patient characteristics

#### Etiology of cirrhosis

All our studied cirrhotic patients with SBP and simple ascites had chronic HCV infection, while only one patient in group I had both chronic HCV and hepatitis B virus infection (Table 1).

**Table 1 Patient characteristics**

	SBP (N=20) [n (%)]	Simple ascites (N=20) [n (%)]	P value
Etiology of cirrhosis			
HCV	19 (95.0)	20 (100.0)	1.000
HBV	0 (0)	0 (0)	
HBV and HCV	1 (5.0)	0 (0.0)	
Grading of ascites			
Mild	1 (5)	0 (0)	0.200
Moderate	7 (35.0)	12 (60.0)	
Tense	12 (60.0)	8 (40.0)	
Child–Pugh class			
A	0 (0)	0 (0)	0.025*
B	8 (40.0)	15 (75.0)	
C	12 (60.0)	5 (25.0)	
MELD score			
Min.–max.	10–23	8–19	0.016*
Median	18.5	13.0	
Type of microorganisms in SBP			
<i>Escherichia coli</i>	16 (80)		
<i>Klebsiella pneumoniae</i>	4 (20)		

P, value for comparing between the two groups. HBV, hepatitis B virus; HCV, hepatitis C virus; max., maximum; MELD, model of end-stage liver disease; min., minimum; SBP, spontaneous bacterial peritonitis. \*Statistically significant at a P value of less than or equal to 0.05.

#### Grading of ascites

In group I patients, 5% had mild ascites; 35% had moderate ascites, and 60% had tense ascites. On the other hand, in group II patients, 60% had moderate ascites and 40% had tense ascites.

#### Staging of liver disease according to Child–Pugh and model for end-stage liver diseases scoring systems

In group I patients, eight (40%) patients were of Child B and 12 (60%) patients were of Child C, while in group II patients, 15 (75%) patients were of Child B and five (25%) patients were of Child C. Regarding MELD score, it ranged between 10 and 23 and 8 and 19 in groups I and II, respectively.

#### Microorganisms causing spontaneous bacterial peritonitis

Among the 53 patients diagnosed with SBP, positive ascitic culture was found in 20 (37.7%) patients, whom we selected for our study. The isolated organisms were Gram-negative bacteria in the 20 patients (16 *Escherichia coli* and four *Klebsiella pneumoniae*). Moreover, PMN cell count in the AF of all the studied patients with SBP was more than or equal to 250 cells/mm<sup>3</sup>.

#### Different studied laboratory parameters

##### Complete blood count

Anemia and thrombocytopenia were evident findings in cirrhotic patients with SBP and simple ascites in

comparison to healthy controls (Table 2). On the other hand, white blood cell (WBC) count showed the highest median in group I patients in comparison to groups II and III ( $8.48 \times 10^3$ ,  $5.24 \times 10^3$ , and  $5.75 \times 10^3$ , respectively), with an evident statistically significant difference between the different studied groups ( $P=0.001$ ).

#### Liver profile

Alanine aminotransferase, aspartate aminotransferase, TSB, and alkaline phosphatase showed significant high values in cirrhotic patients with SBP and simple ascites in comparison to healthy controls. On the other hand, PA and serum ALB showed significant low values in cirrhotic patients with SBP and simple ascites in comparison to healthy controls.

#### \*Vitamin D and LL-37 levels in ascitic fluid of cirrhotic patients with spontaneous bacterial peritonitis and simple ascites

AF Vit-D and LL-37 levels were significantly lower in cirrhotic patients with SBP in comparison to those with simple ascites.

#### Receiver operating characteristic analysis to study the ability of ascitic fluid vitamin D and LL-37 for the diagnosis of spontaneous bacterial peritonitis

In ROC analysis to study the ability of AF Vit-D for the diagnosis of SBP, Vit-D at a cutoff value of less than or equal to 7.5 nmol/l had a sensitivity of 90%, specificity of 85%, positive predictive value of 85.7, negative predictive value of 89.5, and area under the curve of 0.961.

Moreover, AF LL-37 at a cutoff value of less than or equal to 0.6 had a sensitivity of 80%, specificity of 90%, positive predictive value of 88.9, negative predictive value of 81.9, and area under the curve of 0.905 for the diagnosis of SBP (Fig. 1).

#### Correlation between ascitic fluid vitamin D /LL-37 and different studied parameters in cirrhotic patients with spontaneous bacterial peritonitis and simple ascites

In our study, cirrhotic patients with SBP (group I) showed a negative correlation between AF Vit-D and WBCs ( $P=0.007$ ), while no correlation was found between AF LL-37 and different studied parameters.

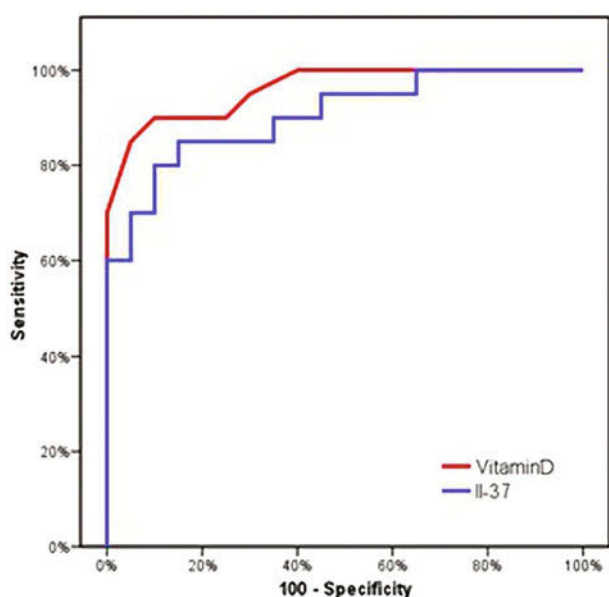
Also, patients with simple ascites (group II), showed no correlation between AF Vit-D and LL-37 with different studied parameters.

In a univariate analysis, lower platelet counts, higher WBC counts, higher TSB, lower PA, lower serum ALB, higher Child–Pugh and MELD scores, lower

**Table 2 Comparison between studied groups according to different laboratory parameters**

	SBP (N=20)	Simple ascites (N=20)	Control (N=20)	P value
Hb (g/dl)	9.62±1.27	9.89±1.96	13.55±0.84	<0.001*
Platelets (×10 <sup>3</sup> )	89.8±19.3	112.2±32.95	244.05±64.28	<0.001*
WBCs (×10 <sup>3</sup> )	8.79±3.98	5.29±2.19	6.33±1.7	<0.001*
ALT (U/l)	35.8±21.87	49.7±34.31	20.4±5.44	0.014*
AST (U/l)	58.2±31.4	53.65±31.79	23.85±5.02	<0.001*
TSB (mg/dl)	39.4±1.3	1.96±1.19	0.52±0.27	<0.001*
ALP (U/l)	131.8±50.5	105.7±40.28	71.0±19.52	<0.001*
PA (%)	36.3±11.2	50.98±11.4	90.1±8.68	<0.001*
Serum ALB (g/dl)	1.8±0.3	2.49±0.7	4.05±0.45	<0.001*
Vit-D (nmol/l)	7.07±0.38	8.3±1.51	–	0.001*
LL-37 normalized to GAPDH gene	0.66±1.19	4.63±4.96	–	<0.001*

P, value for comparing between the two groups. ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hb, hemoglobin; LL-37, cathelicidin protein; PA, prothrombin activity; SBP, spontaneous bacterial peritonitis; TSB, total serum bilirubin; Vit-D, vitamin D; WBCs, white blood cells. \*Statistically significant at a P value of less than or equal to 0.05.

**Figure 1**

ROC curve for ascetic fluid Vit-D and LL-37 to predict SBP. ROC, receiver operating characteristic; SBP, spontaneous bacterial peritonitis; Vit-D, vitamin D.

AF Vit-D and LL-37 levels were significant in patients with SBP (group I) when compared with patients with simple ascites (group II). However, in a multivariate analysis, lower AF Vit-D level was the only significant predictor for SBP ( $P=0.044$ ).

## Discussion

SBP is a common bacterial infection in cirrhotic patients with ascites. It shows a high rate of morbidity and mortality which requires prompt recognition and treatment [22]. Identification of the risk factors for SBP is of paramount importance to improve patient's outcome. There are well-established risk factors for the development of SBP, including: low ascetic fluid protein content (<1 g/dl), elevated serum

bilirubin, advanced liver cirrhosis, variceal hemorrhage, and increased gastric pH with the use of proton pump inhibitors in advanced cirrhosis [23].

Vit-D could increase innate defense and modulate the activation of lymphocytes implicated in the immune response [24], while Vit-D insufficiency might predispose to or increase the risk of bacterial infections and SBP in cirrhotic patients [25].

In the present study, the age showed close medians in different studied groups with no statistically significant difference. Many studies confirmed the effect of menopause on the level of Vit-D [26]; therefore, only male patients were included in our study

In our study, chronic HCV was the main cause of liver cirrhosis among our studied cirrhotic patients with SBP and simple ascites (95 and 100%, respectively). Only one (5%) patient in SBP group I patients had mixed chronic HCV and hepatitis B virus infection. In agreement with our results, it was reported that HCV infection is endemic in Egypt with the highest prevalence in the world; hence, it is the commonest cause of cirrhosis in our population [27].

In the present study, different grades of ascites were reported among our studied cirrhotic patients with SBP and simple ascites with no significant relationship between the severity of ascites and the occurrence of SBP. Similarly, Jeffries *et al.* [28] reported no relation between the grade of ascites and occurrence of SBP among their studied patients. On the other hand, staging of liver disease according to Child-Pugh and MELD scoring systems showed that there was a significant relationship between severity of liver disease and the occurrence of SBP, where patients with SBP showed higher Child-Pugh and MELD



scores than those with simple ascites. This was in agreement with many authors who stated similar results [29–31].

In the present study, the isolated organisms were Gram-negative bacteria in the 20 patients with SBP (16 *E. coli* and four *K. pneumoniae*). In agreement with our results, Koulaouzidis *et al.* [32] found that the microorganisms more commonly isolated from their cases of SBP were *E. coli* (~70%) and *Klebsiella* spp. (~10%).

We demonstrated significant anemia and thrombocytopenia among patients with SBP in comparison to other studied groups. Similar to our results, other studies reported that patients with SBP had significantly lower platelets count; also, low platelet counts might be a predictor for the development of SBP [33,34].

Moreover, our results showed that the WBCs count was significantly high in patients with SBP; this was in accordance with many studies which reported higher levels of WBCs in patients with SBP in comparison to patients without [29,33,35]. SBP triggers the systemic immune response which stimulates the bone marrow to release stored WBCs [36]; however, signs of sepsis in patients with SBP may be masked because of reduced serum PMN cell count caused by hypersplenism [37].

In the present study, disturbed liver profile was evidently reported among cirrhotic patients with SBP. These results can be explained by that the prevalence of SBP depends on the severity of liver dysfunction. Also, our results were in agreement with other authors [38,39].

In our study, AF Vit-D and LL-37 levels were significantly lower in cirrhotic patients with SBP in comparison to those with simple ascites. In agreement with our findings, Zhang *et al.* [20] found that the means of serum and AF Vit-D concentrations in patients with simple ascites were significantly higher than that in patients with SBP. Moreover, Yamshchikov *et al.* [40] and Murdoch *et al.* [41] found that serum Vit-D deficiency was significantly associated with the presence of infectious diseases in their patients.

Also, it has been reported that when Vit-D levels were low, bacteria impaired the VDR and LL-37 pathways in peritoneal macrophages to escape antimicrobial response and that supplementation with Vit-D could enhance peritoneal macrophage VDR and LL-37 expressions [42].

Moreover, Zhang *et al.* [8] reported that AF LL-37 levels in patients with SBP were lower, but statistically insignificant, than in patients with simple ascites. The discrepancy between our results and their results might be because all of our patients were culture-positive neutrocytic SBP, while they included culture-negative neutrocytic SBP patients and the authors themselves reported downregulation of LL-37 in simple ascites patients when cultured with lipopolysaccharide to study the effect of bacteria on noninfected patients. Also, the authors mentioned that there was an evident relationship between Vit-D and LL-37 expression with controversial results and they recommended further studies in this field. On the same territory, it has been documented that the human peptide LL-37 is downregulated during septic shock and then upregulated in the recovery phase of septic shock [43].

The ROC curve was done for both AF Vit-D and LL-37 levels to detect their ability to diagnose SBP. AF Vit-D at a cutoff value of less than or equal to 7.5 nmol/l had a high sensitivity and specificity (90 and 85%, respectively). Also, AF LL-37 at a cutoff value of less than or equal to 0.6 had a high sensitivity and specificity (80 and 90%, respectively).

In our study, based on univariate analysis of different studied parameters, the risk factors of SBP in ascitic cirrhotic patients include: lower platelet counts, higher WBC counts, higher TSB, lower PA, lower serum ALB, higher Child–Pugh and MELD scores, lower AF Vit-D and LL-37 levels. These variables when used for multivariate linear regression analysis, only lower AF Vit-D levels were significantly associated with the occurrence of SBP.

Thus, Vit-D supplementations in deficient cirrhotic individuals may have a role in enhancing peritoneal macrophage VDR and LL-37 expression, which is an important weapon in the fight against SBP (a preventive strategy) [44].

Recently, Buonomo *et al.* [45] found that Vit-D deficiency, MELD more than 15, Child B or C were significantly associated with infection. Also, at a multivariate analysis, Vit-D deficiency remained significantly associated with the risk of infection in HCV-related liver cirrhosis. Added to that, other authors reported that in univariate analysis, higher TSB, higher prothrombin time, and higher Child–Pugh score were significantly associated with mortality in patients with SBP [42].

From our study, we can conclude that both AF Vit-D and LL-37 are deficient in cirrhotic patients with SBP in comparison to patients with simple ascites. However, further research is needed to investigate serum Vit-D and LL-37 levels and correlate this with AF levels which if found to be correlated could be very beneficial as a rapid and noninvasive test for the diagnosis and monitoring therapy of SBP.

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Nil.

#### Conflicts of interest

There are no conflicts of interest.

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