ORIGINAL ARTICLE

Neutrophil Gelatinase-associated Lipocalin-2 and Macrophage antigen -1 in Cirrhotic Patients Infected with Carbapenem Resistant Enterobacteriaceae

[1]Fatma O. Khalil*, [2]Sally W. Elkhadry, [3]Sally S. Mandour, [3]Isis S. Bedira, [4]Amr R. Ibrahim, [5]Neamat A. Abd el mageed , [5]Eman E. El-shemy, [1]Hala A. El-refai

[1]Departments of Microbiology and Immunology, National Liver Institute Menoufia University
[2]Epidemiology and Preventive Medicine, National Liver Institute Menoufia University
[3]Clinical Pathology Department, National Liver Institute Menoufia University
[4]Hepatology and Gastroenterology Department, National Liver Institute Menoufia University and
[5]Department of Hepatogastroentrology & Infectious Diseases, Faculty of Medicine for girls, Al-Azhar University

Key words:
Mac-1, NGAI-2, SBP, PCR, flow cytometer, Cirrhosis

Corresponding Author:
Fatma Omar Khalil
Microbiology & Immunology Department -National Liver Institute- Menoufia University- Menoufia, Egypt
Tel: 0020-01068097624
fatma.khalil@Liver.Menofia.edu.eg

ABSTRACT

Background*: Early detection of bacterial infection in patients with cirrhosis remains a hope for improved prognosis to decrease the morbidity and mortality rate.* ***Objective:*** *To study the diagnostic importance of Neutrophil gelatinase-associated lipocalin-2 and macrophage antigen -1 for early detection of infection in cirrhotic patients and their relationship with carbapenem resistant bacteria.* ***Methodology:*** *150 patients were divided into group I: cirrhotic patients complicated with infection subdivided into three subgroups; A: fifty-five patients with spontaneous bacterial peritonitis (SBP), Subgroup B: fifteen patients with culture-negative neutrocytic ascites (CNNA) and subgroup C: thirty patients with urinary tract infection. Group II: fifty cirrhotic patients without infections. NGAL-2 estimation was done by ELISA, neutrophil MAC-1 by flowcytometry and Carbapenem resistance genes; OXA-48 and KPC-3 were detected by RFLP-PCR analysis* ***Results:*** *Significant increase of ascitic fluid neutrophils and WBCs count in SBP subgroup. Mean fluorescence intensity of Mac-1 and NGAL-2 concentrations were significantly higher in cirrhotic infected than uninfected patients****.*** *The OXA-48 and KPC-3 Carbapenem resistant gram-negative bacteria were prevalent among cirrhotic infected patients. NGAL-2 can detect early infection in cirrhotic patients followed by Mac-1.* ***Conclusion****: NGAL-2 and Mac-1 could be used for early detection of bacterial infection in cirrhotic patients with high specificity and sensitivity.*

INTRODUCTION

Liver cirrhosis is the end stage when the hepatic architecture has been altered[1], it is the first cause of liver-relat[5]ed mortality[2]. Ascites, encephalopathy, increased bilirubin concentration and bleeding esophageal varices are the main presenting signs of liver decompensation[3]. Spontaneous bacteria peritonitis (SBP) due to liver insufficiency occurs in 10-30% of ascitic patients[4]. SBPis subdivided into: Classical SBP: Ascitic fluid polymorphonuclear leucocytes count >250/mm^3 and positive culture. The presence of bacteria in ascitic fluid with neutrophils count less than 250 cell/mm^3 with or without symptoms is known as Monomicrobial non neutrocytic bacterial ascites (MNB). Symptomatizing patients with MNB had the same morbidity and mortality rates like those with SBP or CNNA, while asymptomatic MNB cure without antibiotic therapy[4].

Infection by gram-positive cocci as *Streptococci*, *Enterococci* and gram-negative bacilli as *E. coli* are the commonest etiology of SBP [6]. Neutrophils is activated in response to bacterial infection and release NGAL-2, Human neutrophil lipocalin (HNL) with other chemokines and pathogen-associated molecular patterns to destroy invading bacteria [7]. *Escherichia coli* is the most common Gram-negative enteric pathogen responsible for SBP with cephalosporins as drug of choice [2].

It is important to consider multidrug resistant (MDR) strains in patients with cirrhosis despite the routine use of third generation cephalosporins (cefotaxime, for example) in the suspicion of SBP. Extended spectrum β-lactamase (ESBL)-producing enterobacteria are the most frequent MDR strains in patients with cirrhosis. About 20% of *E. coli and Klebsiella sp* isolates produced ESBL, while 44% of *Staph. aureus* isolates were methicillin-resistant [eight]. The occurrence of infections due to MDR bacteria mostly for those of nosocomial origin [nine] began to increase with rising mortality rates[10].

Mac-1 (Mac-1, CR3) is an α subunit of the β2 integrin family and it is an adhesion molecule expressed on the surface of leukocytes, dendritic cells, macrophages, monocytes, neutrophils, natural killer (NK) cells, and a subset of B, T lymphocytes[11]. Mac-1 expression was increased on exposure to bacterial products to lipopolysaccharide present in wall of gram-negative bacteria such as *E. coli*[12]. Mac-1 has a pivotal role in cellular inflammatory process via mediating signaling pathways[13]

This study was aimed to assess the role of neutrophil MAC-1 and NGAL-2 in bacterial infection complicating liver cirrhosis and to identify the relationship between neutrophil MAC-1, NGAL-2 and Carbapenem resistant bacterial isolates of cirrhotic patients.

METHODOLOGY

The current study was done on 150 subjects from Inpatients and Outpatients Clinics at National Liver Institute, Menoufia University and Hepatogastroenterology & Infectious Diseases Department, Al-Azhar University after patient consent to participate in the study following the research ethical rule of National Liver Institute, Menoufia University under number 00279/2022 and with the 1964 Helsinki declaration and its comparable ethical standards.

Inclusion criteria

Cases were divided into: Group 1: 100 Liver cirrhotic patients with infection, it was sub divided into three subgroups. Subgroup A consists of 55 cirrhotic patients with classical SBP; positive ascitic fluid culture and AF neurophils count ≥ 250 x10^3 cell/µL. Subgroup B consists of 15 Cirrhotic Patients with CNNA (negative AF culture and AF neutrophils leucocytic count ≥ 250 x10^3 cell/Ml). Subgroup C consists of thirty cirrhotic patients with proved UTI. Group II: included fifty liver cirrhotic patients without evident infection. The criteria for involvement in the study were: patients aged 18 years or older, with liver cirrhosis and ascites according to the clinical, biochemical, and imaging markers.

Exclusion criteria:

Antibiotic treated patients, renal failure, hepatocellular carcinoma patients, patients with malignant ascites, septicemia patients, Secondary bacterial peritonitis (obvious or surgical cause of infection), and patients with clinical symptoms of any other infection (tonsillitis, pharyngitis, …..).

Laboratory investigations:

History taking (age, sex, history of blood transfusion and patients with history of any surgery), full clinical examination and abdominal ultrasonography were done for all patients. Investigations were done including: Ascitic fluid examination including AF culture, AF total protein, WBCs and neutrophil counts, AF albumin, and Mac-1 on peripheral blood neutrophils by flow cytometry, CBC, anti HCV and detection of HCV RNA by PCR, HBV serological markers (HBsAg and anti HBc). Kidney and liver profile: alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin, and total bilirubin was done.

NGAL-2 assay:

Serum assessment of lipocalin-2 by enzyme-linked immunosorbent assay (ELISA, DRG, GmbH) was done according to the manual instructions. Bacteriological cultures of ascitic and urinary samples from cirrhotic patients inoculated on blood agar, MacConkey agar, nutrient agar and mannitol salt agar plates and the samples were incubated for 24–48 h at 37°C, the growing organisms were identified, and antibiotic sensitivity test was done according CLSI, 2021[14].

RFLP-PCR analysis

Phenotypic identification of Carbapenem resistant isolates was done using different phenotypic tests and confirmed by RFLP-PCR analysis in three steps: Extraction and purification of total DNA using ThermoScientific Gene JET Genomic extraction and purification kit (Thermofisher, USA), then the extracted DNA underwent amplification by thermal cycler using restriction enzyme and carbapenem genes (KPC-3 & OXA-48) specific forward and reverse primers [15]. Finally, the amplified product was detected using agarose gel electrophoresis and visualized under ultraviolet illumination.

PCR primers:

Target genes	Primers sequences (3`-5`)	PCR Product size (bp)
KPC-3	FW: CGTCTAGTTCTGCTGTCTTG	798
	RV: CTTGTCATCCTTGTTAGGCG	
OXA-48	FW: GCGTGGTTAAGGATGAACAC	438
	RV: CATCAAGTTCAACCCAACCG	

Measurement of MAC-1 on neutrophils by flow cytometery:

EDTA whole peripheral blood samples were collected within 6 h after paracentesis and before antibiotic intake; they were processed within 24 h after collection. Neutrophils were identified by their characteristic forward and side scatter and total of 10.000 cells were examined in each sample. MAC-1 expression and Mean fluorescence intensity (MFI) were assessed on neutrophils by Partec CyFlow® Space Flow

Cytometer in which sample flow one cell at a time through a laser beam that scatters in characteristic way to the focused cells and their components. Labeling of cells with fluorescent markers was done so that light was first absorbed and then emitted in a band of wavelengths that gathered and processed by a computer. Anti-mac-1 antibodies were purchased from Sigma, Aldrich (Germany)

Statistical analysis:

Qualitative data were calculated using number and percent. The normality of the distribution was measured using Kolmogorov-Smirnov. Range (minimum and maximum), mean, standard deviation (SD), median and interquartile range (IQR) was done to describe qualitative data. Significance adjusted at the 5% level. Analysis was done using SPSS version 20

RESULTS

The study was carried out on liver diseased patients They included cirrhotic patients with infection as group I which included one hundred patients and those without infection as group II that included fifty patients. Group I were sub divided into group IA that included 55 Cirrhotic cases with classical SBP, group IB contained 15 Cirrhotic Patients with CNNA and 30 Cirrhotic patients with bacterial UTI

History of hematemesis, SBP, shrunken liver and splenomegaly was evident in group IA than all other studied groups. Also, ascitic tlcx10^3, ascitic neutrophil% and Ascitic neutrphils x10^3 were significantly higher in the same group. Ascitic albumin and total protein was significantly lower in patients with UTI than other groups as in table.1. This table shows ascitic fluid analysis parameter in cirrhotic patients with infection (group I) and those without infection (group II); Ascitic tlcx10^3, ascitic neutrophil% and Ascitic neutrphils x10^3 was significantly higher in patients with SBP other subgroups and more than group II. Ascitic albumin and total protein was significantly lower in group IC those with UTI than other groups.

Table 1: Ascetic Fluid analysis of the studied groups

	Group 1 A	Group 1 B	Group 1 C	Group 2	U test	P value	Post hoc
Ascitic tlcx10^3	4.41±4.22 55 4.30(0.0875-15.2)	2.86±1.37 15 3.0(0.9-4.9)	0.25±0.096 30 0.28(0.0875-0.3750)	0.1614±0.06 50 0.18(0.06-0.29)	87.075	0.0001	P1=0.135 P2=0.0001 P3=0.0001 P4=0.0001 P5=0.0001 P6=0.0001
ascitic neutrophil%	69.40±14.4٢ 55 75.00(40-95)	79.33±9.0٧ 15 84.00(65-93)	57.59±14.39 30 55.00(40-80)	69.66±11.33 50 69.00(50-90)	22.796	0.0001	P1=0.014 P2=0.004 P3=1.000 P4=0.000 P5=0.012 P6=0.002
Ascitic neutrphils x10^3	3.40± 3.24 55 3.3(0.07-9.88)	2.27±1.16 15 2.37(0.784.21)	0.13±0.04 30 0.14(0.07-0.17)	0.11±0.048 50 0.105(0.042-0.232)	72.745	0.0001	P1=0.204 P2=0.0001 P3=0.0001 P4=0.0001 P5=0.0001 P6=0.164
ascitic total protein g/dl	1.16±٠.40 55 1.1(0.5-2.0)	1.14±٠.42 15 1.1(0.5-1.8)	1.024±٠.21 30 1.10(0.7-1.3)	2.32±٠.32 50 2.30(1.50-3.0)	98.527	0.0001	P1=1.000 P2=0.238 P3=0.000 P4=0.907 P5=0.0001 P6=0.0001
Ascitic Albumin g/dl	0.64±0.24 55 0.6(0.2-1.1)	0.68±0.29 15 0.8(0.2-1.1)	0.52±0.14 30 0.6(0.3-0.7)	1.15±0.37 50 1.1(0.3-1.90)	62.685	0.0001	P1=0.997 P2=0.026 P3=0.0001 P4=0.307 P5=0.0001 P6=0.0001
Serum Ascitic Albumin/ Globulin ratio (SAAG)	1.63±0.42 55 1.6(0.40-2.5)	1.91±0.39 15 1.9(1.2-2.5)	1.94±0.48 30 2.1(1.4-2.5)	1.48±0.34 50 1.40(1.1-2.6)	25.715	0.0001	P1=0.131 P2=0.031 P3=0.194 P4=1.000 P5=0.004 P6=0.0001

Abbreviations: Tlc; total leucocytic count, U test; Mann-Whitney test

P1= I-A &I-B P2= I-A &I-C P3= I-A & II P4=1-B&1-C P5=1-B& II P6=1-C&II

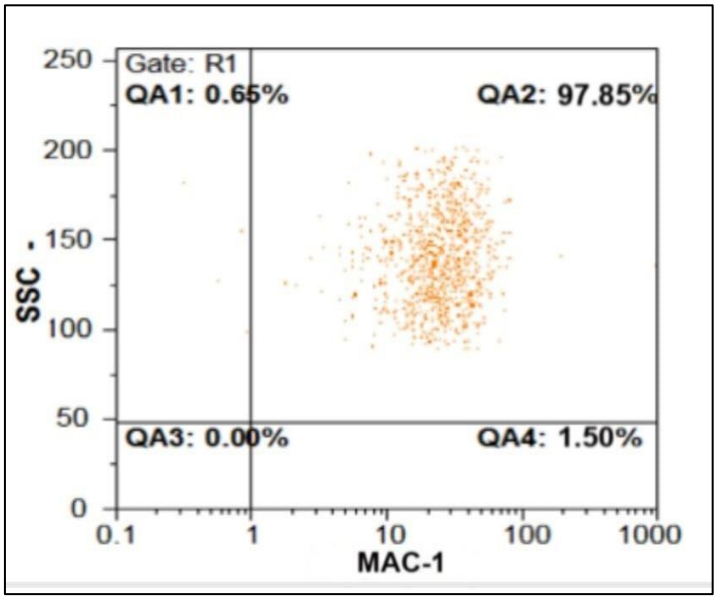

Fig.1: Neutrophil MAC-1 expression in cirrhotic patients with SPP Neutrophil MAC-1 was 97.85%

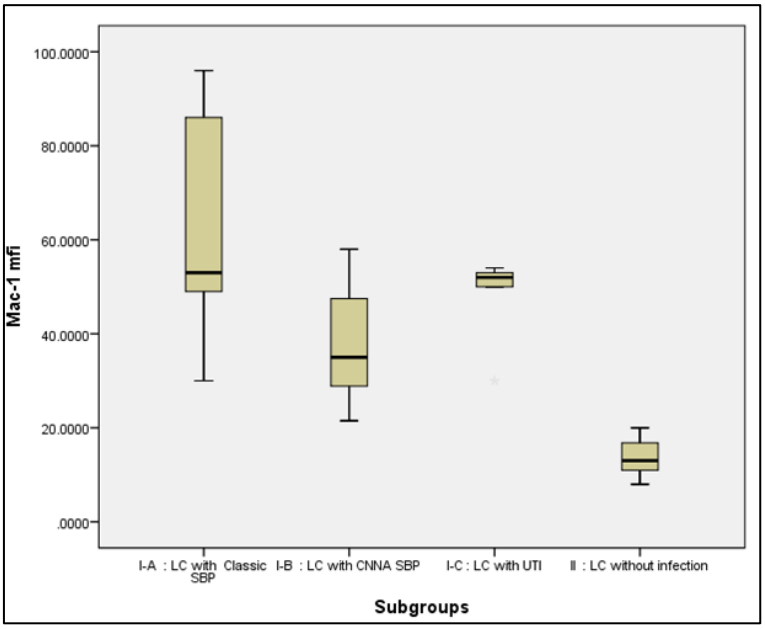

Fig. 2: Mac-1 Mean fluorescence Intensity (MFI) of the studied groups.

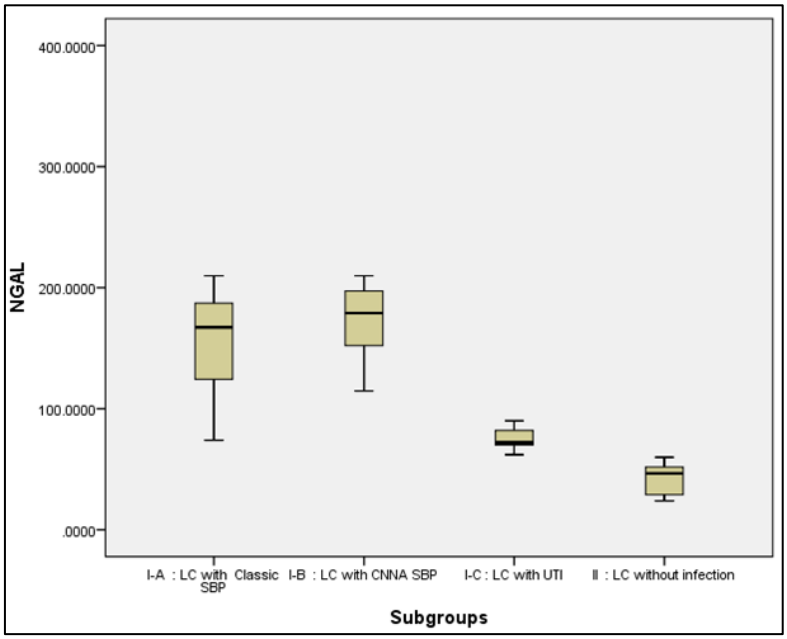

Fig. 3: NGAL-1 concentration in the studied groups

Figures .2 & .3: There was significant increase in NGAL in group I than in group II and significant increase between subgroups IA and IC, and between subgroups IC and Ib. Regarding Mac-1 MFI, there was highly significant increase in group I than II and in SBP subgroup than other subgroups.

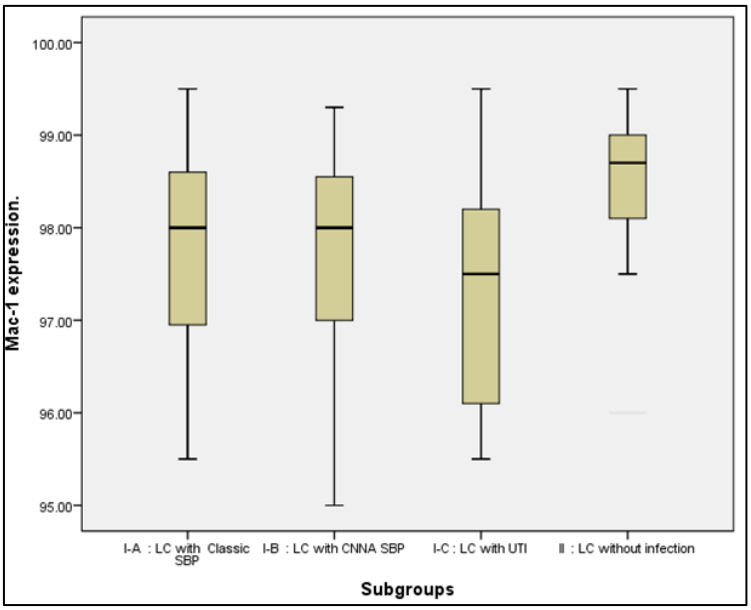

Fig. 4: Mac-1 concentration in the studied groups

This figure showed that Mac-1 was significantly higher in cirrhotic patients' group II than in group I.

Table 2 showed that CRP levels were significantly higher in group I than in group II. Alt, AST, GGT, TP and ALP level was higher in group II than in group I and its subgroups which were not significant. Total and Direct bilirubin was insignificantly higher in cirrhotic with UTI (group IC). Creatinine was significantly higher in group IB.

Table 2: CRP, liver, and kidney functions among the studied groups:

	Group 1 A	Group 1 B	Group 1 C	Group 2	U test	P value	Post hoc
ALT U/L	66.64±40.329 55 56.00(19-200)	48.60±30.071 15 39.00(18-115)	72.41±63.333 30 51.00(19-200)	74.50±39.979 50 64.50(19-145)	6.564	.087	P1=0.204 P2=0.0001 P3=0.0001 P4=0.0001 P5=0.0001 P6=0.164
AST U/L	79.95±40.906 55 71.00(26-198)	60.87±34.542 15 47.00(30-150)	80.00±58.569 30 53.00(30-198)	91.46±46.502 50 77.50(19-163)	7.788	.051	P1=0.396 P2=1.000 P3=0.702 P4=0.699 P5=0.056 P6=0.938
ALP U/L	86.69±56.804 55 66.00(39-260)	52.80±10.080 15 50.00(29-70)	70.48±25.105 30 56.00(45-114)	104.14±176.15 50 60.00(39-950)	5.851	0.119	P1=0.001 P2=0.371 P3=0.985 P4=0.012 P5=0.244 P6=0.717
GGT U/L	44.31±47.038 55 29.00(11-200)	32.93±14.79 15 29.00(15-60)	39.93±24.21 30 36.00(11-80)	48.28±30.653 50 45.00(11-115)	4.320	.229	P1=0.564 P2=0.994 P3=0.996 P4=0.811 P5=0.062 P6=0.708
Total Bilirubin mg/dl	3.89±3.26 55 2.8(1.5-15.0)	3.55±1.12 15 3.30(1.9-5.8)	4.41±3.18 30 3.0(1.5-11.0)	3.024±1.50 50 2.9(0.9-6.0)	4.344	.227	P1=0.987 P2=0.981 P3=0.396 P4=0.732 P5=0.637 P6=0.187
Direct Bilirubin mg/dl	2.83±2.78 55 2.0(0.76-12.0)	2.46±0.93 15 2.30(0.90-4.6)	3.27±2.74 30 2.0(1.0-9.0)	2.196±1.33 50 1.85(0.60-5.0)	3.484	.323	P1=0.956 P2=0.983 P3=0.576 P4=0.646 P5=0.950 P6=0.291
Total Protein g/dl	٥.٧9±0.49 55 ٥.٩(٤.٤- 6.5)	5.74±0.52 15 5.8(5.0-6.8)	5.82±0.32 30 5.6(5.5-6.3)	5.88±0.59 50 5.95(4.3-7.2)	1.280	0.734	P1=0.987 P2=0.981 P3=0.396 P4=0.732 P5=0.637 P6=0.187
Serum Albumin g/dl	2.27±0.38 55 2.3(1.5-2.9)	2.59±0.33 15 2.6(2.1-3.2)	2.46±0.40 30 2.8(2.0-2.9)	2.62±0.47 50 2.80(1.6-3.5)	17.427	0.001	P1=0.022 P2=0.242 P3=0.000 P4=0.814 P5=1.000 P6=0.493
PT (INR) Second	2.32±0.68 55 2.2(1.12-3.5)	2.63±0.66 15 2.6(1.6-3.9)	2.15±0.63 30 2.0(1.3-3.2)	1.59±0.38 50 1.5(1.1-3.5)	46.558	0.0001	P1=0.554 P2=0.820 P3=0.000 P4=0.155 P5=0.0001 P6=0.0001
PTT second	33.71±5.07 55 35.0(22.0-41.0)	34.4±6.84 15 35.0(25.0-48.0)	31.34±6.05 30 29.0(22.0-40.0)	40.41±3.01 50 40.0(33.0-46.0)	55.684	0.0001	P1=1.000 P2=0.388 P3=0.0001 P4=0.641 P5=0.027 P6=0.0001
Urea mg/dl	87.22±54.89 55 75.00(22-235)	87.53±46.89 15 80.00(28-200)	79.97± 22.224 30 78.00(56-120)	80.68±37.275 50 75.00(40-226)	0.862	0.835	P1=1.00 P2=0.951 P3=0.979 P4=0.993 P5=0.996 P6=01.00
Creatinine mg/dl	1.95±1.19 55 1.7(0.7-6.0)	2.28±1.67 15 1.8(0.6-6.0)	1.78±0.73 30 1.6(0.7-3.1)	1.14±0.34 50 1.05(0.6-1.8)	26.818	.0001	P1=0.980 P2=0.965 P3=0.0001 P4=0.865 P5=0.113 P6=0.001
CRP mg/l	84.07±35.07 55 87.92(25.77-169.89)	68.75±30.39 15 63.87(24.55-127.39)	56.61±11.18 30 60.65(41.16-73.74)	68.75±30.39 50 63.87(24.55-127.39)	105.107	0.0001	P1=0.493 P2=0.0001 P3=0.0001 P4=0.634 P5=0.0001 P6=0.0001

Abbreviations: ALT; alanine aminotransferase, AST; aspartate aminotransferase, ALP; alkaline phosphatase, GGT; gamma-glutamyl transferase, PT; prothrombin time, PTT; partial thromboplastin time, CRP; C-reactive protein, U test; Mann-Whitney test
P1= I-A &I-B P2= I-A &I-C P3= I-A & II P4=1-B& 1-C P5=1-B& II P6=1-C& II

The current table; table 3; shows prevalent bacteria detected in SBP isolates from group IA patients in which *E. coli, Klebsiella pneumoni, Pseudomonas aurigonosa, and Enterobacter* species was detected in 47.3%, 38.3%, 3.6% and 1.8% of isolates respectively 38.3% of which having OXA-48 gene and 18.0% having KPC-3 gene of carbapenem resistance. Based on culture and sensitivity tests followed by RFLP-PCR , our study showed that the prevalent bacteria in SBP isolates from group IA patients were E. coli, Klebsiella pneumoni , Pseudomonas aurigonosa, and Enterobacter species in 47.3%, 38.3% , 3.6% and 1.8% of isolates respectively 38.3% of which having OXA-48 gene and 18.0% having KPC-3 gene of carbapenem resistance.

Table 3: Type of infection and organism distribution among the studied groups:

	Group 1 A	Group 1 B	Group 1 C	Group 2	X2 test	P Value
Type of infection						
SBP	55(100.0%)	15(100.0%)	0(0.0%)		100.0	0.0001
UTI	0 (0.0%)	0(0.0%)	30(100.0%))			
microorganism						
E. coli	26(47.3%)		10(33.3%)			
Klebsiella pneumoni	21(38.2%)		13(43.3%)		9.45	0.221 F
Pseudomonas aeruginosa	2(3.6%)		2(6.7%)			
Enterobacter	1(1.8%)		0(0.0%)			
Streptococcus pneumoni	3(5.5%)		0(0.0%)			
Staphylococcus aureus	2(3.6%)		2(6.7%)			
Proteus vulgaris	0(0.0%)		2(6.7%)			
Candida albicans	0(0.0%)		1(3.3%)			
OXA-48						
Negative	29(61.7%)		14(58.3%)		0.075	0.784
positive	18(38.3%)		10(41.7%)			
KPC-3						
Negative	41(82.0%)		17(70.8%)		1.193	0.275
positive	9(18.0%)		7(29.2%)			

Abbreviations: SPP; spontaneous bacterial peritonitis, X2 test; chi-squared test

In table 4; NGAL-2 concentration was the highest in Cirrhotic Patients with CNNA and was significantly higher in the same group than in group II. Regarding Mac-1, there was significant increase in Mac-1 mean fluorescence intensity (MFI) and concentration in group I than in group II Table (4). Moreover, NGAL-2 at a cut-off of 110.72 ng/ml with AUROC 0.899 can differentiate infection in cirrhotic patients with 84% specificity and 92.7% sensitivity (p <0.0001**) and Mac-1 MFI with high significance with AUROC of 0.859, sensitivity of 70.9% and 72% specificity.

Table 4: Results of NGAL, Mac-11 and MFI among the studied groups:

	Group 1			Group 2	U test	P value	Post hoc
	A	B	C				
NGAL-2	163.38±50.84 55 167.24(73.96-341.89)	181.74±53.911 15 178.96(114.69-341.89)	73.93±8.76 30 71.0(62.0-90.0)	41.62±11.86 50 46.6(23.91-59.86)	126.393	0.0001	P1=0.822 P2=0.0001 P3=0.0001 P4=0.0001 P5=0.0001 P6=0.0001
MAC 1	97.75±1.165 55 98.0(95.50-99.50)	97.76±1.148 15 98.0(95.00-99.30)	97.41±1.19 30 97.55(95.50-99.50)	98.52±0.59 50 98.65(96.0-99.5)	22.986	0.0001	P1=1.0 P2=0.750 P3=0.0001 P4=0.920 P5=0.147 P6=.000
MFI	62.73±20.59 55 53.0(30.0-96.0)	37.65±11.69 15 35.0(21.5-58.0)	48.52±8.69 30 52.0(30.0-54.0)	13.79±3.25 50 13.0(8.0-20.0)	108.064	0.0001	P1=0.0001 P2=0.0001 P3=0.0001 P4=0.026 P5=0.0001 P6=0.0001

P1= I-A &I-B P2= I-A &I-C P3= I-A & II P4=1-B&1-C P5=1-B& II P6=1-C&II
Abbreviations: MFI; mean fluorescence intensity

In table 5; NGAL-2 at a cut-off of 110.72 ng/ml with AUROC 0.899 can differentiate infection in cirrhotic patients with 84% specificity and 92.7% sensitivity (p <0.0001**) and Mac-1 MFI with high significance with AUROC of 0.859, sensitivity of 70.9% and 72% specificity

Table 5: Sensitivity and specificity of NGAL & Mac-1 MFI to discriminate cirrhosis with bacterial infection from without infection:

Test Result Variable (s)	Cut-off	Area	Std. Error	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval		Sensitivity	Specificity	Accuracy	PPV	NPV
					Lower Bound	Upper Bound					
NGAL-2	110.72	.899	.026	0.0001**	.848	.951	92.7% [0.82, 0.98]	84% [0.75, 0.91]	87% [0.81, 0.92]	0.77 [0.65,0.86]	0.95 [0.88,0.98]
Mac-1 MFI	49.5	.859	.029	0.0001**	.802	.916	70.9% [0.57, 0.82]	72% [0.62,0.81]	72% [0.64,0.79]	0.60 [0.47,0.72]	0.81 [0.71,0.88]

The test result variable(s): NGAL, Mac-1 mfi has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased. a. Under the nonparametric assumption b. Null hypothesis: true area = 0.5

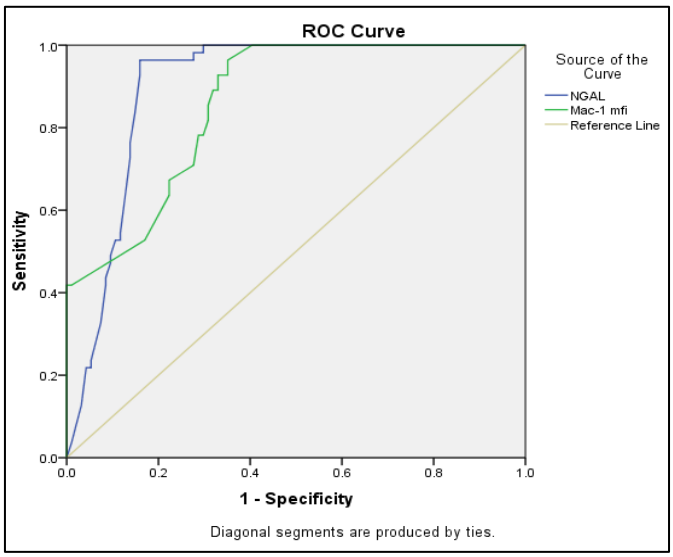

Fig. 5: ROC curve for NGAL-2, Mac-1 MFI to diagnose infection in cirrhotic patients

DISCUSSION

Neutrophils are the most common leukocytes in peripheral circulation and are well-known for their function in the host's defense against bacteria, fungi, and viruses, as well as their influence on adaptive immunity[16]. The major activators of neutrophils are damage- and pathogen-associated molecular patterns generated by aseptic inflammatory or infected tissue, respectively[17&18]. In vivo, NGAL-2 produced by neutrophils are thought to be key mediators in the fight against bacterial infection because they mediate neutrophil adherence and migration by interacting with adhesion molecules[16]. Elevated levels of proinflammatory cytokines, disruption to the intestinal barrier, microflora translocation, portal shunt, circulatory system endotoxins, and the buildup of additional PAMPs all contribute to neutrophil dysfunction during liver cirrhosis [15].

Our study showed that NGAL-2 level was significantly higher in cirrhotic patients suffering from infection than those without infection, these results were in accordance with Liu; et. al, [15] who found that not only the baseline ascetic NGAL-2 levels were significantly increased in the SBP than non SBP patients of decompensated cirrhosis cases, but also ascitic NGAL-2 levels were positively correlated with PMN, which is a classic diagnostic marker of SBP. Another study by Jonsson; et. al,[6] proved that plasma NGAL-2 was associated with presence of bacterial infections and that following antibiotic therapy, NGAL-2 level was markedly decreased.

In terms of MFI of Mac-1, it was much increased in group I than group II, and the SBP subgroup was significantly higher than the other group I subgroups. These findings corroborated those of Prince; et. al.,[19], who found that neutrophil Mac-1 expression rises on the neutrophil surface after 5 minutes of contact to bacteria, making Mac-1 a diagnostic biomarker for neonatal sepsis. LL-37 is a ligand for the main integrin receptor Mac-1 on the surface of myeloid cells, according to Lishko; et. al,[20] It protects the host against infections by directly killing pathogens and activating a variety of responses from immunological and other host cells. Mac-1-mediated phagocytosis of Gram-negative and Gram-positive bacteria is enhanced when bacteria are coated with LL-37 [21]. Stalhammar; et. al.,[13] published research that agreed with our findings, during early-onset sepsis, the expression of complement receptor 3 (CR3) increases in adults and newborn newborns, according to the researchers. The study by ***Prince; et. al,***[19]demonstrated that a deficiency of Mac-1 increases mortality following intra-peritoneal inoculation of *S. pneumonia* which was secondary to overwhelming sepsis. A greater number of Mac-1-deficient animals were having bacteremia by 24 h compared with controls.

Group I and its subgroups had significantly higher ascetic neutrophil counts than group II. Similarly, ***Saadietal, et. al.,***[22] explained that a higher neutrophil count in ascitic fluid is an accurate and earlier indicator of SBP when utilizing an index of >250/mm3 ascitic neutrophils.

The present study shows that patients with SBP had a more severe liver disease evidenced by that history and clinical findings including hematemesis, previous SBP, portal hypertension, shrunken liver and splenomegaly. This was also mentioned by Such and Runyon, and Runyon and Hoefs,[23, 24] who stated that, most SBP episodes occur in patients with advanced cirrhosis because of significant hepatic dysfunction, and a single episode of ascitic fluid infection has been regarded a justification for liver transplantation.

Our results were slightly different from Runyon and Hoefs,[23]who demonstrated that in patients with CNNA, clinical signs and symptoms were like those with culture positive SBP. The mortality rate of CNNA (50%) was like that of SBP (70%). Two out of 17 CNNA patients have previously experienced SBP.

The present study shows that ascitic TLC x103, relative and absolute neutrophil count were significantly higher in patients with SBP than other subgroups and group II, and that ascetic albumin and total proteins are higher in group II. These results are in concordance with *Runyon and Hoefs*,[24] who observed that, ascitic fluid TLC and PMN counts were generally higher in bacterial peritonitis, while SBP was only seen in individuals with very low ascitic fluid total protein concentrations. Low protein cirrhotic ascites has lower bactericidal and phagocytic activity than high protein peritoneal fluid, which explains the difference.

Our results shows that CRP levels was increased in group I than in group II agreeing with Runyon and Hoefs,[23] who proved that The SBP group exhibited strikingly increased CRP levels. While, ALT, AST, GGT, TP and ALP level was was non-significantly higher in group II than in group I and its subgroups. In a study by ***Syed et al,***[25], they confirmed that mean levels of serum ALT and AST were significantly lower among SBP group compared to non-SBP patients. Regarding urea and creatinine, they were higher in infected than non-infected patients. However, Preto-Zamperlini et al. [26]found that there are no differences in serum urea or serum creatinine in the SBP and NIA groups, which was explained by severe renal complication, may not happen so frequently in children having cirrhosis and SBP.

Our study shows that *E. coli* was the highest isolated organism (47.3%) from patients with SBP followed by *Klebsiella pneumonia* (38.2%) then *Pseudomonas aurigonosa* (3.6%), and *Enterobacter* species (1.8%). This agree with Ding et al.[5] who documented that *E. coli* was the most common pathogen (24.3%) of 334 pathogens identified from the ascetic fluid samples of patients with SBP, followed by *Klebsiella pneumoniae* (12.0%). Also, EASL,[5] stated that Gram-negative

bacteria (GNB), most often *Escherichia coli*, are the most prevalent pathogens when SBP ascitic culture is positive.

Furthermore, 18.0% of cirrhotic individuals with SBP had the carbapenem resistance gene KPC-3. This agreed with Alexopoulou et al.[27] who discovered that meropenem had a drug resistance rate of 30.7 percent among 130 patients with SBP. Li et al.[28] investigated thirty-one individuals with SBP, four of whom had KPC-3 and two of whom had E. coli that was resistant to meropenem.

At the present study NGAL-2 at a cut off 110.7272 ng/ml with AUROC 0.899 can differentiate infection in cirrhotic patients with 84% specificity and 92.7% sensitivity, these results were closely related to results proved by Balkhy et al, [29] who mentioned that ascitic NGAL-2 as a marker for SBP diagnosis at a cut-off value of 100.8 (ng/ml), sensitivity is 97.62%, specificity is 97.67%, and the area under the curve (AUC) is 0.974. Liu et al., found that an ascitic NGAL-2 of 108.95 ng/ml was performed as a cutoff value, showed a sensitivity of 76.9% and a specificity of 45.1% [15].

CONCLUSION

Finally, we concluded that end stage liver disease patients are vulnerable group for bacterial infection especially SBP which may be life threatening infection due to the nature of drug resistant organisms. The non-invasive biological markers as NGAL-2 and Mac-1 MFI could be good markers with for early detection of SBP with a high specificity and sensitivity. More studies with larger population are recommended to find a pathological role these markers and to be target for therapy.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

Abbreviations: NGAL-2, neutrophil gelatinase-associated lipocalin-2; MAC-1, macrophage antigen -1; SBP, spontaneous bacterial peritonitis; CNNA, culture-negative neutrocytic ascites, UTI; urinary tract infection, MNB; Monomicrobial non neutrocytic bacterial ascites, HNL; Human neutrophil lipocalin, MDR, multidrug resistance, ESBL, Extended spectrum β-lactamase, AF, ascetic fluid, MFI; mean fluorescence intensity.

Authors' contributions:
Khalil F.O. and El-refai H.A. contributed to the design of the work, conduction of study analysis, and interpretation of the data. Mandour S.S. and Bedira I.S. contributed to the laboratory analysis and acquisition of the data, statistical analysis was done by Elkhadry S.W. Inclusion of study participants and collection of their data was conducted by *Ibrahim A.R., Abd el mageed N.A. and El-shemy E.E*. All authors have read and approved the final manuscript.

Funding: None

Availability of data and materials
Please contact the corresponding author for data requests

Competing interests:
The authors declare that they have no competing interests

REFERENCES

1. Asrani SK, Devarbhavi H, Eaton J, Kamath PS. Burden of liver diseases in the world. J Hepatol. 2019;70(1):151-71.
2. Asrani SK, Hall L, Hagan M, Sharma S, Yeramaneni S, Trotter J, et al. Trends in Chronic Liver Disease-Related Hospitalizations: A Population-Based Study. Am J Gastroenterol. 2019;114(1):98-106.
3. Dever JB, Sheikh MY. Editorial: spontaneous bacterial peritonitis--bacteriology, diagnosis, treatment, risk factors and prevention. Authors' reply. Aliment Pharmacol Ther. 2015;41(12):1298.
4. Dever JB, Sheikh MY. Review article: spontaneous bacterial peritonitis--bacteriology, diagnosis, treatment, risk factors and prevention. Aliment Pharmacol Ther. 2015;41(11):1116-31.
5. Ding X, Yu Y, Chen M, Wang C, Kang Y, Lou J. Causative agents and outcome of spontaneous bacterial peritonitis in cirrhotic patients: community-acquired versus nosocomial infections. BMC Infect Dis. 2019;19(1):463.
6. Mattos AA, Wiltgen D, Jotz RF, Dornelles CMR, Fernandes MV, Mattos AZ. Spontaneous bacterial peritonitis and extraperitoneal infections in patients with cirrhosis. Ann Hepatol. 2020;19(5):451-7.
7. Beste LA, Leipertz SL, Green PK, Dominitz JA, Ross D, Ioannou GN. Trends in burden of cirrhosis and hepatocellular carcinoma by underlying liver disease in US veterans, 2001-2013. Gastroenterology. 2015;149(6):1471-82 e5; quiz e17-8.
8. Jonsson N, Gille-Johnson P, Martling CR, Xu S, Venge P, Martensson J. Performance of plasma measurement of neutrophil gelatinase-associated

lipocalin as a biomarker of bacterial infections in the intensive care unit. J Crit Care. 2019;53:264-70.

9. Lari AR, Azimi L, Rahbar M, Alaghehbandan R, Sattarzadeh-Tabrizi M. First report of Klebsiella pneumonia carbapenemase-producing Pseudomonas aeruginosa isolated from burn patients in Iran : phenotypic and genotypic methods. GMS Hyg Infect Control. 2014;9(1):Doc06.
10. Khan SQ, Khan I, Gupta V. CD11b Activity Modulates Pathogenesis of Lupus Nephritis. Front Med (Lausanne). 2018;5:52.
11. Qiu X, Li J, Yang X, Tang J, Shi J, Tong Y, et al. Is neutrophil CD11b a special marker for the early diagnosis of sepsis in neonates? A systematic review and meta-analysis. BMJ Open. 2019;9(4):e025222.
12. Stalhammar ME, Douhan Hakansson L, Jonzon A, Sindelar R. Differential neutrophil chemotactic response towards IL-8 and bacterial N-formyl peptides in term newborn infants. Ups J Med Sci. 2017;122(1):35-42.
13. Stalhammar ME, Sindelar R, Douhan Hakansson L. Neutrophil Receptor Response to Bacterial N-formyl Peptides is Similar in Term Newborn Infants and Adults in Contrast to IL-8. Scand J Immunol. 2016;84(6):332-7.
14. Azimi L, Nordmann P, Lari AR, Bonnin RA. First report of OXA-48-producing Klebsiella pneumoniae strains in Iran. GMS Hyg Infect Control. 2014;9(1):Doc07.
15. Liu H, Zhu P, Nie C, Ye Q, Gao Y, Liu H, et al. The value of ascitic neutrophil gelatinase-associated lipocalin in decompensated liver cirrhosis with spontaneous bacterial peritonitis. J Clin Lab Anal. 2020;34(6):e23247.
16. Heymann F, Tacke F. Immunology in the liver--from homeostasis to disease. Nat Rev Gastroenterol Hepatol. 2016;13(2):88-110.
17. Xu Y, Zhang Q, Zhao Y. The functional diversity of neutrophils and clustered polarization of immunity. Cell Mol Immunol. 2020;17(11):1212-4.
18. Taylor NJ, Manakkat Vijay GK, Abeles RD, Auzinger G, Bernal W, Ma Y, et al. The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality. Aliment Pharmacol Ther. 2014;40(6):705-15.
19. Prince JE, Brayton CF, Fossett MC, Durand JA, Kaplan SL, Smith CW, et al. The differential roles of LFA-1 and Mac-1 in host defense against systemic infection with Streptococcus pneumoniae. J Immunol. 2001;166(12):7362-9.
20. Lishko VK, Moreno B, Podolnikova NP, Ugarova TP. Identification of Human Cathelicidin Peptide LL-37 as a Ligand for Macrophage Integrin alphaMbeta2 (Mac-1, CD11b/CD18) that Promotes Phagocytosis by Opsonizing Bacteria. Res Rep Biochem. 2016;2016(6):39-55.
21. Such J, Runyon BA. Spontaneous bacterial peritonitis. Clin Infect Dis. 1998; 27(4):669-74; quiz 675-6.
22. Saadi T, Khoury S, Veitsman E, Baruch Y, Raz-Pasteur A. Spontaneous bacterial peritonitis with a very high leukocyte count in ascitic fluid caused by Haemophilus influenzae. Int J Gen Med. 2013;6:689-91.
23. Runyon BA, Hoefs JC. Culture-negative neutrocytic ascites: a variant of spontaneous bacterial peritonitis. Hepatology. 1984;4(6):1209-11.
24. Runyon BA, Hoefs JC. Ascitic fluid analysis in the differentiation of spontaneous bacterial peritonitis from gastrointestinal tract perforation into ascitic fluid. Hepatology. 1984;4(3):447-50.
25. Syed VA, Ansari JA, Karki P, Regmi M, Khanal B. Spontaneous bacterial peritonitis (SBP) in cirrhotic ascites: a prospective study in a tertiary care hospital, Nepal. Kathmandu Univ Med J (KUMJ). 2007;5(1):48-59.
26. Preto-Zamperlini M, Farhat SC, Perondi MB, Pestana AP, Cunha PS, Pugliese RP, et al. Elevated C-reactive protein and spontaneous bacterial peritonitis in children with chronic liver disease and ascites. J Pediatr Gastroenterol Nutr. 2014;58(1):96-8.
27. Alexopoulou A, Vasilieva L, Agiasotelli D, Siranidi K, Pouriki S, Tsiriga A, et al. Extensively drug-resistant bacteria are an independent predictive factor of mortality in 130 patients with spontaneous bacterial peritonitis or spontaneous bacteremia. World J Gastroenterol. 2016;22(15):4049-56.
28. Li YT, Yu CB, Huang JR, Qin ZJ, Li LJ. Pathogen profile and drug resistance analysis of spontaneous peritonitis in cirrhotic patients. World J Gastroenterol. 2015;21(36):10409-17.
29. Balkhy HH, El-Saed A, Alshamrani MM, Alsaedi A, Nasser WA, Gammal AE, et al. High Burden of Resistant Gram Negative Pathogens Causing Device-associated Healthcare Infections in a Tertiary Care Setting in Saudi Arabia, 2008-2016. J Glob Antimicrob Resist. 2020;23:26-32.