Monocyte Chemotactic Protein-1 Gene Expression in Blood and Ascitic Fluid of Cirrhotic Patients with Spontaneous Bacterial Peritonitis

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Background and study aim: Cirrhotic patients with ascites show a higher susceptibility to bacterial infections, monocyte chemotactic protein-1 (MCP-1) secretion is up-regulated during chronic hepatitis and correlates with the severity of hepatic inflammation. The aim of this work is to determine the level of expression of MCP-1 gene in blood and ascitic fluid in cirrhotic patients with and without spontaneous bacterial peritonitis (SBP) to evaluate its role in pathogenesis of SBP and its role in diagnosis.

Patients and Methods: This study included 15 healthy subjects served as control group in addition to 35 cirrhotic patients due to HCV infection with ascites; classified into two groups, cirrhosis without SBP (15 patients) and cirrhosis with SBP (20 patients). All groups were subjected to quantitative estimation of MCP-1 gene expression in blood by real time PCR. In SBP and non SBP groups the gene expression were assessed in ascitic fluid also at diagnosis and reassessed in SBP group after treatment.

Results: Blood and ascitic fluid expression of MCP-1 gene were significant higher in SBP group than non SBP group and control group. SBP group showed a significant decrease in level of MCP-1 gene expression in blood and ascitic fluid after resolution of infection by appropriate treatment of SBP.

Conclusion: MCP-1 gene expression in both blood and ascitic fluid may be related to pathophysiology and course of SBP and can be used as a marker for diagnosis.

INTRODUCTION

Liver cirrhosis is the clinical end stage of different entities of chronic liver disease **[1]**. Ascites is the most common complication; about 60% of patients with compensated cirrhosis develop ascites within 10 years of disease onset **[2]**. Patients with cirrhosis and ascites show higher susceptibility to bacterial infections, because of inadequate defense mechanisms **[3,4]**. Spontaneous bacterial peritonitis (SBP) is a common and potentially life-threatening complication in patients with cirrhosis. It is a prototypical infective disease in cirrhotic patients characterized by peritoneal neutrophil infiltration, which also serves as a diagnostic criterion for SBP (e.g. an ascites neutrophil count ≥250 cell/mm^3) **[5]**. Factors influencing the development of SBP in patients with liver cirrhosis are poorly understood **[6]**. SBP can be caused by many reasons due to alterations of the immune system that are very common in patients with end-stage liver disease and associated with an increased risk of infection and death **[7,8]**. Consequently, elevated concentrations of pro-inflammatory cytokines are found in ascitic fluid of these patients **[9, 10].** In addition, hepatitis C virus (HCV) infection is associated with increased hepatic expression of monocyte chemotactic protein-1 [MCP-1also known as chemotatic cytokine ligand 2 (CCL2)] **[11]**. CCL2 is the first discovered human CC chemokine located on chromosome 17 (chr.17, q11.2).Human MCP-1 is composed of 76 amino acids and is 13

kDa in size **[12]**. Chemotactic cytokines are known to be critical mediators of inflammatory cell trafficking into sites of injury and are crucial for the modulation of tissue injury, inflammation and repair **[13]**. MCP-1 is one of the most potent chemokines for monocytes/macrophages and activated lymphocytes during infections **[14]**. In addition, several studies have shown that neutrophil infiltration is affected either directly or indirectly via MCP-1 **[15,16]**. The aim of this study was to investigate the expression of MCP-1 gene in blood and ascitic fluid of patients with decompensated cirrhosis with and without SBP to evaluate its role in pathogenesis of SBP.

PATIENTS AND METHODS

A prospective case-control study was conducted on (35) cirrhotic patients with ascites attending to Hepatology, Gastroenterology and Infectious Diseases Department in addition to (15) health subjects in the period from September 2015 to April 2016, samples of blood from studied groups were analyzed at Molecular Biology Unit, Faculty of Medicine, Benha University.

The studied subjects were classified into three groups; Control group: 15 healthy subjects (11 males and 4 females; mean age was 28.60±4.12 years), Cirrhosis without SBP: 15 cirrhotic patients without SBP (7 males and 8 females; mean age was 61.60±9.75 years) and Cirrhosis with SBP: 20 cirrhotic patients with SBP (13 males and 7 females; mean age was 55.55± 8.94years). SBP was diagnosed by ascitic fluid poly morphonuclear leukocyte (PMN) count ≥ 250 cells/mm^3.

Patients with malignant ascites, tuberculous ascites, evidence for secondary peritonitis, alcoholic liver cirrhosis, HBV infection, antibiotic treatment before paracentesis were excluded from this study. All groups were subjected to full history taking, thorough clinical examination and routine laboratory investigations (complete blood picture, liver profile tests and kidney function tests).

Assessment of MCP-1 gene expression by real time PCR using sybr green:

MCP-1 gene expression was performed in blood for the control group and in both blood and ascitic fluid for all patients at the time of diagnosis and after 5 days of treatment for SBP (cefotaxime administrated 2g IV/8 hours, recommended treatment of SBP) **[17]**.

Total RNA Extraction

Total RNA extraction from 100µl EDTA blood and from 100µlasciticfluidfor each subject was performed using Direct-zol™ RNA MiniPrep from Zymo Research according to the manufacturer instructions, with addition of 300µl Trizolreagent to each sample to be extracted.

Spectrophotometric Quantification of RNA

Total RNA concentration was measured by Nanodrop spectrophotometer 2000 (USA) at A260 and A280. To ensure significance, A260 readings should be greater than 0.15. An absorbance of 1 unit at 260nm corresponds to 44µg of RNA per mL **[18]**. The ratio of the reading at (A260/A280) provides an estimate of the purity of RNA. Pure RNA has an A260/A280 ratio of 1.9 to 2.3.

Two Steps RT-PCR

1st step:The 1st step RT-PCR was for conversion of RNA into complementary DNA (cDNA) in a Veriti™ Thermal Cycler (Applied Biosystems), using Sensi FAST™ cDNA Synthesis Kit (Bioline Reagents Ltd, United Kingdom). PCR mix for cDNA included Total RNA (5µl), 5x TransAmp Buffer (4µl),Reverse Transcriptase (1µl) and up to 20µl nuclease free-water with the thermal profile; 25°C for 10min, 42°C for 15min and 85°C for 5min.

2nd step: RT-PCR for quantitation of MCP-1 gene expression was done using ABI7900HT fast real time PCR, (Applied Biosystem, USA). Single plex reactions were done. This step was performed using Sensi FAST™ Sybr Hi-Rox Kit (Bioline Reagents Ltd, United Kingdom). The primers sequences were human MCP-1; FP: 5´-AACTGAAGCTCGCACTCTCG-3´, RP: 5´-TCAGCACAGATCTCCTTGGC-3´) and human b-actin (FP: 5´-GACTACCTCATGAAGATC-3´, RP: 5´-GATCCACATCTGCTGGAA-3´) **[19]**. A single plex real time PCR reaction was performed with addition of 2x SensiFAST SYBR Hi-ROX Mix (10µl), FP (0.8µl), RP (0.8µl), cDNA (2µl) and up to 20µl nuclease free water. The thermal cycling conditions were 95°C for 5min (holding), cycling (40 cycles: denaturation; 95°C for 15sec, annealing; 56°C for 1min and extension; 72°C for 20sec). Melting curve analysis was performed in each run to confirm specificity of real-time PCR assay (95°C for 15sec, 60°C for 1min and 95°C for 15sec).

Data Analysis

The data, produced as sigmoid-shaped amplification plots (the cycle number is plotted against fluorescence on the linear scale), were analyzed by the RQ manager program 1.2 ABI SDS software (ABI 7900HT). Because the control samples are used as calibrators, their expression levels are set to 1. But because the expression levels were plotted as log10 values (log10 of 1 is 0), the expression level of the control samples appear as 0 in the graph.

Because the relative quantities of the MCP-1 gene are normalized against the relative quantities of the endogenous control β-actin gene, β-actin has no bars in the graph. Fold expression changes are calculated using the equation $2^{-\Delta\Delta CT}$ **[20]**.

Statistical analysis:

The results were analyzed using the SPSS software package version 20 (Chicago, IL, USA) and Microsoft office Excel. Quantitative data are expressed as mean±SD. ANOVA test was used to test the significance of difference between the mean values of more than two groups. Differences between two groups were compared by the studied t-test. The comparison of categorical variables was determined by x^2 test. Correlations between data were performed using Pearson correlation tests as required. Roc curve was used to detect the diagnostic performance of MCP-1 gene expression in both blood and ascitic fluid in diagnosis of SBP. Differences were considered significant at $p<0.05$.

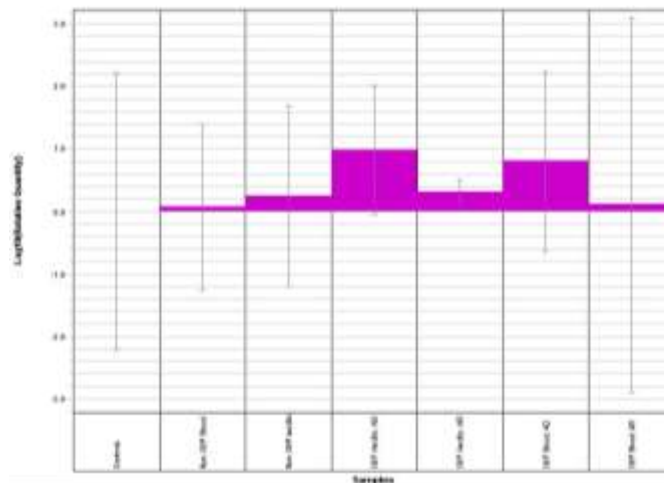

Figure (1): Gene expression plot of MCP in the studied groups

Relative quantitation of MCP-1 mRNA gene expression for all samples (AD: at diagnosis, AR: after resolution), represented as Log10. The expression level of the control samples appear 0 in the graph because the log10 for 1 is 0. The relative quantities for MCP-1 are normalized against relative quantities of GAPDH (endogenous control).

RESULTS

In the current study, the majority of studied patients in SBP group were males (65%), there was very highly statistically significant difference between the studied groups as regard age which was higher in group II (without SBP) than SBP and control groups (p=0.000), also there was very highly statistically significant difference as regard DM (p=0.000). Regarding clinical data there was highly statistically significant difference between SBP group and non SBP group regarding jaundice (p=0.000), which more common in SBP (80% vs 53.3% respectively), and both Child-Pugh and MELD score (p=0.036, 0.034 respectively) (Table 1).

There was highly statistically significant difference between studied groups regarding CBC, liver functions tests, serum creatinine. Between SBP and non-SBP group there was highly statistically significant difference regarding SAAG (p=0.024), mean level of MCP-1 gene expression in blood was higher in SBP group than non-SBP group and control group with very highly statistically significant difference (p=0.000), also MCP-1 gene expression in ascitic fluid was higher in SBP than non SBP group (p=0.021) (Table 2).

Within SBP group the level of expression of MCP-1 gene in both blood and ascitic fluid was decreased after treatment of SBP (in blood; 4.93

±0.320 vs 4.31±0.0472 before and after treatment, respectively and in ascitic fluid; 5.11 ± 0.323 vs 4.50 ± 0.0438 before and after treatment, respectively) with highly statistically significant difference (p=0.001 for both) (Figure 2).

Regarding correlation studies we found that there was significant positive correlation between MCP-1 gene expression in blood and the expression in ascitic fluid in both non-SBP and SBP (r=0.739, p=0.002 and r=0.985, p=0.000 respectively), also there was significant positive correlation between MCP-1 gene expression in blood and Child-Pugh score in non SBP group. While there was significant positive correlation between MCP-1 gene expression in ascitic fluid and platelet count in non-SBP group (r=0.56, p=0.049), with gene expression in blood in both SBP and non SBP (r=0.985, p=0.000 and r=0.739, p=0.002 respectively) also there was significant positive correlation with Child-Pugh in SBP group (r= 0.842, p= 0.000) (Table 3).

Ascitic expression of MCP-1 gene at cutoff 4.51 had higher sensitivity, specificity, PPV and NPV than its blood expression (90 %, 80%, 85.7%, 85.7%vs85 %, 76.7%, 70.83%, 88.5% respectively) with AUC was 0.913 and 0.892 (p<0.001) (Table 4, Figure 3).

Table (1): Baseline demographic and clinical characteristics of studied groups

Variables	Controls	Non-SBP Cirrhosis	SBP Cirrhosis	Test	p
	n.=15	n.=15	n.=20		
Sex (♂/♀) (n., %)	11(73.3%)/ 4(26.7%)	7(46.7%)/ 8(53.3%)	13(65%)/ 7(35%)	2.391[#]	0.300
Age (years) (mean±SD)	28.60±4.12	61.60±9.75[a]	55.55±8.94[a,b]	72.171[‡]	0.000
Diabetes mellitus	0 (0%)	11 (73.3%)	8 (40%)	17.176[#]	0.000
Hypertension	0 (0%)	1 (6.7%)	1 (5%)	0.955[#]	0.62
Jaundice	0 (0%)	8 (53.3%)	16 (80%)	22.22[#]	0.000
Gasterointestinal bleeding	0 (0%)	6 (40%)	9 (45%)	0.088[#]	0.767
Hepatic encephalopathy	0 (0%)	0 (0%)	5 (25%)	8.333[#]	0.016
Child-Pugh score(B/C)	-	6(40%)/ 9(60%)	2(10%)/18(90%)	4.375[#]	0.036
MELD score (mean±SD)	-	15.378±3.58	24.324±6.362	4.907[^]	0.034

[#]: X^2 test, [^]: t test, [‡]: Anova test

[a]: significant against controls, [b]: significant against Non-SBP, significant p values are in bald

Table (2): Baseline laboratory characteristics of studied groups

Variables	Controls	Non-SBP Cirrhosis	SBP Cirrhosis	Test	p
	n.=15	n.=15	n.=20		
Hemoglobin (g/dl)	13.65±1.185	9.373±2.04[a]	8.715±2.350[a]	29.724[‡:]	0.000
Platelets×10³ (cell/mm³)	297.933±	97.668±33.375[a]	118.85±37.82[a]	116.89[‡]	0.000
Total leukocyte count×10³ (cell/mm³)	7.302±1.729	6.598±1.96	12.72±6.72[a,b]	9.927[‡]	0.000
Aspartate Aminotransferase (IU/L)	26.26±5.93	45.053±20.38	83.25±79.66[a]	3.72[‡]	0.032
Alanine Aminotransferase (IU/L)	24.60±7.423	45.73±17.09	70.80±61.60[a]	5.68[‡]	0.006
Albumin (g/dl)	4.113±0.42	2.9400±0.354[a]	2.615±0.424[a,b]	62.28[‡]	0.000
Total Bilirubin (mg/dl)	0.622±0.307	2.72±1.77	7.57±5.25[a,b]	18.59[‡]	0.000
Prothrombin time (sec)	12.97±0.652	15.62±1.94[a]	17.61±3.404[a,b]	15.51[‡]	0.000
Creatinine (mg/dl)	0.855±0.207	1.426±0.44	2.18±1.611[a,b]	6.86[‡]	0.002
SAAG(g/dl)	-	2.25±0.554	1.85±0.45	5.57[^]	0.024
MCP-1 gene expression in blood (log10 RU)	4.199±0.019	4.27±0.0399	4.93±0.320[a,b]	69.70[‡:]	0.000
MCP-1 gene expression in Ascitic fluid (log10 RU)	-	4.44±0.094	5.11±0.323[b]	60.17[^]	0.021

[#]: X^2 test, [^]: t test, [‡:] anova test
[a]: significant against controls, [b]: significant against Non-SBP, significant p values are in bald

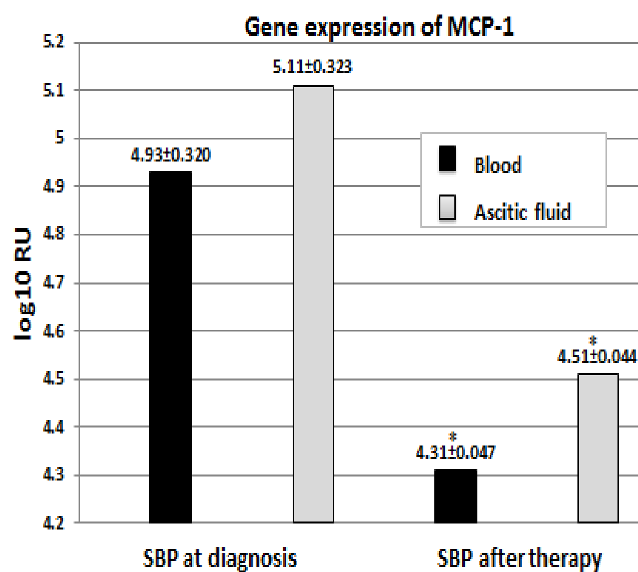

Figure (2): MCP-1 gene expression in blood and ascitic fluid in SBP before and after treatment

Table (3): Correlation between MCP-1 gene expression in blood and ascitic fluidat diagnosis and some studied parameters in both SBP and non-SBP groups

Variables	MCP-1 gene expression in blood				MCP-1 gene expression in ascitic fluid			
	Non-SBP		SBP		Non-SBP		SBP	
	r	p	r	p	r	p	r	p
Hemoglobin(mg/dl)	-0.307	0.189	-0.155	0.581	-0.078	0.783	-0.284	0.225
Platelets×10^3 (cell/mm^3)	0.0155	0.515	0.375	0.168	0.516	0.049	0.179	0.450
Total leukocyte count×10^3 (cell/mm^3)	0.175	0.461	0.403	0.136	0.423	0.116	0.219	0.354
Aspartate Aminotransferase (IU/L)	-0.038	0.875	-0.449	0.093	-0.163	0.567	-0.059	0.804
Alanine Aminotransferase (IU/L)	-0.035	0.882	-0.282	0.309	-0.091	0.747	-0.050	0.835
Albumin (g/dl)	-0.148	0.532	0.414	0.125	0.113	0.688	-0.216	0.361
Total Bilirubin (mg/dl)	-0.082	0.354	-0.410	0.129	-0.406	0.134	0.243	0.301
Prothrombin time (sec)	-0.082	0.731	-0.036	0.899	0.049	0.863	-0.012	0.958
Creatinine (mg/dl)	0.263	0.261	0.306	0.267	0.244	0.382	0.253	0.281
SAAG (g/dl)	-0.341	0.141	0.351	0.199	0.147	0.535	-0.346	0.135
MCP-1 gene expression in Ascitic fluid (log10 RU)	0.739	0.002	0.985	0.000	-	-	-	-
MCP-1 gene expression in blood (log10 RU)	-	-	-	-	0.739	0.002	0.985	0.000
Child–Pugh score	0.791	0.000	-0.479	0.073	-0.415	0.124	0.842	0.000
MELD score	0.410	0.072	-0.194	0.488	-0.147	0.602	0.442	0.051

Table (4): Diagnostic performance of MCP-1 gene expression in blood and ascitic fluid for diagnosis of SBP

Variables	Cutoff	Sensitivity	Specificity	PPV	NPV	AUC	Accuracy	95% CI	p
MCP-1 gene expression in blood (log10 RU)	4.28	85 %	76.7%	70.8%	88.5%	0.892	80%	0.77-1.00	<0.001
MCP-1 gene expression in Ascitic fluid (log10 RU)	4.51	90 %	80%	85.7%	85.7%	0.913	85.7%	0.80-1.00	<0.001

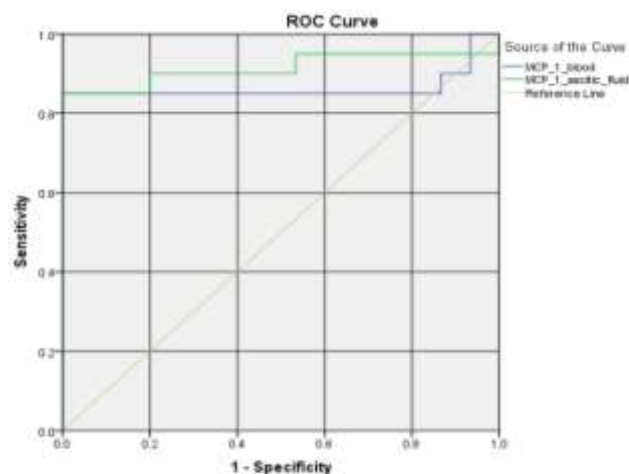

Figure (3): Roc curve for performance of MCP-1 gene expression in blood and ascitic fluid for diagnosis of SBP.

DISCUSSION

SBP is the most frequent infection in patients with liver cirrhosis. In these patients, SBP bacterial protein is recognized, and proinflammatory cytokines are released to blood and ascites **[21]**. In the current study, we found that SBP was common in males than females (65% vs 35% respectively) with mean age (55.55±8.94 years) which lower than non SBP and higher than control groups (p=0.000), this was coincided with **Syed et al., [22]** who found that SBP occurs more with higher ages due to more chance of infection in those patients also these results were in agreement with the study of Kim et al. **[23]** and Salama et al. **[6]** which showed that the majority of studied SBP patients were males (70% for first study and 72% for second study) with mean age was (53.3± 8.8, 51.24±9.3 years) respectively**.**On the same hand, these results were in line with those obtained by Kasztelan-Szczerbinska et al. **[24]** and Mostafa et al. **[25]** who found that SBP was more frequently among individuals of the masculine sex in percentages ranging from 72.8% to 83.7% and the mean age observed between the individuals with SBP ranging from 52.8 to 58.4 years. Regarding the clinical data, the present work found that, jaundice was more evident in SBP than non-SBP (80% vs 53.3% respectively) with highly significant difference between both groups (p=0.000). This was in accordance with that elicited by Thiele et al. **[26],** who found that jaundice was the most common complication of SBP (73.3%) but with insignificant difference between SBP and non SBP (p=0.336). In the present work we noted that the majority of SBP cases were Child-Pugh class C (90%) with statistically significant difference between SBP and non-SBP (p=0.036). This result matches with that reported by Cirera et al. **[27]**, Syed et al. **[22]** and Paul et al. **[28]** who elicited that 70%, 85%,80% respectively of SBP patients had Child class C. There was a significant increase of MELD score in SBP (p=0.034) with mean value (24.324±6.362). This coincided with Thiele et al. **[26],** who found higher MELD score in SBP than non-SBPwith mean (22.2±7.6 vs 17.9 ± 6.7). Also, Kraja et al. **[29]** and Gayatri et al. **[30]** observed that individuals with moderate to high MELD score present a substantially greater risk for SBP development.SBP patients in this study had lower mean SAAG value (1.85±0.45 g/dl) as compared to non-SBP patients (2.25± 0.554 g/dl). Similar results were reported by Thiele et al. **[26]** as mean value of SAAG in SBP was 1.3 g/dl and in non-SBP was 1.7 g/dl. This can be explained by Tarn and Lapworth **[31]** who stated that SBP is advanced liver disease is associated with low serum albumin concentration and so on lower SAAG than cirrhotic patients without SBP, and reinforced by Albillos et al. **[32]** who reported that SAAG should be the test of choice with the addition of an ascitic fluid PMN count to diagnose/exclude bacterial peritonitis. In contrast to these findings Nouman et al. **[33]** observed a lower mean SAAG value (1.2 g/dl) in non-SBP patients as compared to SBP patients (1.5 g/dl), this difference may be related to different numbers of studied patients.MCP-1 is one of the key chemokines that participate in the recruitment of inflammatory cells and is highly expressed under inflammatory conditions **[34]**. MCP-1 acts as a chemotactic factor for monocytes and macrophages, thus, these cells migrate to the ascetic fluid. These monocytes and macrophages release TNF-α and other cytokines, which in turn induces the expression of adhesion molecules on endothelial cells, therapy mediating a systemic reaction to the infection **[23]**. There was significant increase, reported in the present work, in the mean of level of MCP-1 gene expression in both blood and ascitic fluid in SBP than non-SBP which was in agreement with Gabele et al**. [13]**. There was significant decrease in MCP-1 expression in both blood and ascitic fluid after SBP treatment. This finding was in agreement with Kim et al. **[23]** who reported a change in various cytokines levels after treatment of SBP as decrease in MCP-1 and interleukin-10 levels on follow up after treatment. Our results could suggest that this chemokine (MCP-1) may play a pathophysiological role during the course SBP**.** Also this study found that MCP-1 gene expression in both blood and ascitic fluid can be used to diagnose SBP but ascitic expression had higher sensitivity, specificity than blood expression (90%, 80% vs 85%, 76.7% respectively) and we not found any literatures discus this point.

CONCLUSION

MCP-1 gene expression in both blood and ascitic fluid may be related to development and course of SBP, and can be used as a marker for diagnosis of SBP.

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Conflicts of interest: None.
Ethical approval: The protocol of this study was approved by ethical committee of Benha

University and written informed consent was taken from all patients for participation in this work.

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