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Original Article

Evaluation of Serum and Ascetic Fluid Calprotectin for Detection of Hepatocellular Carcinoma in Cirrhotic Patients

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ABSTRACT

Background: Hepatocellular carcinoma [HCC] is a leading cause of cancer-related deaths worldwide, particularly in Egypt, where it ranks as the most common liver malignancy. Early detection of HCC is crucial for improving survival rates. Alpha-fetoprotein [AFP], the traditional biomarker for HCC, has limitations in sensitivity and specificity, particularly in early-stage detection. Calprotectin, a protein complex involved in inflammation and immune response, has shown potential as a novel biomarker, but its role in HCC diagnosis remains underexplored.

Aim of work: This study aims to evaluate the diagnostic value of serum and ascitic fluid calprotectin levels for the early detection of hepatocellular carcinoma in patients with liver cirrhosis.

Patients and methods: This prospective observational study included patients with liver cirrhosis, divided into two groups: those diagnosed with HCC and those without HCC. Serum and ascitic fluid calprotectin levels were measured using enzyme-linked immunosorbent assay [ELISA]. The diagnostic accuracy of calprotectin was assessed using receiver operating characteristic [ROC] curve analysis, and its performance was compared to AFP.

Results: The study included 100 cirrhotic patients, 50 with HCC and 50 without. Calprotectin levels were significantly higher in both serum and ascitic fluid of HCC patients compared to non-HCC controls [$p < 0.001$]. ROC curve analysis demonstrated that ascitic calprotectin had a higher diagnostic accuracy [AUC 0.89, sensitivity 85%, specificity 88%] than serum calprotectin [AUC 0.85, sensitivity 80%, specificity 85%]. These findings indicate that calprotectin, particularly in ascitic fluid, is a more sensitive and specific biomarker for early HCC detection compared to AFP.

Conclusion: Calprotectin levels in serum and ascitic fluid are promising biomarkers for the early detection of hepatocellular carcinoma in cirrhotic patients. Their superior diagnostic accuracy compared to AFP suggests potential utility in clinical practice, warranting further large-scale validation studies.

Keywords: Hepatocellular carcinoma; Calprotectin; Liver cirrhosis; Alpha-fetoprotein.

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INTRODUCTION

Hepatocellular carcinoma [HCC] is a significant global health burden and the most common primary liver malignancy, accounting for a substantial number of cancer-related deaths worldwide. In Egypt, HCC represents one of the leading causes of cancer morbidity and mortality, closely linked to the high prevalence of chronic liver diseases, primarily hepatitis C virus [HCV] and hepatitis B virus [HBV] infections. Other risk factors, such as liver cirrhosis, non-alcoholic fatty liver disease [NAFLD], and environmental exposures, further amplify the disease burden. Early detection of HCC is critical for improving patient outcomes, as therapeutic options are more effective during the initial stages of the disease [1].

Despite advancements in imaging techniques and the development of biomarkers, such as alpha-fetoprotein [AFP], current diagnostic tools often fall short in sensitivity and specificity, especially in detecting early-stage HCC. AFP, the most widely used biomarker, is associated with limited sensitivity and frequent false-negative results in small tumors. Additionally, its levels can be elevated in non-cancerous conditions, such as advanced cirrhosis and acute hepatitis, reducing its diagnostic reliability. These limitations highlight the urgent need for novel, reliable biomarkers to enhance early diagnosis and improve the treatment outcomes [2].

Calprotectin, a member of the S100 protein family, is a heterodimeric complex consisting of S100A8 and S100A9 proteins. It is primarily released by neutrophils and macrophages during inflammatory responses and acts as a damage-associated molecular pattern [DAMP]. Calprotectin has been extensively studied in inflammatory conditions, such as inflammatory bowel disease and spontaneous bacterial peritonitis, but its role in malignancy, particularly HCC, remains underexplored. Recent evidence suggests that calprotectin may contribute to tumorigenesis by promoting inflammation, immune evasion, and tumor progression [3].

This study investigates the diagnostic potential of serum and ascitic fluid calprotectin levels in cirrhotic patients for the early detection of HCC. By comparing calprotectin levels between HCC patients and non-HCC cirrhotic controls, the study aims to evaluate its diagnostic accuracy and assess its utility as a complementary biomarker to AFP. Early identification of HCC using such biomarkers could pave the way for timely intervention, ultimately improving patient survival and reducing the overall disease burden.

PATIENTS AND METHODS

This was a prospective cohort study designed to assess the efficiency of ascitic fluid calprotectin in the early detection of hepatocellular carcinoma [HCC]. The study included 60 cirrhotic patients with ascites who had normal alpha-fetoprotein [AFP] levels and no detectable findings for HCC on baseline CT scans. The study was conducted at Al-Azhar University Hospital in New Damietta between July 2022 and July 2023. All procedures followed the ethical principles outlined in the Helsinki Declaration, and ethical approval was obtained from the institution's ethics committee.

Study Design: All eligible patients were followed up every two months for 12 months with repeated abdominal ultrasound and AFP testing. Based on the occurrence of HCC during follow-up, patients were categorized into two groups. Baseline ascitic calprotectin levels were compared between these groups to evaluate its utility in early HCC detection. Written informed consent was obtained from all participants at the time of recruitment.

The inclusion criteria for the study comprise patients with cirrhotic ascites and high-risk factors for hepatocellular carcinoma [HCC], which include advanced age, diabetes, thrombocytopenia, or liver cirrhosis secondary to hepatitis C virus [HCV] or hepatitis B virus [HBV]. In contrast, **the exclusion criteria** consisted of individuals who are using regular immunosuppressive drugs, those receiving prophylactic antibiotics for spontaneous bacterial peritonitis [SBP], patients with autoimmune diseases, individuals with conditions associated with increased calprotectin levels such as SBP, and those diagnosed with abdominal malignancies, including gastric, pancreatic, colorectal, ovarian cancers, cholangiocarcinoma, liver metastases, or peritoneal cancers.

History Taking: Comprehensive medical histories were obtained, with a specific focus on recent surgeries and comorbid conditions such as peptic ulcer disease.

Clinical Examination: Detailed physical examinations were conducted, which included assessments for portal hypertension, evidenced by signs such as dilated abdominal veins and splenomegaly, as well as indicators of liver failure, including jaundice, ascites, edema, flapping tremor, and spider angiomata.

Laboratory Investigations: The laboratory investigations comprised several key components. Liver function tests involved measuring levels of aminotransferases, bilirubin, albumin, total protein, and alkaline phosphatase. Additionally, a coagulation profile was assessed, which included prothrombin time [PT], partial thromboplastin time [PTT], and the international normalized ratio [INR]. Renal function tests evaluated serum creatinine and blood urea nitrogen levels. Furthermore, a diagnostic paracentesis was performed under sterile conditions to collect 30 mL of ascitic fluid for analysis. This analysis included tests for cell counts, albumin, total protein, glucose, lactate, lactate dehydrogenase [LDH], amylase, and carcinoembryonic antigen [CEA] levels, along with the calculation of the serum-ascites albumin gradient [SAAG]. A 5 mL sample of the ascitic fluid was specifically analyzed for calprotectin levels using an enzyme-linked immunosorbent assay [ELISA] provided by Immundiagnostik AG, Germany

Abdominal Ultrasonography was performed after a 6-hour fast to evaluate several key features, including signs of portal hypertension such as portal vein diameter, splenic bipolar diameter, and splenic vein diameter. The examination also assessed the severity of liver cirrhosis and the extent of ascites, as well as identifying hepatic focal lesions, portal vein thrombosis, peritoneal deposits, and intra-abdominal masses.

Diagnosis of HCC: All patients were followed for six months with abdominal USG and AFP levels assessed every two months to detect HCC, confirmed by AFP and Triphasic CT. Patients were stratified according to the Barcelona Clinic Liver Cancer [BCLC] staging system, which considers tumor burden, liver function [Child-Turcotte-Pugh score], and patient performance status. This system classifies survival prognosis into stages 0, A, B, C, and D.

Statistical Analysis: Statistical analysis was conducted using SPSS version 26 [SPSS Inc., Chicago, IL, USA]. The Kolmogorov-Smirnov test was used to assess data normality. Categorical variables were expressed as numbers and percentages, and comparisons were made using the chi-square test. Continuous variables were expressed as mean ± standard deviation [SD], and comparisons between the two groups were performed using independent t-tests. A p-value of <0.05 was considered statistically significant. Receiver operating characteristic [ROC] curve analysis and calculation of the area under the curve [AUC] were performed to assess the predictive accuracy of ascitic calprotectin for HCC detection.

RESULTS

In our study, the mean age of the included patients was 60.8±6.2 years. Forty-three patients [71.6%] were males and 17 patients were female. The chronic disease conditions showed that, 58.3% were diabetic, 28.3 % were hypertensive, 8.3% had chronic kidney disease [CKD], and 5 % had ischemic heart disease [IHD]. In terms of the cause of liver cirrhosis, 83.3% were HCV, and 16.7% were HBV. Furthermore, portal vein thrombosis was detected in 48.3% of the studied patients [Table 1].

The laboratory investigation results were documented in table [2]. These data reflected liver function derangement. For example, there was increased levels of total bilirubin and reduction of albumin than the normal levels. In addition, the hemoglobin concentrations were highly reduced [anemia of chronic disease] with thrombocytopenia and increased levels of Alfa-fetoprotein.

Table [3] Showed the results of ascitic fluid investigations. Mainly, the mean ascitic calprotectin level was 143.5 ± 115 ng/ml, while the serum level was 1.6 ± 0.4 ng/ml.

Table [4] showed that, 19 patients [31.7%] developed HCC after a mean time of 3.1 ± 1.6 months. In addition, AFP levels were significantly higher in HCC patients compared to non-HCC [p = 0.0001]. HCC patients also showed significantly elevated ascitic calprotectin levels [227.6 ± 139.4 ng/ml vs. 107.2±71.9 ng/ml, p = 0.0001] and a higher number of focal lesions [2.5±1.2]. However, there was no significant difference between HCC and non-HCC groups regarding serum calprotectin levels or CTP score [p = 0.1–0.7].

Based on the ROC that was applied [Figure 1] to determine the cut-off value of ascitic Calprotectin associated with the development of HCC. The following cut off value was detected as shown in table [5].

Table [1]: Demographic data, chronic diseases and basic clinical characteristics of the study group

Variables		Total [n=60]
Age	Mean ± SD	60.8 ± 6.2
	Range	50 – 77
Gender [n,%]	Male	43 [71.6%]
	Female	17 [28.3%]
Chronic diseases	DM	35 [58.3%]
	HTN	17 [28.3%]
	CKD	5 [8.3%]
	IHD	3 [5%]
Cusses of liver cirrhosis	HCV	47 [78.3%]
	HBV	13 [21.7%]
Portal vein thrombosis		29 [48.3%]

DM: Diabetes mellitus, HTN: Hypertension, CKD: Chronic kidney disease, IHD: Ischemic heart disease

Table [2]: Laboratory investigations of the studied cases

Variables	Results [mean ±SD]
AST IU/L	29.9 ± 14
ALT IU/L	40.3 ± 21.8
Total bilirubin [mg/dl]	2.1 ± 0.5
Albumin [g/dl]	2.6 ± 0.3
INR	1.9 ± 0.3
Creatinine [mg/dl]	1 ± 0.4
WBC /mcL	3.6 ± 2
HGB [g/dl]	8.7 ± 1.6
PLT/Ul	84.5 ± 34
FBS [mg/dl]	115 ± 36
AFP [ug/ml]	5.4 ± 2.1
CEA [ng/ml]	1.6 ± 0.5

AST: Aspartate aminotransferase, ALT: Alanine transaminase, INR: international normalized ratio, WBC: White Blood Count, HGB: hemoglobin, PLT: platelet, FBS: fasting Blood sugar, AFP: Alfa fetoprotein, CEA: carcinoembryonic antigen.

Table [3]: Ascitic fluid parameter of the studied cases

Variables		Total [n=60]
Ascitic TLC [cell /mm$_3$]		123 ± 61
Ascitic PNL [cell /mm^3]		99 ± 48.8
Ascitic protein [gm/dl]		2.07 ± 0.1
Ascitic Albumin [gm/dl]		0.94 ± 0.1
LDH [U/l]		206 ± 17.5
Glucose [mg/dl]		133 ± 22.5
SAAG		1.9 ± 0.4
CEA [ng/ml]		1.5 ± .6
Ascitic Calprotectin [ng/ml]	Mean±SD	143.5 ± 115
	Min. - Max.	14 – 452
Serum Calprotectin [ng/ml]	Mean±SD	1.6 ± 0.4
	Min. - Max.	1.1 – 2.07

SAAG: Serum Ascites Albumin Gradient, PNL: pleomorphic neutrophils lymphocytes, LDH: lactate dehydrogenase, CEA: Carcinoembryonic antigen

Table [4]: characteristics of the HCC and no-HCC subgroups.

Variables		Total [n=60]		P value
		HCC	**Non-HCC**	
Incidence of HCC		19 [31.7%]	41[68.3]	**0.001***
Gender [n,% of total]	Female	5 [8.3 %]	12[20%]	**0.04***
	Male	14 [23.4%]	29 [48.3%]	
Age [mean ± SD]		60.3 ± 6.5	60.7 ± 5.9	**0.9**
Ascetic Calprotectin [ng/ml]	Mean±SD Min. – Max.	227.6 ± 139.4 [55 – 452]	107.2 ± 71.9 [14 – 415]	**0.0001***
Serum calprotectin [ng/ml]	Mean±SD Min. – Max.	1.6 ± 0.3 [1.1- 2.07]	1.4 ± 0.2 [1.1 – 1.9]	**0.**15
Alfa fetoprotein [AFP] [ug/ml]	Mean±SD Min. – Max.	668.6 ± 208.5 [25.7 – 888]	22.8 ± 13.5 [5.3 – 39]	**0.0001***
No of focal lesion	Mean±SD Min. – Max.	2.5 ± 1.2 [1-5]	-	**0.0001***
Child-Turcotte-Pugh [CTP] score	Mean±SD	9.5 ± 2.8	9.8 ± 2.6	0.7
Time to diagnosis [months]	Mean±SD	3.1 ± 1.6	-	**0.0001***

Table [5]: AUC and cut-off values of ascitic Calprotectin associated with the development of HCC.

AUC Ascitic Calprotectin	Standard Error	P-value	95% CI[Lower–Upper]
0.802	0.06	0.0001	0.69 – 0.94
Cut-off Values [ng/ml]	**Sensitivity [%]**	**Specificity [%]**	
113	84.2	70.1	
119	79.1	74.2	
122.5	78.9	78	
127	73.7	81	

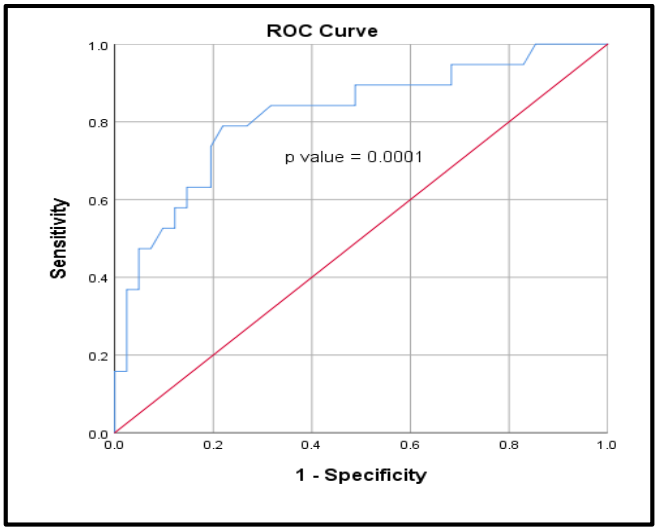

Figure [1]: Receiver operating characteristics analysis of the serum Calprotectin to identify risk of occurrence of HCC.

DISCUSSION

Calprotectin is mainly used as a marker of disease activity in different inflammatory bowel disease [IBD]. It differentiates IBD from functional gastrointestinal [GIT] disorders [4,5]. However, its role in diagnosis of malignancy is rarely studied before. It is expected to be locally elevated due to introduction of inflammatory cells and influx to malignant tissue. In addition, it had an apopteic actions on the malignant cells [6].

Furthermore, the detection of Calprotectin in the ascitic fluid is not a common investigation. It was previously evaluated in cirrhotic patients with systemic bacterial peritonitis [SBP] and a significant correlation was reported with the count of polymorphnuclear leucocytes [PNLs] and can be used as a predictor of SBP [7,8].

This was the basis for conduction of this study to test the value of ascitic calprotectin in HCC. This study highlights the diagnostic potential of ascitic calprotectin as a biomarker for the early detection of hepatocellular carcinoma [HCC] in patients with liver cirrhosis. Elevated levels of ascitic calprotectin were significantly associated with the development of HCC, with better sensitivity and specificity compared to the traditional biomarker, alpha-fetoprotein [AFP]. These findings align

with previous research suggesting the role of calprotectin in inflammation-driven malignancies.

Hanafy *et al.* demonstrated that ascitic calprotectin levels are significantly higher in patients with HCC compared to those with cirrhotic ascites without HCC. Their study reported a sensitivity of 93.3% and specificity of 94% at a cutoff value of 126 ng/mL, emphasizing its value as an early predictor of HCC in high-risk cirrhotic patients [9]. This supports the findings of our study, which also observed a high diagnostic accuracy of ascitic calprotectin, with an AUC of 0.802 and significant sensitivity and specificity.

Further supporting evidence comes from **Sun *et al.*** who observed elevated levels of ascitic calprotectin and other neutrophil extracellular trap [NET] markers in malignant ascites compared to benign ascites. This suggests that calprotectin may also be useful in differentiating malignant from benign conditions in HCC patients, further broadening its potential clinical application [10].

While serum calprotectin did not show significant differences between HCC and non-HCC groups in our cohort, its localized elevation in ascitic fluid likely reflects the role of localized inflammatory processes in HCC pathogenesis. Similar findings have been reported in other studies investigating calprotectin's role in inflammatory conditions such as spontaneous bacterial peritonitis and inflammatory bowel disease [11-13].

This study has **limitations**, including the relatively small sample size and short follow-up duration, which may limit the generalizability of the findings. Additionally, factors such as baseline inflammation and the heterogeneity of liver cirrhosis etiologies could influence calprotectin levels, potentially confounding the results.

Conclusion:

Ascitic calprotectin demonstrates significant potential as a biomarker for the early detection of hepatocellular carcinoma [HCC] in patients with liver cirrhosis. Its superior diagnostic accuracy compared to alpha-fetoprotein [AFP] highlights its clinical value, particularly in high-risk patients. While further large-scale studies are needed to validate these findings, incorporating ascitic calprotectin into diagnostic protocols could improve early detection and timely intervention, ultimately enhancing patient outcomes and survival rates.

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