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Association between serum level of pentraxin-3 and interleukin-40 with HCC disease in Egypt

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ABSTRACT

Background Hepatocellular carcinoma (HCC) is a global public health problem that lacks efficient methods for early diagnosis. The inflammation of the hepatocytes due to the cirrhosis caused by HCC can be reflected by the disturbance in the level of some proteins secreted by the inflammatory immune cells. We aimed to study pentraxin-3 (PTX3) and interleukin-40 (IL-40) blood levels in patients with HCC **Material and method** This study included 46 of HCC patients and 24 of control with no history of malignancy. All the volunteers are subjected to complete clinical examination and routine laboratory analysis including CBC, coagulation profile tests, liver and kidney function tests. Serum levels of AFP, interleukin-40, and pentraxin-3 were measured by the ELISA technique **Results** Both PTX3 and IL-40 blood levels were significantly higher in HCC patients compared to healthy controls ($P < 0.05$). In addition both of them high AUC under ROC curve with significant value (AUC= 0.97, P <0.001; AUC=0.745, P= 0.016) for PTX3 and IL-40 respectively. Almost no studies have discussed the role of IL-40, which is new characterized cytokine, with HCC disease. **Conclusions:** This study suggested that patients with HCC showed increased presence of PTX3 and IL-40 serum level which may suggest a potential relationship of both of them with HCC. Although the role of IL-40 in HCC pathogenesis

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and development is still unclear but several studies have revealed that HCC progression is solely dependent on the extent of liver inflammation, hence, the balance between pro-inflammatory and anti-inflammatory cytokines is the key ingredient for controlling the disease progression.

Introduction

HCC incidence rates have been rising in the past 3 decades, and these trends are expected to remain through 2030 [1]. According to the World Health Organization (WHO), Several important risk factors have been identified, including chronic HBV infection, chronic HCV infection, alcoholic liver disease, hereditary hemochromatosis, and any other causes for cirrhosis [2].

HCC staging system is designed with data from two sources. Firstly, prognostic biomarkers for cancer and cirrhosis. Secondly, treatment-dependent variables. In oncology, the standard classification of cancer is based on the TNM staging in accordance with the American Joint Committee on Cancer [3, 4].

Till now, most of screening tests for HCC diagnosis, such as AFP serum level, have not excellent sensitivity and specificity. More ever, some liver nodules may not release AFP, and also patients with chronic active hepatitis or liver cirrhosis may have high levels of AFP [5].

The treatment options for HCC include surgury, radiofrequency ablation, high-intensity focused ultrasound, targeted molecular therapy such as sorafenib and more rarely liver transplantation. The success of these treatments could be seriously improved by early cancer detection and effective post-treatment monitoring [6].

In the early stage of HCC, surgical resection, liver transplant, local ablation and other curative therapies can improve patient´s survival[7]. However, the 5-year recurrence rate is very high, it may reach as high as 80%-90% even the HCC patients have received potentially curative therapies [8]. It has been already advanced stage for most people when HCC was diagnosed [9].

Currently, well-tolerated combinations of direct-acting antivirals (DAAs) have largely replaced IFN-based therapy [10, 11]. More recent and larger studies have demonstrated that successful DAA therapy is associated with a 71% reduction in HCC risk [12].

IL-40 is the last cytokine to be discovered in October 2017 . It is one of the new characterized genes related to the immune system. It is mainly expressed by fetal liver, bone marrow, and activated B cells [13].

The gene encoding IL-40 is annotated in the human genome as C17orf 99, and encodes a small secreted protein (27 kDa) of 265 amino acids, including a 20-amino-acid signal peptide [13]. The latter observations suggest that it may play a role in the pathogenesis of certain human diseases [14].

Remarkably, IL-40 is not structurally related to any other cytokine family, indicating that it likely has unique evolutionary history [13].

PTX-3 is one of the pentraxin superfamily, and is produced by many cell types, such as phagocytes, DCs, fibroblasts and endothelial cells in response to primary inflammatory signals. PTX3 is acute-phase inflammatory protein. So, it is a new candidate prognostic marker for the mortality in various inflammatory diseases [15].

C-reactive protein (CRP) and serum amyloid P component (SAP) are short pentraxin. On contrast, Pentraxin 3 (PTX3) is long pentraxin. Gene targeted mice and epigenetic studies in humans suggest that PTX3 plays essential role in innate immunity and inflammation as well as in tissue remodeling [16].

In this study, we aimed to evaluate the role of two new inflammatory biomarkers (PTX3 and IL-40) in the diagnosis of HCC.

Patients and Methods

Patients

This study is composed of two groups; the first included 46 of HCC volunteers while the second group included 24 of age matched healthy volunteers as controls. All The healthy volunteers are chosen from patients out clinics in Zagazig university hospitals and patients were selected from those admitted at Gastroenterology and Hepatology unit, Zagazig university hospitals in the period of October 2017 till March 2018.

all the volunteers are subjected to complete full history about their living standards, family history of disease and Clinical Pathological Features **(Table1).**

Clinical parameters of HCC patients including tumor number, size, site, presence of metastasis, and portal vein thrombosis were recorded. None of patients received neoadjuvant chemotherapy, radiotherapy, or immunotherapy. Liver cirrhosis diagnosis was established on basis of clinical, laboratory, and imaging investigations. Patients suffering from diabetes mellitus (DM), obesity, dyslipidemia, chronic HBV infection or any other identifiable cause for chronic hepatitis other than HCV were excluded from this study. Additionally, heart failure, renal failure, proteinuria, active bacterial infections, and evidence of endocrine disorder or receiving hormone replacement therapy were also excluded.

The study protocol was approved by the Ethics Committees of Faculty of Medicine, Zagazig University. Informed written consent was obtained from each individual.

Sample Collection

Blood samples were collected under complete aseptic conditions by clean venipuncture using sterile disposable syringes. We divided blood sample into 2 portions: 1 ml of whole blood was collected into tubes containing EDTA for complete blood count (CBC) including hemoglobin, platelet, and total leukocyte counts. 2.5 ml of whole blood was collected into tubes containing citrate for PT,PTT,INR. About 5 ml of blood were withdrawn from each patient as well as controls. Blood was delivered into clean dry test tubes and allowed to clot at room temperature. 5 ml of whole blood was centrifuged at 1600 rpm for 5 min and the serum was aliquoted into 1.7 ml eppendorf tubes. Serum samples were stored in tightly closed vials at -80oC until used for miRNA extraction, pentraxin and biochemical analyses.

Routine laboratory analysis

Prothrombin time and international normalized ratio (INR) were performed for all patients. ALP,ALT, AST, albumin, total protein,total bilirubin, urea, and creatinine were measured in serum by routine enzymatic methods (spinreact).

Serum alpha fetoprotein (AFP) concentration was measured by ELISA (kit provided by Biosource Europe S.A, Belgium).

All patients were tested for anti-HCV antibodies in sera by ELISA, using third generation kits (Dia Sorin, Italy) according to the manufacturer's instructions.

Detection of Interleukin 40(IL-40) levels by ELISA

IL-40 assay were performed by Enzyme-Linked Immunosorbent Assay with serum samples collected, separated and stored in a freezer at -80 °C. The concentration of IL-40 in sera was detected with ELISA kit (NOVA, Bioneovan company; Synergy HT, biotec) following the manufacturers instruction.

PTX3 determination

PTX3 assay were performed by Enzyme-Linked Immunosorbent Assay with serum samples collected, separated and stored in a freezer at -80 °C. The concentration of PTX3 in sera was detected with ELISA kit (Sun Red Company; Synergy HT, biotec) following the manufacturers instruction.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS version 20.0). Qualitative date was represented as number and percentage, quantitative data were represented by mean ± SD, and the following tests were used to test for significance of mean differences between different groups. Differences between quantitative independent groups were tested by t test or Mann Whitney. Correlation between parameters was done by Pearson's correlation or Spearman's. ROC analysis was used to estimate the sensitivity and specificity of PTX3 and IL-40 as a diagnostic biomarker for HCC. P value was set at <0.05 for significant results &<0.001 for high significant result.

Results

Our study included two groups of HCC and healthy Egyptian volunteers. They are all subjected to the same measurements including routine laboratory analysis, serum IL-40 and PTX3 levels. Our data using statistical analysis showed a variety of the results. Some of the parameters were associated with HCC risk while others were not.

Routine laboratory measurements

Patients had a significant lower HB than controls (9.80, 12.65) respectively (*P* <0.001). In addition, they had a significant decrease in RBCs count than control (3.38, 4.53) respectively (*P* <0.001). on contract, patients had a higher WBCs level than the control group (9.13, 7.82) respectively but with no significance power (p = 0.987). Platelet count were significant lower in patients than controls (115.73, 241.83) respectively (*P* <0.001). PT, PTT and INR were significant higher in patients than control (*P* <0.001, 0.044, <0.001) respectively) **(Table 2).**

AFP were significant higher in patients rather than control (431.7, 2.73) respectively (*P* <0.001). While total protein was significant higher in control than patients (7.41, 5.99) respectively (*P*<0.001). Albumin as total protein was higher in control than patients (3.93, 2.29) respectively (*P*<0.001). Total bilirubin, AST and ALT were significant higher in patients than control (*P*<0.001, *P*<0.001, *P*<0.01). on contrast, ALP is higher in patients than control but with no significant power (*P*=0.604).

For kidney function tests, Urea level in patients were statistically significant than control (42.31, 11.14) respectively (*P*<0.001). Also, creatinine were statistically significant among patients than control (1.81, 0.81) respectively p value<0.002 **(Table2).**

Pentraxin assay and correlation with other biochemical parameters

Our results showed that Pentraxin was higher in patients than control (21.64, 7.35) respectively (*P*<0.001) **(Table 2).**

Pentraxin was excellent negative correlated with HB, RBCs and PLTs (*P*<0.001 <0.001, 0.003) respectively. Pentraxin had a positive excellent correlation with PT and INR (*P* <0.001.). There were no significant correlation between pentraxin and both WBCs and PTT (*P*> 0.05) **(Table 3).**

Pentraxin was good negative correlated with Total protein, total

Bilirubin and Albumin (P<0.001). Pentraxin had a positive excellent correlation with AFP (P<0.001). There was a good positive correlation between pentraxin, AST and ALT (P <0.001,<0.01). While not significant correlated with alkaline phosphatase (P> 0.05) **(Table 3).**

Pentraxin was good positively correlated with urea and creatinine (P <0.001, <0.01) **(Table 3).**

Interleukin-40 assay and correlation with other biochemical parameters

Our results showed that IL-40 was higher in patients than control (8.068, 3.173) respectively **(Table 2).**

IL-40 was excellent negative correlated with HB (P =0.085). On contrast, there was no correlation with other hematological parameters including RBCs, WBCs, PLTs, PT, PTT, and INR (P> 0.05) **(Table 3)**.

IL-40 was negative correlated with Albumin significantly (P = 0.025) and positive correlated with Total protein (p=0.036) **(Table 3).**

No correlation was found between IL-40 and other biochemical parameters including AFP, ALP, AST, ALT,total bilirubin, urea, creatinine (P> 0.05) **(Table 3).**

Correlation between interleukin-40 and pentraxin-3

There was significant positive correlation between IL-40 and PTX-3 (P=0.028) **(Table 3).**

Receiver operating characteristic curve

Receiver operating characteristic curve (ROC) analyses were carried out to evaluate the diagnostic value of serum interleukin-40 and pentraxin-3 in comparison to AFP.

The ROC curve analysis (AUC) value of AFP was (0.975, P< 0.001), with a sensitivity of 96.2% and a specificity of 0.100 % and cut off value of 41.05 **(Table 4, Fig 1)**.

The ROC curve analysis (AUC) value of pentraxin-3 was (0.994, P< 0.001), with a sensitivity of 96.2% and a specificity of 0.97.1% and cut off value of 19.45 **(Table 4, Fig 2).**

The ROC curve analysis (AUC) value of interleukin-40 was (0.745, P=0.016), with a sensitivity of 73.1% and a specificity of 0.66 % and cut off value of 6.497 **(Table 4, Fig 3)**.

Association between PTX-3 and IL-40 with disease severity

PTX-3 Serum levels are positively correlated with disease severity by comparing between presence and absence of ACLF (Fig.4), it was observed that patients with ACLF showed higher PTX3 medians than those without ACLF (8.0 vs.3.1 ng/mL; p < 0.001). When comparing PTX3 levels according to MELD score (Fig.5), it was observed that patients with MELD higher than 20 had higher median PTX3 levels compared to others (6.7 vs.3.4 ng/mL; p = 0.002).

For IL-40, there was no association between its level in blood and disease severity (p>0.05).

Discussion

Due to the absence of efficient biomarkers for early HCC diagnosis, we aimed in our study to evaluate the diagnostic impact of two proteins which are related to the inflammation caused by HCC and liver cirrhosis. We suggested the relation between PTX-3 and IL-40 serum levels with HCC pathogenesis and development.

The role of PTX-3 in cancer progression was discussed in several types of cancer [17-19]. However, limited studies discussed its role on HCC progression. Our study showed significant increase in PTX-3 level in HCC patients compared to control. This was in agreement with Carmo and his colleagues study (2016) who examined the PTX3 polymorphisms and its plasma levels in chronic hepatitis C patients and they found increase in

plasma PTX3 level in HCC patients infected with HCV than those with mild or severe fibrosis. And they revealed a remarkable correlation between PTX3 polymorphisms (rs1840680 and rs2305619) and HCC occurrence [20].

PTX3 plays an important role in pathogen invasion, inflammatory response, and clearance of apoptotic cells. In normal conditions, the serum level of PTX3 is very low. While in the inflammatory environment such as infection, autoimmune, or metabolic disease, it increases sharply [21].

More interesting, our results showed that PTX-3 Serum levels are positively correlated with disease severity by comparing between presence and absence of ACLF (Fig.4), it was observed that patients with ACLF showed higher PTX3 medians than those without ACLF (8.0 vs.3.1 ng/mL; $p < 0.001$). When comparing PTX3 levels according to MELD score (Fig.5), it was observed that patients with MELD higher than 20 had higher median PTX3 levels compared to others (6.7 vs.3.4 ng/mL; $p = 0.002$). This was supported by the results found by Pereira and his team (2017) who found correlation between PTX-3 serum level and HCC severity.

For IL-40, our results showed significant increase in IL-40 serum level in HCC patients compared to control. The relation between IL-40 and cancer development is still in its infantile stage since there has not been a comprehensive cross-reference and/or systematic analysis made for solid establishment of an oncological concept for its role. This interleukin is generally more difficult to identify (because they do not share structural/genetic similarities to other cytokines), so they were generally identified through screening of genomic databases or other bioinformatics-based screening methods [14].

Researchers analyzed the expression of IL-40 by activated B cells. They found that IL-40 can be produced by spleen B cells upon activation with anti-IgM, anti-CD-40, and IL-4, but that their ability to produce IL-40 increases significantly if the B cells are polarized in vitro with TGF-b[13]. In addition, they observed that human B cell lymphoma cell lines (OCI-Ly1) constitutively express IL-40, suggesting a potential role of IL-40 in human B cell-associated diseases [13].

It is worth noting that our results showed higher diagnostic impact for PTX-3 with AUC 0.994 ($p<0.001$) than AFP. In addition, our results showed good diagnostic impact for IL-40 with AUC 0.745 ($p=0.016$). This was supported by the positive correlation between PTX-3 and IL-40 ($P=0.028$). This indicates that the combination between the three biomarkers (AFP, PTX-3 and IL-40) can give high scoring for diagnosis strategy of HCC.

Conclusion

In our study higher levels of PTX-3 and IL-40 are observed in HCC patients compared to healthy. Serum PTX3 is related to the severity of the disease. These results are promising and indicate a potential use for PTX3 and IL-40 as an inflammatory and prognostic biomarker for patients with HCC and liver cirrhosis.

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Table 1: Clinical Pathological Features of HCC Patients

Clinical stage	**n= 46**
Stage I/II	**21**
Stage III/IV	**25**
Tumor size	
< 5 cm	**20**
> 5 cm	**26**
Lymph node metastasis	
Absent	**27**
Present	**19**
Distant metastasis	
Absent	**22**
Present	**24**
Portal vein thrombosis	
Negative	**35**
Positive	**11**
Number of tumor lesions	
Single	**24**
Multiple	**22**
Site of lesions	
Right lobe	**11**
Left lobe	**13**
Both	**22**

Table (2): Biochemical parameters measured in both HCC patients and control

Variables	HCC patients n=46 Mean ±SD (Min-Max)	Control n=24 Mean ±SD (Min-Max)	Test [a]	P
HB g/dl	9.80±1.90 (5.80-13.60)	12.65±1.53 (10.60-15.50)	-4.937	<0.001***
RBCs (10^6/µl)	3.38±0.97 (2.10-6.20)	4.53±0.47 (3.50-5.20)	-3.890	<0.001***
WBCs (10^3/ µl)	9.13±6.80 (2.0-28.90)	7.82±1.53 (5.70-11.30)	-0.016	0.987
PLT (10^3/ µl)	115.73±100.32 (24.0-491.0)	241.83±77.24 (158.0-388.0)	-3.895	<0.001***
PT (sec)	19.5±2.8 (13.8-27.1)	13.7±1.8 (11.2-16.7)	7.71	<0.001***
PTT (sec)	46.5±12.2 (30.9-83.3)	38.0±1.8 (36.1-39.7)	-2.012	0.044*
INR (%)	1.73±0.27 (1.19-2.60)	1.15±0.16 (0.97-1.42)	8.145	<0.001***
AFP (ng/ml)	431.70±390.14 (78.0-1280.0)	2.73±0.95 (1.10-4.10)	-3.956	<0.001***
Total protein (g/dl)	5.99±1.08 (4.32-9.74)	7.41±0.70 (5.53-8.16)	-4.868	<0.001***
Total Bilirubin (mg/dl)	5.16±4.23 (0.92-15.70)	0.55±0.33 (0.21-1.14)	-4.863	<0.001***
Albumin (g/dl)	2.29±0.31 (1.68-2.90)	3.93±0.38 (3.27-4.46)	-13.020	<0.001***
AST (U/L)	86.61±70.02 (17.30-337.20)	22.31±5.44 (16.20-35.40)	-4.303	<0.001***
ALT (U/L)	44.18±32.99 (8.80-171.10)	20.44±5.76 (12.00-34.40)	-2.952	0.003**
Urea (mg/dl)	42.31±28.89 (8.30-110.10)	11.14±4.19 (6.80-19.0)	-3.863	<0.001***
Creatinne (mg/dl)	1.81±1.36 (0.49-5.73)	0.81±0.15 (0.60-1.02)	-3.11	0.002**
ALP U/L	103.08±43.88 (0.00-197.0)	92.33±24.62 (51.0-133.0)	-0.518	0.604
PTX-3 (ng/ml)	21.64±2.44 (19.15-28.0)	7.35±3.81 (2.18-13.29)	-4.899	<0.001***
IL-40 (pg/ml)	8.068±3.46 (0.205-11.746)	3.173±4.61 (0.043-14.81)	-2.405	0.016*

Data are represented as mean ± SD

a Mann Whitney test

* $P < 0.05$ is significant, ** $P < 0.01$ is very high significant, *** $P < 0.001$ is very high significant

HB= hemoglobin, RBCs= red blood cells, WBCs= white blood cells, PLTs= platelets, PT=prothrombin time, PPT= partial thromboplastin time, INR= international concentration,, ALT= alanine transaminase, AST= aspartate transaminase, ALP= alkaline phosphatase, AFP= alpha feto protein, PTX-3= pentraxin-3, IL-40= interleukin-40

Table 3: Correlation between both pentraxin-3 and Interleukin-40 with other biochemical parameter in HCC patients

Variables	r[a]	P	r[a]	P
	Pentraxin-3		Interleukin-40	
HB	-0.963	<0.001***	-0.284	0.085*
RBCs	-0.574	<0.001***	-0.143	0.393
WBCs	0.149	0.373	-0.098	0.557
PLT	-0.474	0.003**	-0.241	0.145
PT	0.751	<0.001***	0.146	0.382
PTT	0.454	0.051	0.050	0.838
INR	0.725	0.001**	0.190	0.252
AFP	0.800	<0.001***	0.108	0.633
Total protein	-0.649	<0.001***	0.341	0.036*
Total Bilirubin	-0.691	<0.001***	0.228	0.169
Albumin	-0.669	<0.001***	-0.363	0.025*
AST	0.625	<0.001***	0.257	0.120
ALT	0.505	0.001**	0.153	0.358
ALP	0.159	0.340	-0.235	0.155
Urea	0.658	<0.001***	0.253	0.125
Creatinine	0.523	0.001**	0.306	0.062
PTX-3	-	-	0.357	0.028*
IL-40	0.357	0.028*	-	-

a Pearson's correlation

* $P < 0.05$ is significant, ** $P < 0.01$ is very high significant, *** $P < 0.001$ is very high significant

HB= hemoglobin, RBCs= red blood cells, WBCs= white blood cells, PLTs= platelets, PT=prothrombin time, PPT= partial thromboplastin time, INR= international concentration,, ALT= alanine transaminase, AST= aspartate transaminase, ALP= alkaline phosphatase, AFP= alpha feto protein, PTX-3= pentraxin-3, IL-40= interleukin-40

Table (4): ROC Curve for AFP, Pentraxin-3 and interleukin-40

Parameter	AUC	SE	*P*	Cut Off	Sn	Sp	PPV	NPV	95% CI	
									Lower Bound	Upper Bound
AFP	0.975	0.007	<0.001***	41.05	90.9%	100.0%	100.0%	91.7%	0.918	1.000
PTX-3	0.994	0.009	<0.001***	19.45	96.2%	91.7%	97.2%	92.8%	0.997	1.000
IL-40	0.745	0.097	0.016*	6.497	73.1%	66.7%	82.6%	53.3%	0.556	0.935

* $P < 0.05$ is significant, ** $P < 0.01$ is very high significant, *** $P < 0.001$ is very high significant

AFP= alpha feto protein, PTX-3= pentraxin-3, IL-40= interleukin-40

Sn= sensitivity, Sp= specificity, PPV= positive predictive value, NPV= negative predictive value, AUC= area under curve, SE= standard error

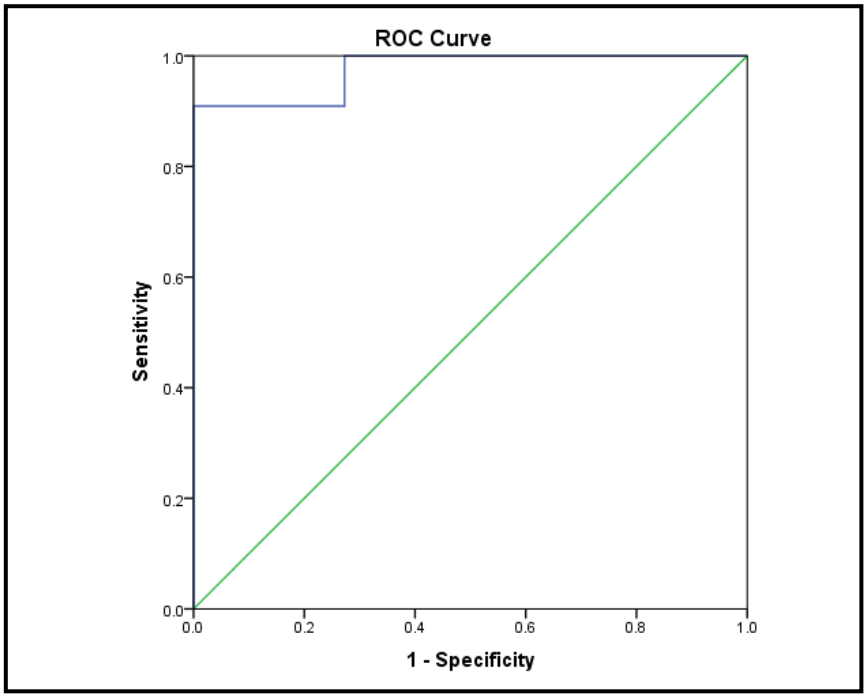

Fig (1): ROC curve for AFP in HCC patients

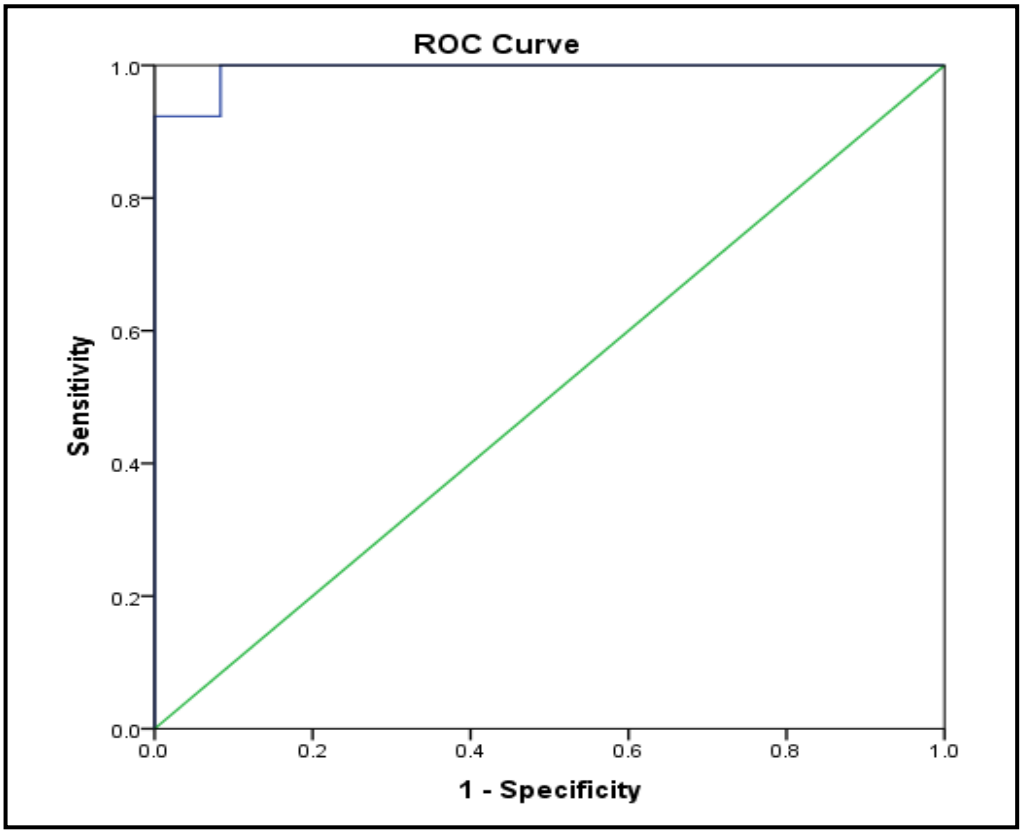

Fig (2): ROC curve for Pentraxin-3 in HCC patients.

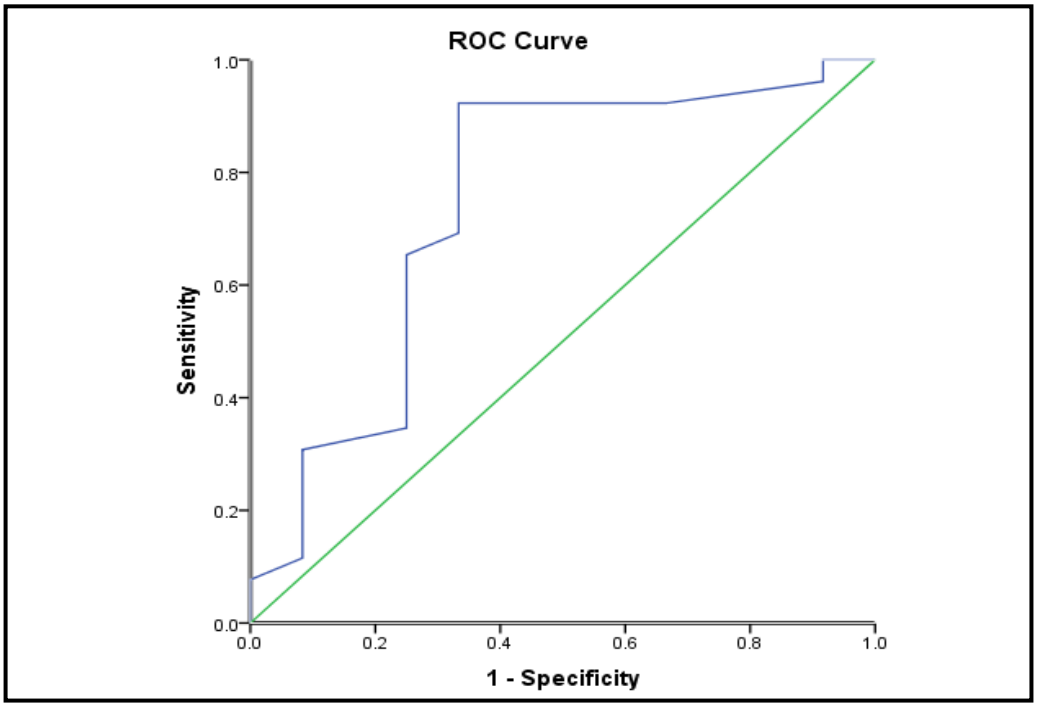

Fig (3): ROC curve for Interleukin-40 in HCC patients.

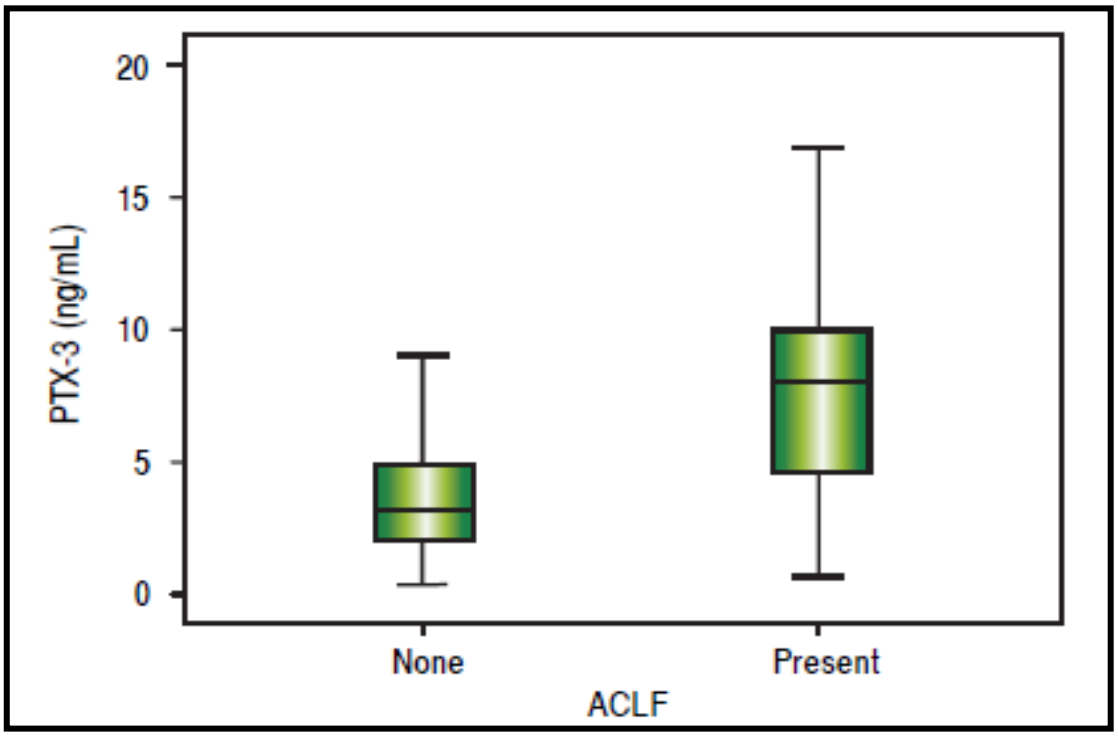

Fig (4): Pentraxin 3 levels according to the presence or absence of Acute-on-Chronic Liver Failure (ACLF)

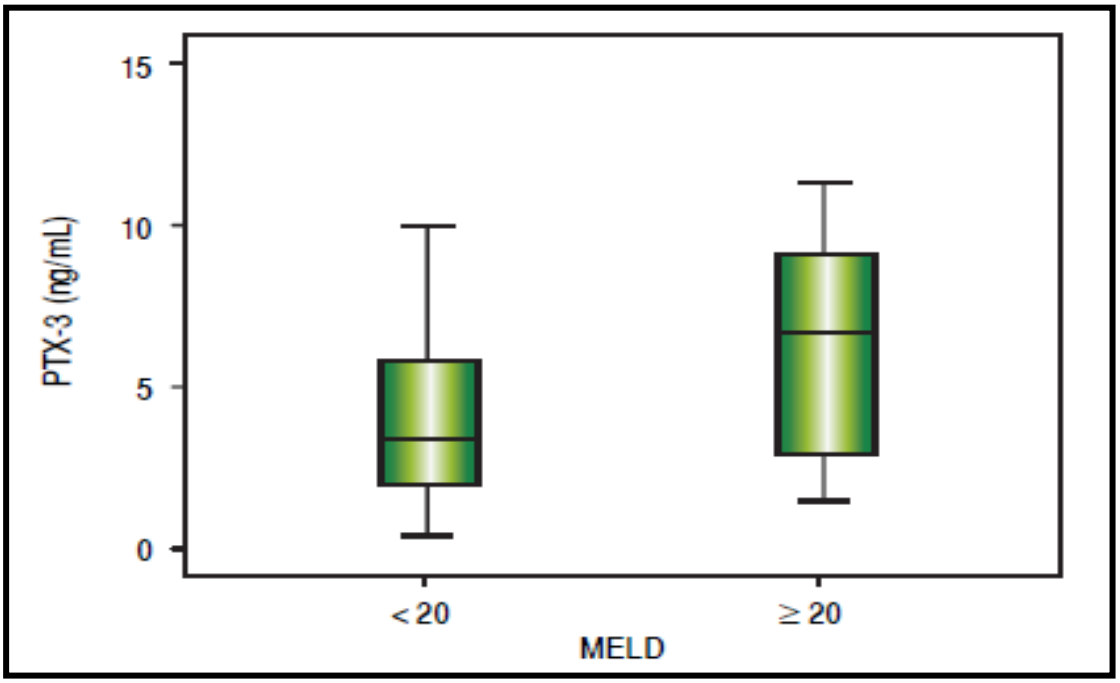

Fig (5): Pentraxin-3 levels according to Model for End-Stage Liver Disease (MELD)

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