The Egyptian Journal of Hospital Medicine (October2023) Vol. 93, Page 6970-6979

Clinical Significance of Serum Angiopoietin 2 in Patients with Hepatocellular Carcinoma

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ABSTRACT
Background: Angiopoietin 2 (ANG-2) is essential for blood vessel development, stability, and remodeling.
Objective: We aimed to find out the potential use of serum ANG-2 as a diagnostic indicator for early detection of hepatocellular carcinoma (HCC).
Patients and Methods: The present investigation included 80 participants who were categorized as follows: 30 individuals diagnosed with liver cirrhosis, 30 individuals diagnosed with HCC, and 20 healthy individuals serving as control group. The ages of participants ranged between 35 and 65 years.
Results: ANG-2 levels demonstrated a significant increase within the HCC group compared to the other two study groups and also in the liver cirrhosis group if compared to the healthy individuals. Age, bilirubin, aspartate aminotransferase, Child-Pugh score, alanine transaminase, Barcelona Clinic Liver Classification stages, and tumor characteristics were significantly positively correlated with ANG-2 in the HCC group. A statistically significant negative connection was observed between the levels of albumin and ANG-2, as well as between ANG-2 and platelets.
Conclusions: ANG-2 has the potential to serve as a diagnostic indicator in HCC and may also play an important function in controlling the advancement of liver disease. Additionally, it has been demonstrated to be very valuable in identifying individuals with aggressive tumors**.**
Keywords: Serum angiopoietin, Hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer. Its prevalence is rising in Egypt as a result of potential increased risk factors such as viral hepatitis C and hepatitis B [(1)]. HCC is considered one of the hyper-vascular tumors, and the mechanism of its proliferation is mainly associated with the angiogenesis process. Tumor angiogenesis is considered the hallmark of cancer that promotes tumor growth and metastasis. Factors involved in tumor angiogenesis are mainly angiopoietins as (Ang 2), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) [(2)].

Ang 2 has an important marker in the angiogenesis process during the progression of different human malignancies such as HCC, lung cancer, and breast cancer [(3)]. Ang 2 contains a total of 478 amino acids. It mainly has two domains, one coiled-coil domain (aa 166-248) that is responsible for mediating multimerization in addition to a C-terminal fibrinogen-like domain (aa 275-495) that is responsible for mediating receptor binding. The gene Ang 2 is significantly expressed within HCC. A tyrosine kinase receptor family member, who is known as Tie 2, is responsible for Ang 2 action. HCC neovascularization and development are both significantly influenced by the Ang 2-Tie 2 pathway [(4)].

Through this study, we aimed to find out if serum ANG 2 might be used as a diagnostic tool for early diagnosis of (HCC).

PATIENTS AND METHODS

The present investigation included about 80 participants assigned as the following: 30 liver cirrhosis patients as G II, 30 Hepatic cell carcinoma patients as G III, and 20 healthy people as the control group, all of whom were between the ages of 35 and 65.

Our investigation was authorized by the Tanta University Hospitals Ethical Committee in Egypt. Before being included in the research, patients or their family provided an informed written agreement.

Exclusion criteria: Mixed HCC–cholangiocellular carcinoma or other extrahepatic malignancies, previous HCC ablation history, and other comorbidities.
All patients had a full clinical examination, computed tomography (CT), magnetic resonance imaging (MRI), abdominal ultrasonography, radiological HCC studies, and real-time abdominal ultrasound (US).

Child Pugh scoring of HCC and liver cirrhosis: The scoring system used bilirubin, serum albumin, neurological disorder, ascites and INR. The severity of cirrhosis using the **Child-Pugh system:** Child-Pugh A gets from five to six points, child-Pugh B gets about seven to nine points, and child-Pugh C gets from ten to fifteen points.

Encephalopathy scoring system: None gets one point, Grades one & two get two points, and Grades three & six get three points. **Ascites scoring system:** None gets one point, slight gets two points, and moderate gets three points. **INR scoring system:** below 1.7 gets 1 point, from 1.7 to 2.2 gets two points, INR more than 2.2 gets three points.

Albumin scoring system: higher than 3.5 mg/ml gets one point, between 2.8 to 3.5 mg/ml gets two points, and lower than 2.8 mg/ml gets three points. **Bilirubin**

Received: 25/06/2023
Accepted: 25/08/2023

scoring system: below 2 mg/ml gets one point, from 1 to 3 mg/ml gets two points, and more than 3 mg/ml gets three points.

Barcelona Clinic Liver Cancer (BCLC) HCC staging system:

Stage 0 (Very Early Stage) refers to a tumor that is less than two centimeters in size, has an excellent performance status (PS 0), and the liver is functioning properly (Child-Pugh A). Stage A (Early Stage) refers to either just a single tumor lesion of any size or up to 3 tumor lesions that are all smaller than three centimeters in size. Stage B (Intermediate Stage): The liver contains several tumors, but its performance status is good and active (PS 0), and the liver is functioning normally (Child-Pugh A or B). Stage C (Advanced Stage): In this case, the cancer has undergone metastasis to the lymph nodes, blood arteries, or other anatomical sites inside the body and the liver is less active with no excellent performance status (PS one or two) but still operating well (Child-Pugh A or B). Stage D: Severe liver damage (Child-Pugh C), not good performance status (PS three or four). Routine laboratory tests: Complete blood cell count by automatic blood cell counter (ERMA), liver function tests by full automated chemistry analyzer (konelab Thermoscientific prime 60), alpha feto protein (AFP) by TOSHO AIA -360 automated immunoassay analyzer, virology of HCV by Murex anti-HCV ELISA kit and virology of HBV by Murex HBsAg ELISA kit. Specific laboratory investigations: Measurement of Ang 2 by ELISA technique.

Measurement of Ang_2 using ELISA technique: 50µl standard was added followed by about 50µl Streptavidin –HRP in standard wells, to test well: 40 µl from each sample was added, and then about 50µl of Streptavidin –HRP + 10ul of (ANG-2) antibody were both added into the plate, after that, the sealing membrane was firmly sealed, shacked gently & incubated at temp., 37°C for 60 minutes. In order to prepare a washing solution, it was 30 times diluted using distilled water. After gently removing the membrane, the liquid was drained, and any residual water was thrown away. Each well of the plates received 50 l of Chromogen A and 50 l of Chromogen B. And then mixed gently and also incubated at a temp of 37 °C for 10 minutes without light. 50 µl of the Each well of the (ANG -2) plates were filled with stop solution. in order to stop the reaction, which resulted in the blue color instantly converting to yellow.

Final measurement: The optical density (OD) was measured under 450 nm wavelength around 15 minutes after the stop solution was poured, using the blank well as the standard. While, the equation of standard curve linear regression had been calculated based on the samples' OD values, the standard concentration, and the associated OD values were then used within the regression equation to estimate the concentration of the relevant sample.

Ethical Approval: The research was authorized by Tanta University's Ethics Board, and the patients were provided all of the information they needed regarding the experiment. Each participant in the research provided written informed permission. This experiment was done in compliance with the World Medical Association's Code of Ethics (Declaration of Helsinki) for human research.

Statistics Analysis

The statistical analysis was carried out using SPSS version 22. Quantitative measures were compared for the same group using a paired Student's t-test. These measures were presented in terms of mean ± standard deviations (SD), while percentage (%) and frequency were used to present the qualitative measures. In order to compare more than two different averages, the ANOVA test was carried out. To assess the two groups qualitatively in respect to one another, the chi-square test was used. The goal of regression analysis is to investigate the effect of one or more independent variables on a dependent variable. A statistically significant level was decided to be a two-tailed $P \leq 0.05$.

RESULTS

Patients diagnosed with HCC tended to be older than those diagnosed with cirrhosis and those in the healthy group, resulting in a highly important difference between the three groups. The average value of ALT, AFP, total bilirubin, AST, and direct bilirubin in HCC group showed a highly statistically significant increase in comparison with other research groups. The mean value of albumin was found to be considerably lower in both HCC and liver cirrhosis groups when compared to the study control group (Table 1).

Table (1): Age, sex, AFP, Hemoglobin levels, direct bilirubin, total bilirubin and platelets count, AST, Albumin, ALT, and Total leucocytic count of the studied groups.

		Control	Cirrhosis	HCC	F. test	p. value	
Age (in years)		50.60±3.82	50.27±4.25	53.23±4.70	`4.060	0.021*	P1=0.790 P2=0.038* P3=0.010*
Sex	**Men**	14(70.0%)	17(56.7%)	17(56.7%)	**X^2=1.111**	**0.574**	
	Women	6(30.0%)	13(43.3%)	13(43.3%)			
HB (g/dl)		12.69±0.71	10.42±0.67	10.07±1.00	67.634	0.001*	P1=0.001* P2=0.001* P3=0.106
WBCs (10^3 / µl)		6.8±1.024	4.47±0.75	4.42±0.63	56.051	0.001*	P1=0.001* P2=0.001* P3=0.846
Platelets (10^3 / µl)		244.4±61.34	112.9±15.24	77.0±9.25	139.356	0.001*	P1=0.001* P2=0.001* P3=0.001*
ALT (IU/l)		22.15±4.00	85.30±11.98	91.37±12.14	291.864	0.001*	P1=0.001* P2=0.001* P3=0.030*
AST (IU/l)		22.70±3.81	65.13±11.02	85.23±10.58	258.985	0.001*	P1=0.001* P2=0.001* P3=0.001*
Albumin (g / dl)		4.52±0.65	2.48±0.37	2.24±0.22	204.483	0.001*	P1=0.001* P2=0.001* P3=0.026*
Total bilirubin (mg/dl)		0.85±0.17	2.13±0.51	3.14±0.27	238.777	0.001*	P1=0.001* P2=0.001* P3=0.001*
Direct bilirubin (mg/ dl)		0.28±0.09	1.05±0.16	1.21±0.25	158.403	0.001*	P1=0.001* P2=0.001* P3=0.002*
AFP		4.58±0.88	31.00±4.04	351.30±11.14	221.951	0.001*	P1=0.183 P2=0.001* P3=0.001*

The investigations are demonstrated as standard deviation ± average. HB stands for hemoglobin, while Aspartate aminotransferase is abbreviated as AST. ALT stands for alanine transaminase. Alpha-fetoprotein is abbreviated as AFP. There was a notable and statistically significant variation in HCV virology across the study groups. While, concerning the Child-Pugh classification, there was a substantial difference between the groups with hepatic cirrhosis and HCC (Table 2).

Table (2): Classification of research groups based on HCV virology, using the Child-Pugh classification

		Control	Cirrhosis	HCC	Total	X^2	P-value
Virology HCV	**Positive**	0	30	30	60	**80.001**	**0.001***
		0	100.0%	100.0%	75.0%		
	Negative	20	0	0	20		
		100.0%	0.0%	0.0%	25.0%		
Child Pugh	**A**	-	27	20	47	4.812	0.028
			90.0%	66.7%	78.3%		
	B	-	3	10	13		
			10.0%	33.3%	21.7%		

Data showed as frequency (%). HCV: Hepatitis C virus

Table (3) showed the BCLC stages of the HCC group and tumor characteristics.

Table (3): Stages of BCLC in the HCC group

	N	%
BCLC stages		
O – A	19	63.3
B	9	30
Tumor numbers		
≤ 2	26	86.7
> 2	4	13.3
Total	30	100
Tumor diameter		
≤ 5 cm	26	86.7
> 5 cm	4	13.3
Total	30	100
Macrovascular invasion		
No	25	83.3
Yes	5	16.7
Total	30	100
Extrahepatic metastasis		
M 0	26	86.7
M 1	4	13.3
Total	30	100
Lymph node metastasis		
N 0	25	83.3
N 1	5	16.7
Total	30	100

Data are presented as frequency (%). BCLC: Barcelona Clinic Liver Classification.

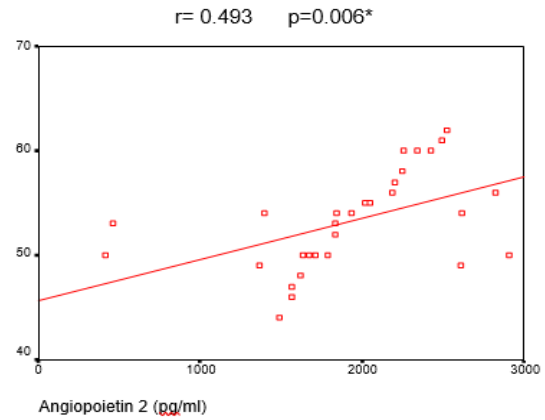
When compared to the other research groups, the spike in the average value of Ang 2 in the HCC group was highly statistically significant. as compared to the healthy study group, the rise in the average value of Ang 2 in the liver cirrhosis group was statistically significant (Table 4).

Table 4: Ang 2 in the studied groups

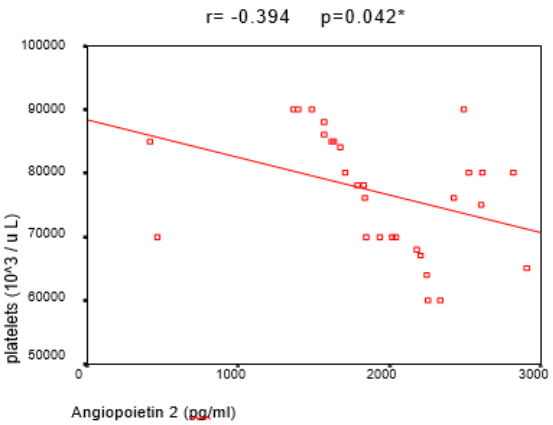
	Control	Cirrhosis	HCC	F. test	p. value	
(ANG-2) (Pg /ml)	148.29±6.62	514.45±47.75	1927.93±86.51	168.086	0.001*	P1=0.013* P2=0.001* P3=0.001*

Data are presented as mean ±SD

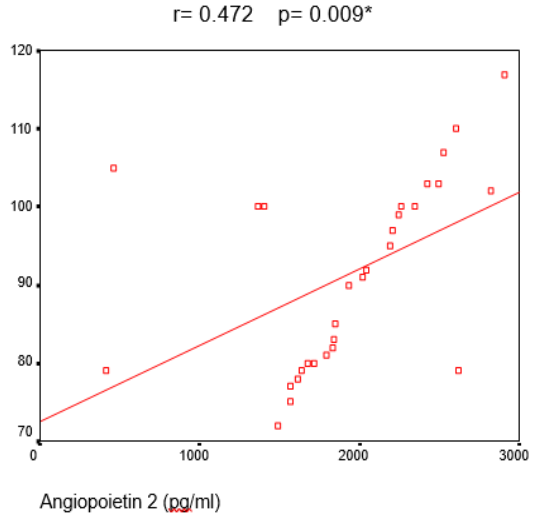
There was no connection identified between sex and Ang 2. While, a positive significant correlation between Ang 2 and AST, ALT, total bilirubin, direct bilirubin, age, Child-Pugh score, tumour characteristics, and BCLC stages was observed in the HCC group. Finally, a negative significant association was discovered between Ang 2 and albumin, as well as platelets (Figures 1 & 2 and table 5).


r= 0.493 p=0.006*
Angiopoietin 2 (pg/ml)

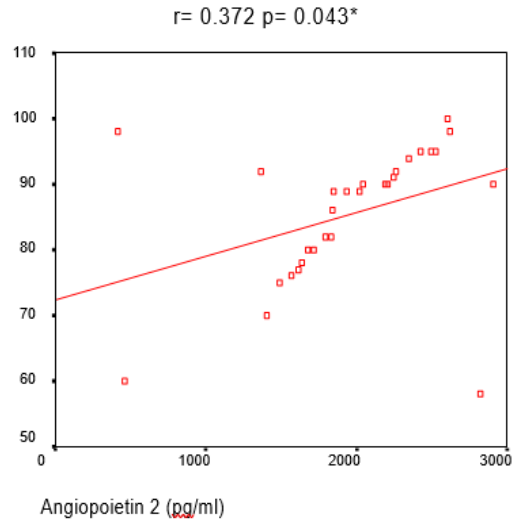
A


r= -0.394 p=0.042*
platelets (10^3 / u L)
Angiopoietin 2 (pg/ml)

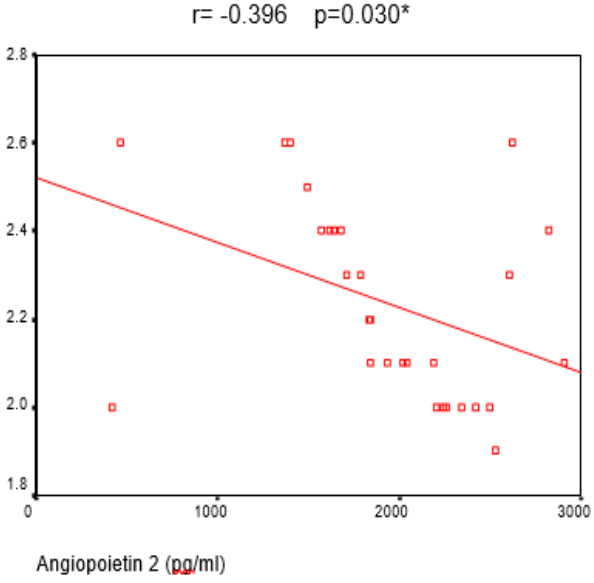
B


r= 0.472 p= 0.009*
Angiopoietin 2 (pg/ml)

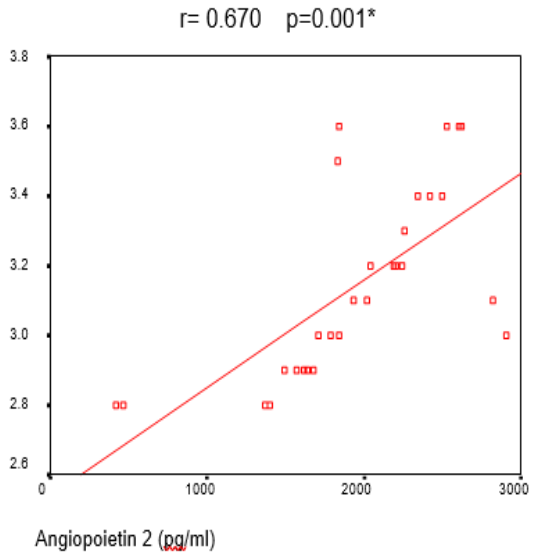
C


r= 0.372 p= 0.043*
Angiopoietin 2 (pg/ml)

D


r= -0.396 p=0.030*
Angiopoietin 2 (pg/ml)

E


r= 0.670 p=0.001*
Angiopoietin 2 (pg/ml)

F

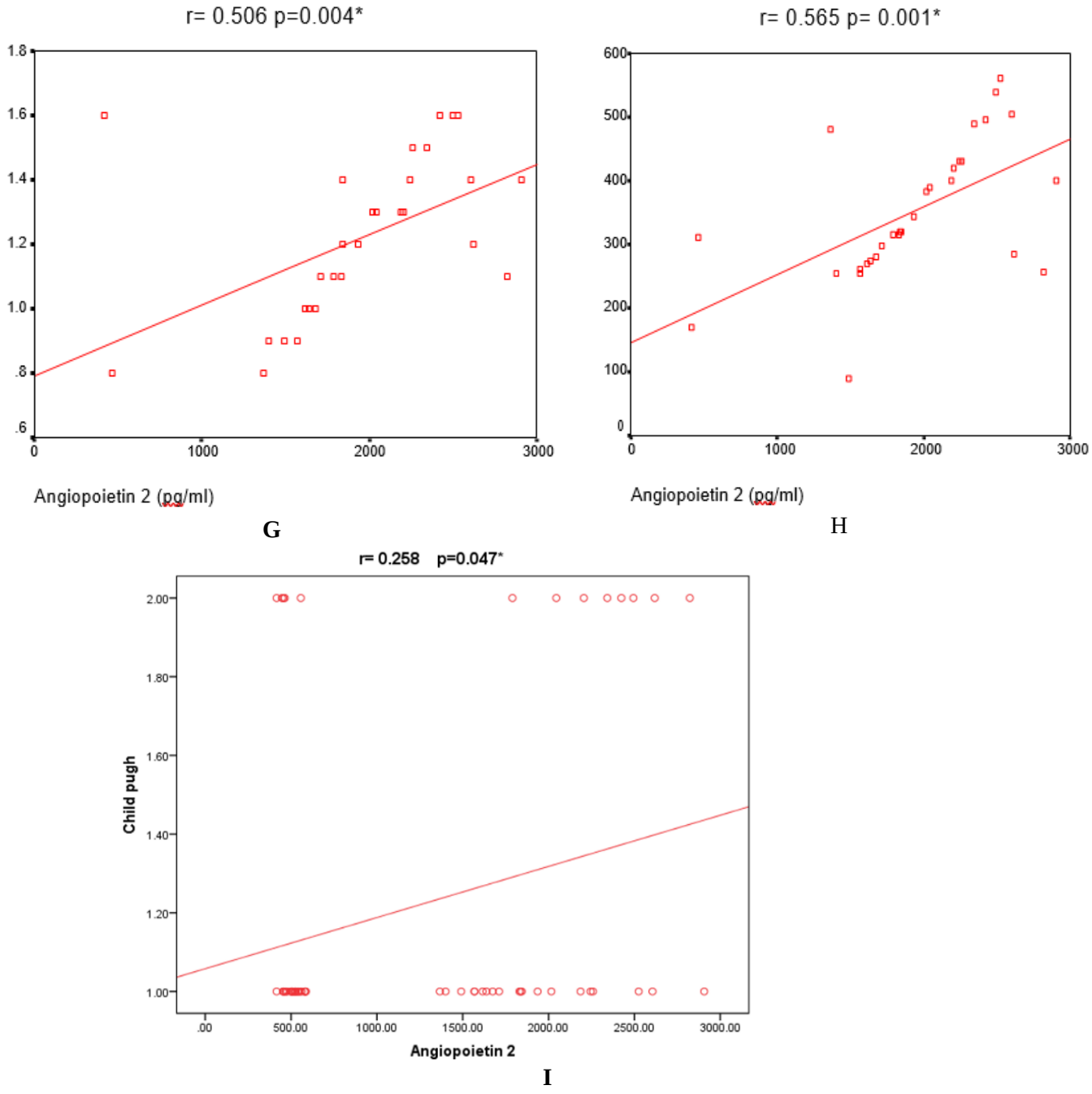


Figure (1) : Positive correlation between (ANG 2) and (D) AST, (A) age,(C) ALT, (G) direct bilirubin, (F) total bilirubin, (H) AFP & (i) Child-Pugh sc. A negative correlation of (ANG 2) with (B) platelet count & (E) albumin.

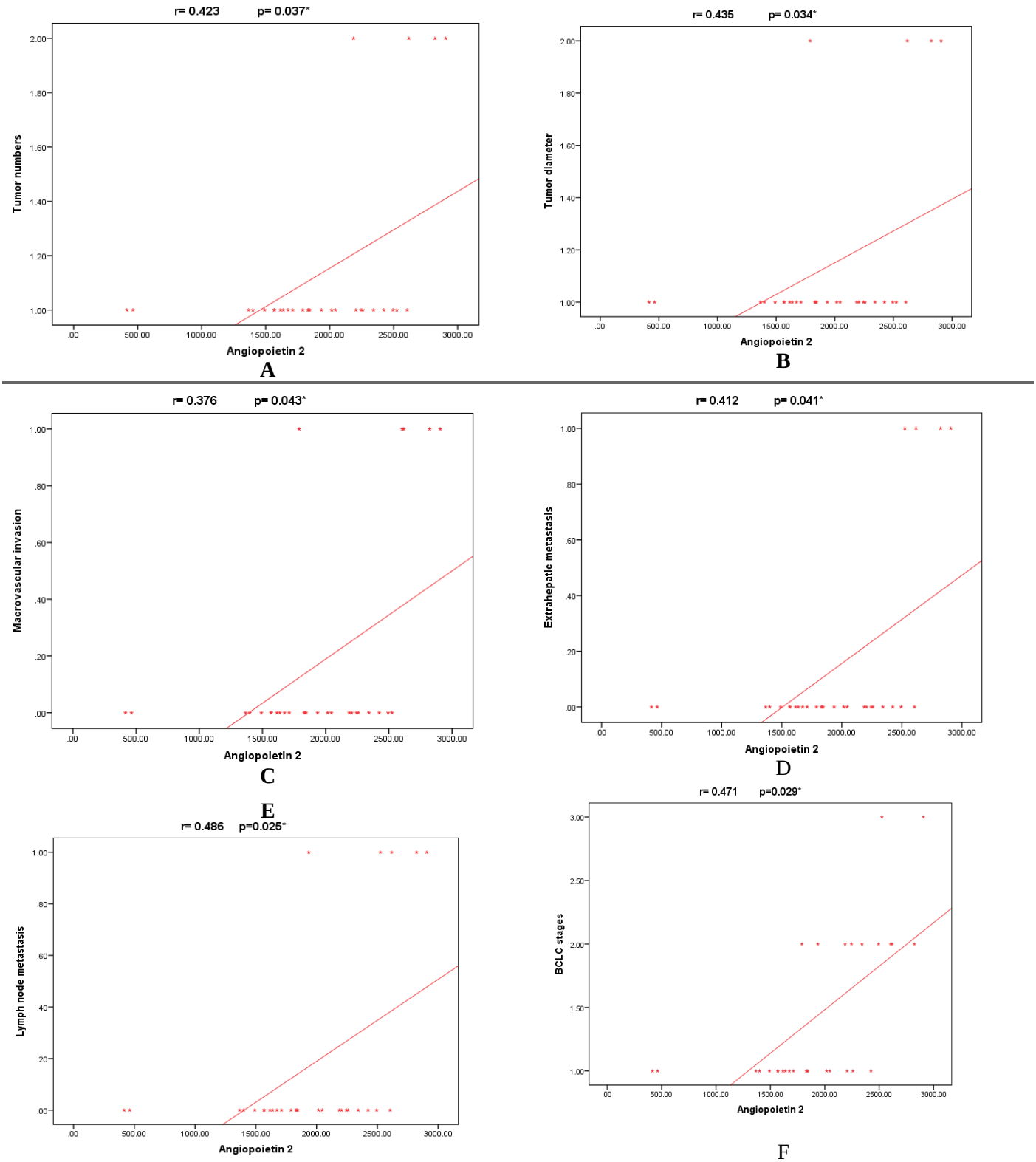


Figure 2: A positive correlation of ANG 2 with tumor (A) numbers, (B) diameter, (C) Macrovascular invasion, (D) extrahepatic metastasis, (E) lymph node metastasis & (F) BCLC stage.

The best cut-off level for ANG 2 in discriminating all patients from healthy, HCC from the research control group, and HCC from cirrhosis group was 420 pg/ml, 430 pg/ml and 580 pg/ml with an AUC of 0.998, 0.964 & 0.940 yielding a sensitivity of 97%, 93% & 90%, specificity of 95%, 90% & 83%, positive predictive value (PPV) of 98%, 91% & 84%, negative predictive value (NPV) of 90%, 93% & 89% and accuracy of 96%, 92% & 87% respectively (Figure 3).

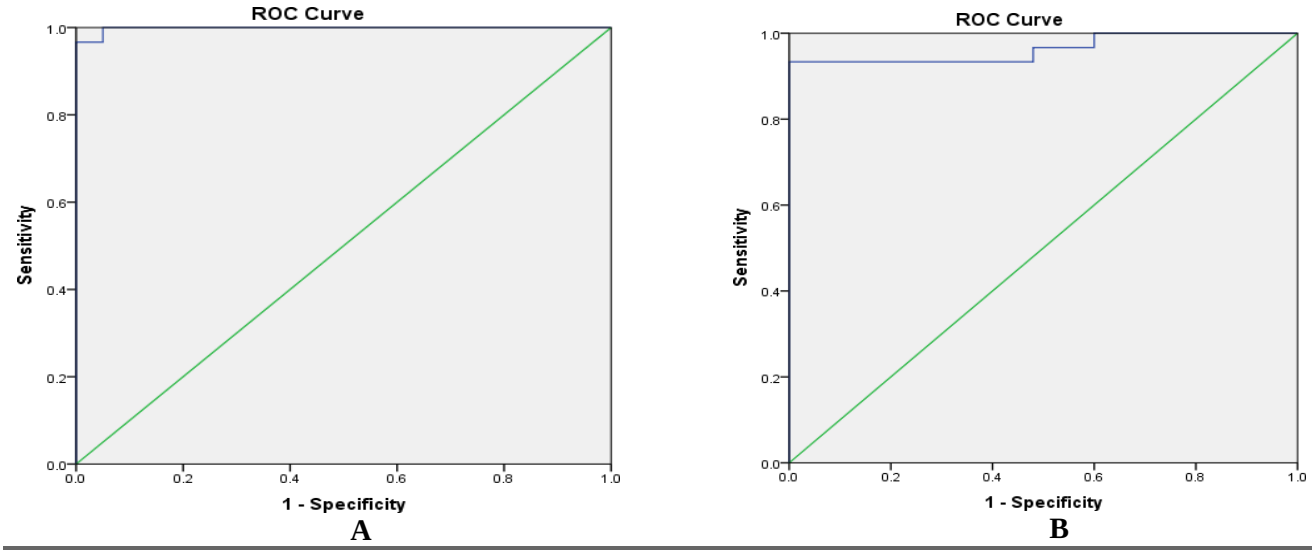


Figure 3: ROC curve of (ANG-2)2 in (A) the patients and control group, (B) HCC and control group, (C) HCC and Cirrhosis group.

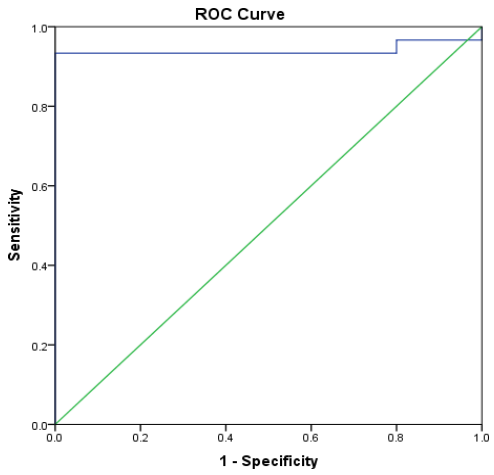


Table 5: Relation between (ANG 2) and other variables

	Univariate		Multivariate	
	HR (95% CI)	**P value**	**HR (95% CI)**	**P value**
Age	0.534 (0.354 – 0.745)	0.018*	0.624 (0.284 – 3.814)	0.163
Sex	0.754 (0.418 – 2.631)	0.327		
HB	0.531 (0.308 – 1.942)	0.216		
WBCs	0.854 (0.413 – 1.325)	0.521		
Platelets	2.012 (1.541 – 5.631)	0.038*	3.524 (0.574 – 6.824)	0.542
ALT	0.754 (0.574 – 0.983)	0.040*	0.659 (0.408 – 2.106)	0.184
AST	0.472 (0.138 – 4.520)	0.108		
Albumin	2.531 (0.478 – 6.513)	0.314		
Total bilirubin	0.514 (0.258 – 0.842)	0.021*	0.397 (0.214 – 2.854)	0.287
Direct bilirubin	0.487 (0.138 – 0.745)	0.010*	0.542 (0.315 – 1.759)	0.193
AFP	0.745 (0.521 – 0.896)	0.014*	0.658 (0.218 – 3.874)	0.267
Child Pugh	0.458 (0.215 – 0.683)	0.037*	0.654 (0.325 – 2.769)	0.347
Tumor numbers	0.486 (0.269 – 0.731)	0.024*	0.659 (0.304 – 2.631)	0.163
Tumor diameter	0.591 (0.218 – 0.695)	0.034*	0.325 (0.067 – 1.754)	0.227
Macrovascular invasion	0.774 (0.426 – 0.864	0.039*	0.475 (0.176 – 1.652)	0.451
Extrahepatic metastasis	0.548 (0.315 – 0.729)	0.027*	0.674 (0.438 – 4.632)	0.107
BCLC stages	0.458 (0.361 – 0.697)	0.016*	0.846 (0.249 – 4.218)	0.176
Lymph node metastasis	0.754 (0.357 – 0.952)	0.028*	0.486 (0.298 – 2.651)	0.138

DISCUSSION

Our primary results demonstrated that those with HCC had a considerably greater average age than other participants in the study. Our results are consistent with those of **Abdel-Razek *et al.*** [5], who found that the mean age of HCC patients was 58 years.

In the present work, most patients with HCC and cirrhosis had considerably worse outcomes in both hemoglobin and platelet counts compared to the healthy control group. These results align with the research conducted by **Aljada *et al.*** [6], which suggests that while elevated levels of serum erythropoietin (EPO) may be present in approximately 23% of HCC cases, it is uncommon to observe increased levels in packed cell volume or hemoglobin concentration. Additionally, the majority of patients are diagnosed with anemia during the initial stages of HCC due to the tumor's various effects. The total leucocytic count and platelets decreased significantly within all research groups when compared to the control group. This is confirmed by **Pratt and Kaplan** [6] who claimed that the drop in total leucocytic count perceived with liver cirrhosis could be because of hypersplenism with splenic margination, whereas thrombocytopenia is mainly caused by portal hypertension with attendant congestive splenomegaly. This is consistent with earlier research by **AbdelGhafer *et al.*** [7] and **Aljada *et al.*** [8] who noticed that liver function tests were considerably higher in patients with HCC as compared to other groups, which included a significant increase in direct bilirubin, ALT, total bilirubin, as well as AST in the HCC group.

In earlier study of **Durazo *et al.*** [9], there was a very significant decrease in serum albumen, which is consistent with our findings.

The current study found that HCC group possessed the highest AFP level when compared with the other investigation groups, showing a statistically significant difference between those in the HCC group and other research groups, a conclusion found to be in accordance with the findings of previous investigation [10].

Due to the high incidence of HCV in our country, 60 patients were chosen for anti-HCV seropositivity in the study that we conducted, which is consistent with **Nair *et al.*** [11] who discovered that 80% of HCC patients proved to be HCV-positive. Cirrhosis was observed in 50% of the patients within the investigation, making it the most prominent clinical risk factor for HCC. This correspond with the findings of **Blum *et al.*** [12] who found that HCC developed in 90% of patients in addition to cirrhosis.

The group diagnosed with HCC had the highest average blood Ang 2 levels, in comparison with both the cirrhosis group and the healthy control group included in the study. These findings come in accordance with previous studies done by **Abdel Ghafar *et al.*** [7] and another study that was carried out by **Ao *et al.*** [13]. They discovered that HCC patients had a statistically significant and considerably elevated level of Ang 2 when compared to healthy control group and liver cirrhosis patients. In contrast to the results obtained in the present investigation, **Pestana *et al.*** [14] observed no statistically significant difference in blood Ang 2 levels when comparing liver cirrhosis and HCC groups. Moreover, the results of this investigation demonstrated a statistically significant elevation in the average serum Ang 2 concentration among individuals with liver cirrhosis, in comparison with the control group. These results concur with those of **Pestana *et al.*** [14] and **Scholz *et al.*** [15]. In contrast to this result **Abdel Ghafar *et al.*** [7] mentioned that no significant difference was observed in the levels of serum Ang 2 between both cirrhosis patients and research healthy controls.

In our research, we discovered that serum Ang 2 had significant positive associations with AST, direct bilirubin, total bilirubin, and ALT, but a negative correlation was observed with both serum albumin and platelets. The Earlier investigation conducted by **Osawa *et al.*** [16] found that there was positive correlation between liver function and Ang 2 , but there was a negative correlation existed between Ang 2, platelets and albumin. These findings are in accordance with our results.

In recent research, AFP and Ang 2 were shown to have a statistically significant relationship with one another. According to **Pestana *et al.*** [14] who reported a significant positive association between serum Ang 2 levels and AFP. Our findings are consistent with their findings. **Scholz *et al.*** [15] reported that they found no significant correlation between serum Ang 2 levels and AFP levels in HCC patients as well as in individuals with cirrhosis. The results of our present research investigation differ from these findings.

There was a significant relationship between serum Ang 2 and Child-Pugh classification in the current recent investigation. These results are consistent with those of **Choi *et al.*** [17]. **Scholz *et al.*** [15] found no association between Child-Pugh classification and serum Ang_2 levels, which is in contrast to the current study's findings. There was a significant association between HCC & BCLC and Ang 2 stages in the recent research. This result agrees with **Diaz-Sanchez *et al.*** [18] who found that increased Ang 2 levels were substantially associated with the advanced level of BCLC.

A significant association between tumor features and Ang 2 was found in current research. These findings confirm those of **Choi *et al.*** [17] and **Ao *et al.*** [13] who also discovered a connection between Ang 2 levels and tumor features**.** In contrast to **Scholz *et al.*** [15] who said that there wasn't any correlation found between tumor features and Ang 2 serum levels.

The optimal threshold was identified by analyzing ROC curves for ANG 2 in discriminating all patients from healthy, HCC from healthy group, and HCC from the cirrhosis group was 420 pg/ml, 430 pg/ml & 580 pg/ml with an AUC of 0.998, 0.964 & 0.940 yielding sensitivity of 97%, 93% & 90%, specificity of 95%,

90% & 83%, (PPV) of 98 %,91 % & 84 %, (NPV) of 90%, 93% & 89% and accuracy of 96%, 92%& 87% respectively.

Hence, Ang 2 may act as a diagnostic marker discriminating between healthy individuals and liver disease. Also, angiopoietin may be a valuable diagnostic marker for distinguishing HCC patients. These outcomes agree with **Nouh *et al*. [19]** who found that sensitivity and specificity were of 80% and 70% respectively. And also, in line with **Scholz *et al*. [15]** who stated a specificity of 73.28 % and sensitivity of 70.56 %. Hence, Ang 2 may act as a tool evaluating the severity and advancement of liver disease.

We recommend that further studies on a large number of patients for more comprehensive statistical analysis are needed. Samples from different multicenter are recommended. Further studies with the application of anti-angiopoietin as a therapeutic target are recommended and thus it can be applied as a treatment marker in HCC.

CONCLUSIONS

ANG 2 has the potential to serve as a diagnostic indicator in HCC and may also play an important function in controlling the advancement of liver disease. Additionally, it has been demonstrated to be very valuable in identifying individuals with aggressive tumors.

DECLARATIONS

- **Consent for publication:** All authors agreed to submit the work.
- **Availability of data and material:** Available
- **Competing interests:** None
- **Funding:** No fund
- **Conflicts of interest:** No conflicts of interest.

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