AIM OF THE WORK

The aim of this work was to study the significance of serum clusterin (CLU) level as a serological tumor marker in hepatocellular carcinoma (HCC) on top of HCV related hepatic cirrhosis. Also, to correlate its level in different studied groups with clinical and laboratory findings.

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PATIENTS

This study was conducted on 71 patients admitted to Hepatobiliary Unit, Internal Medicine Department, Alexandria Main University Hospital.

They were divided into the following groups:

Group I: 25 patients with chronic HCV with no evidence of liver cirrhosis as diagnosed by laboratory tests and abdominal ultrasound

Group II: 25 patients with chronic HCV related liver cirrhosis as diagnosed by laboratory tests and abdominal ultrasound.

Group III: 21 patients with chronic HCV related liver cirrhosis and proved hepatocellular carcinoma (HCC). HCC was diagnosed by two of the following criteria:

- The presence of hepatic focal lesion in cirrhotic patient by ultrasonography. This was confirmed by the unique dynamic radiological pattern of HCC by using triphasic CT in which HCC showed contrast uptake in the arterial phase and rapid wash out in the venous phase.
- High serum AFP level.
- Liver biopsy for patients with atypical triphasic CT or normal AFP levels (whenever needed).

Control group IV: 10 healthy control subjects with matched age and sex and with normal liver function

In the present study, patients with hepatitis B virus infection, cholestatic liver diseases, alcoholic cirrhosis, any form of cancer other than HCC were excluded.

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METHODS

All patients in this study were subjected to:

1) History taking; including:

- Personal data: age, sex, occupation, residence and special habits as smoking and alcohol consumption.
- Patients' complaints: right hypochondrial pain, weight loss, jaundice, fatigue, hematemesis and melena.

2) Clinical examination; including:

- General examination focusing on: jaundice, hepatic encephalopathy, lower limb edema, palmar erythema and spider angioma.
- Local examination to assess hepatic and splenic sizes as well as the presence or absence of ascites and its degree.

3) Sample collection:

Blood samples were obtained by venipuncture using sterile disposable plastic syringes. About 9 ml of venous blood were withdrawn aseptically after disinfection of the skin using 70% alcohol, and divided into the following:

- Two mL were delivered into an EDTA (ethelenediamine tetra-acetic acid) vacutainer for CBC.
- About 3 mL were delivered into a sodium citrated vacutainer for PT.
- Four mL were delivered to a serum separator tube (SST) and the samples were allowed to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Serum was separated, aliquoted and stored at ≤ -20 °C for clusterin assay, AFP assay, viral markers and for liver function tests. Repeated freeze-thaw cycles were avoided.

4) Biochemical studies; including:

- Complete blood picture (using the automated cell counter ADVIA 2120). [137]
- Liver profile: (using the dimension RxL max clinical chemistry autoanalyzer).
  - Serum Alanine aminotransferases (ALT), serum Aspartate aminotransferases (AST) and serum alkaline phosphatase levels. [138]
  - Serum albumin level. [139]
  - Prothrombin activity (using sysmex). [140]
  - Total serum bilirubin level. [141]

- Viral markers including serum HCV antibodies and hepatitis B surface antigen (using chemiluminescent technique by Cobas e 411). [142]

- Serum alpha-fetoprotein (AFP) levels using chemiluminescent technique by ADVIA centaur XP. [142]

- Serum clusterin (CLU) level using quantitative sandwitch enzyme immunoassay technique (ELISA). [142]

5) Severity of liver disease was graded according to the clinic-biochemical classification of Child-Pugh et al [143] as follows:

Measurments	1 point	2 points	3 points
Encephalopathy	None	Slight to mild	Moderate to sever
Ascites	None	Slight to mild	Moderate to sever
Serum albumin(g/dl)	>3.5	2.8-3.5	<2.8
Serum bilirubin(g/dl)	<2	2.1-3	>3
Prothrombin activity	>70%	40-70%	<40%

Score was calculated and accordingly the patients were divided into class A, B and C:

Risk group	Numerical score
Child Pugh class A	5 and 6
Child Pugh class B	7 to 9
Child Pugh class C	10 to 15

6) performance status

Score 0: No cancer related symptoms. Normal lifestyle.

Score 1: Minor symptoms related to cancer. Capable of nonstrenuous activity. Fully ambulatory.

Score 2: Confined to bed or chair less than 50% of waking hours.

Score 3: Confined to bed or chair more than 50% of waking hours.

Score 4: Totally confined to bed or chair.

Score 5: Dead.

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7) HCC staging; according to Barcelona Clinic Liver Cancer staging (BCLC) [144] as follows:

BCLC stage	Performance status(PS)	Tumor features	Severity of liver disease
Stage 0 (very early stage)	0	Single <2cm Carcinoma in situ	Child-Pugh A
Stage A (early stage)	0	Single <5cm Or 3 tumors <3cm each	Child-Pugh A
Stage B (intermediate stage)	0	Large multinodular	Child-Pugh class A-B
Stage C (advanced stage)	1-2	Vascular invasion or metastases	Child-Pugh class A-B
Stage D (terminal stage)	>2	Any	Child-Pugh C

8) Imaging evaluation:

- Abdominal ultrasound: to assess liver (size, echogenicity, presence of focal lesion; its size and site), splenic size, portal circulation and its patency as well as the presence of ascites. [145, 146]
- Triphasic CT abdomen; focused on: detection of hyper-vascular liver tumor(s) with stress on the size, site, number of lesion(s), where focal hepatic tumor(s) showing enhancement in the hepatic arterial phase and decreased attenuation in portal venous phase. [147, 148]

9) Alpha-fetoprotein (AFP) assay: [142]

Principle of assay:

The ADVIA centaur AFP assay is a two-site sandwich immunoassay system using direct chemiluminometric technology, which uses constant amounts of two antibodies. The first antibody, in the lite reagent, is an affinity purified polyclonal rabbit anti-AFP antibody labeled with acridinum ester, the second antibody, is the solid phase, is a monoclonal mouse anti-AFP antibody covalently coupled to paramagnetic particles.

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10) Clusterin (CLU) assay: [142]

Principle of assay:

This assay employes the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for CLU had been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any CLU present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for CLU was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of CLU bound in the initial step. The color development was stopped and the intensity of the color was measured.

Reagents:

- CLU microplates: polystyrene microplates coated with a mouse monoclonal antibody against clusterin.
- CLU standard: 1 µg/vial of recombinant human Clusterin in a buffered protein base with preservatives; lyophilized.
- CLU conjugate: 21 mL/vial of monoclonal antibody against Clusterin conjugated to horseradish peroxidase with preservatives.
- Assay Diluent RD1-19: 11 mL/vial of a buffered protein base with preservatives.
- Calibrator Diluent RD5T: 21 mL/vial of a buffered protein base with preservatives.
- Wash Buffer Concentrate: 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative.
- Color Reagent A: 12 mL/vial of stabilized hydrogen peroxide.
- Color Reagent B: 12 mL/vial of stabilized chromogen (tetramethylbenzidine).
- Stop Solution: 6 mL/vial of 2 N sulfuric acid.

Sample collection and storage:

Sample preparation:

Serum samples were diluted 2000-fold (10 µL of sample + 390 µL of Calibrator Diluent RD5T). Then the dilution was Completed to the 2000-fold by adding (10 µL of the diluted samples to 490 µL of Calibrator Diluent RD5T).

Reagent preparation:

All reagents were brought to room temperature before use.

Wash Buffer - when crystals had formed in the concentrate, warming to room temperature and mixing gently until the crystals had completely dissolved was done. 20 mL of Wash Buffer Concentrate were diluted into deionized or distilled water to prepare 500 mL of Wash Buffer.

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Substrate Solution - Color Reagents A and B were mixed together in equal volumes within 15 minutes of use, protected from light. 200 µL of the resultant mixture were required per well.

CLU Standard - The CLU Standard was reconstituted with 1.0 mL of deionized or distilled water. This reconstitution produced a stock solution of 1000 ng/mL. The standard was mixed to ensure complete reconstitution and the standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. 400 µL of Calibrator Diluent RD5T were pipetted into the 200 ng/mL tube. 200 µL of Calibrator Diluent RD5T were pipetted into the remaining tubes. The stock solution was used to produce a dilution series (below). Each tube was mixed thoroughly before the next transfer. The 200 ng/mL standard served as the high standard. Calibrator Diluent RD5T served as the zero standard (0 ng/mL).

Assay procedures:

All reagents were brought to room temperature before use.

1. All reagents, working standards, and samples were prepared as directed in the previous sections.
2. Excess microplate strips were removed from the plate frame, returned to the foil pouch containing the desiccant pack, and resealed.
3. 100 µL of Assay Diluent RD1-19 were added to each well.
4. 50 µL of Standard, control, or sample were added per well, covered with the adhesive strip provided, incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Each well was aspirated and washed, the process were repeated three times for a total of four washes using autowasher. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.
6. 200 µL of CLU Conjugate were added to each well, covered with a new adhesive strip, incubated for 2 hours at room temperature on the shaker.
7. The aspiration/wash was repeated as in step 5.
8. 200 µL of Substrate Solution were added to each well, incubated for 30 minutes at room temperature on the benchtop, protected from light.
9. 50 µL of Stop Solution were added to each well. The color in the wells was changed from blue to yellow.
10. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.
11. A standard curve was constructed by plotting absorbance value against concentration of the standards on log-log paper.
12. Concentrations of the unknown samples were determined using this standard curve.

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The standard curve was created as follows:

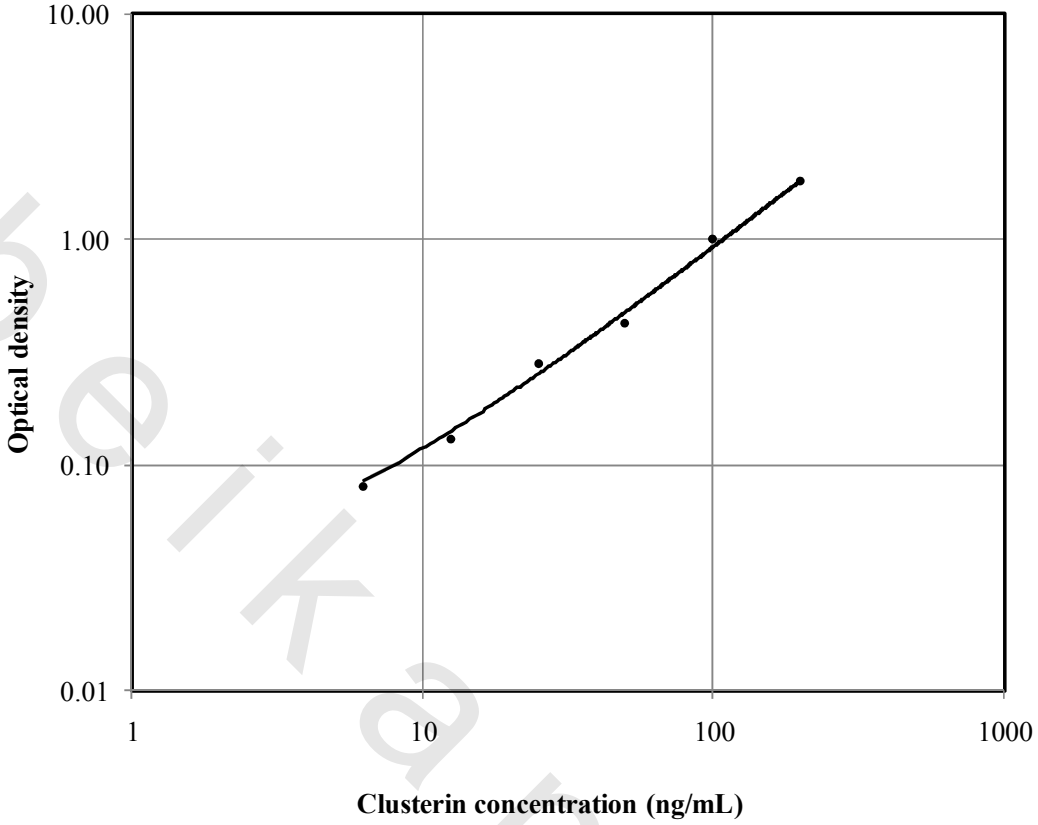

Figure (2): Standard curve of clusterin.

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STATISTICAL ANALYSIS

Statistical analysis of the data [149]

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. [150]

Qualitative data were described using number and percent. Quantitative data were described using minimum and maximum, mean and standard deviation and median.

Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Monte Carlo correction.

The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used.

For normally distributed data, comparison between two independent population were done using independent t-test while more than two population were analyzed F-test (ANOVA) to be used and Post Hoc test (Scheffe). Correlations between two quantitative variables were assessed using Pearson coefficient. For abnormally distributed data Kruskal Wallis test was used to compare between different groups and pair wise comparison was assessed using Mann-Whitney test. Correlations between quantitative and ordinal variables were assessed using Spearman coefficient.

Diagnostic performance was used and was expressed in sensitivity, specificity, positive predictive value, negative predictive value and accuracy. Receiver operating characteristic curve (ROC) was plotted to analyse a recommended cutoff, the area under the ROC curve denotes the diagnostic performance of the test. Area more than 50% gives acceptable performance and area about 100% is the best performance for the test.

Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

1- Mean value $(\overline{X}) = \frac{X}{n}$.

Where X = the sum of all observations.

n = the number of observations.

2- The standard deviation S.D. = $\sqrt{\frac{\sum (X - \overline{X})^2}{n - 1}}$

Where

$\Sigma (Xi - X)^2$ = the sum of squares of differences of observations from the mean.

3- Chi-Square test:

It tests the association between qualitative nominal variables, it is performed mainly on frequencies. It determines whether the observed frequencies differ significantly from expected frequencies.

$$\text{Computed} \quad X^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

Where E = expected frequency

O = observed frequency

$$E = \frac{\text{Raw total x Column total}}{\text{Grand total}}$$

4- Fisher's exact test and Monte Carlo test

It is used whenever the expected frequency in any of the cells of 2×2 table falls below 5. This test involves the calculation of the P value directly, without the use of particular test statistic.

$$P = \frac{a+b!\,c+d!\,a+c!\,b+d!}{n!\,a!\,b!\,c!\,d!}$$

a, b, c, d: are the numbers in each cell

n: the total sample size.

!: factorial = successive multiplication of the integers in descending order.

In case of an r x c table the "MonteCalo". test was used.

5- Student (Unpaired-sample) "t" test:

It is used during comparison between the means of different sample groups. The "t" is calculated as follows:

$$\mathbf{t} = \frac{X_1 - X_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where

X_1 = Mean of first group.

X_2 = Mean of second group.

S_1 = Standard deviation of the first group.

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S_2 = Standard deviation of the second group.

n_1 = Sample size of the first group.

n_2 = Sample size of the second group.

6- One way analysis of variance (ANOVA) was performed for comparison between more than two groups

Variance ratio F was computed by the formula.

$$F_{(r-1),(n-1)} = \frac{\text{Means quare between classes}}{\text{Mean square within classes}}$$

Where r = number of groups

n = total sample size

7- Correlation coefficient (r):

$$\mathbf{r} = \frac{\left(\sum X_i Y_i\right) - \frac{\left(\sum X_i\right)\left(\sum Y_i\right)}{n}}{\sqrt{\left[\sum X_i^2 - \frac{\left(\sum X_i\right)^2}{n}\right]\left[\sum Y_i^2 - \frac{\left(\sum Y_i\right)^2}{n}\right]}}$$

Where X and Y are the values of the first and second observations in the same individual correlation can be significant at $p < 0.05$.

10- Receiver operating characteristic curve (ROC):

It is generated by plotting sensitivity (TP) on Y axis versus 1-specificity (FP) on X axis at different cut off values. The area under the ROC curve denotes the diagnostic performance of the test. Area more than 50% gives acceptable performance and area about 100% is the best performance for the test. The ROC curve allows also a comparison of performance between two tests.

- Diagnostic Sensitivity = Positivity in diseased patients, expressed as percent = $\frac{TP}{TP + FN} \times 100$

- Diagnostic Specificity = Negativity in non-diseased subjects, expressed as percent = $\frac{TN}{FP + TN} \times 100$

- Predictive value of positive results (PV^+) = Percent of subjects with positive results who are diseased = $\frac{TP}{TP+FP}\times 100$

- Predictive value of negative results (PV) = Percent of subjects with negative test results who are non diseased $= \frac{TN}{TN+FN}\times 100$

- Accuracy: Rate of Agreement = (True positives + True negatives) / Total tested x 100

- Calculation of diagnostic sensitivity and specificity were also chosen at the optimal cut off which has a highest positive likelihood ratio (^+LR)

  where $^+LR = = \frac{\text{Sensitivity}}{1-\text{specificity}} = \frac{\text{TP rate}}{\text{FP rate}}$

N.B. $^+LR > 1$ denotes good performance of a test

TP = True positive (number of diseased patients correctly classified by the test)

FP =False positives (number of nondiseased patients misclassified by the test)

FN =False negatives (number of diseased patients misclassified by the test)

TN =True negatives (number of non diseased patients correctly classified by the test)

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RESULTS

This study was performed on 71 patients, who were admitted to Hepatobiliary Unit, Internal Medicine Department, Alexandria Main University Hospital. The patients were classified into three groups:

Group I: (n=25): HCV positive patients without liver cirrhosis.

Group II: (n=25): HCV positive patients with liver cirrhosis.

Group III: (n=21): HCV positive patients with liver cirrhosis and HCC.

Group IV: 10 age and sex matched healthy subjects were included as a control group

Age and sex:

In HCV positive patients without liver cirrhosis **(Group I)**; the mean age was 49.80 ± 9.90 years. On the other hand, the mean age for HCV positive patients with liver cirrhosis **(Group II)** was 51.60 ± 4.78 years. While, it was 53.05 ± 7.41 years and 49.80 ± 8.22 years for **Group III** and **IV** respectively. No significant difference was reported between different studied groups. **Table (1)**

As regards sex, males predominated females in all studied groups, with no statistically significant difference between different groups. **Table (1)**

Table (1): Comparison between different studied groups according to demographic data.

	HCV (I) (n = 25)		Cirrhosis (II) (n = 25)		HCC (III) (n = 21)		Control (IV) (n = 10)		Test of sig.	p
	No.	%	No.	%	No.	%	No.	%		
Age (years)										
Min. – Max.	39.0 – 75.0		41.0 – 62.0		45.0 – 75.0		36.0 – 62.0			
Mean ± SD	49.80 ± 9.90		51.60 ± 4.78		53.05 ± 7.41		49.80 ± 8.22		F=0.805	0.495
Median	50.0		52.0		54.0		50.0			
Sex										
Male	13	52.0	13	52.0	14	66.7	7	70.0	χ^2=3.719	0.293
Female	12	48.0	12	48.0	7	33.3	3	30.0		

χ^2: Chi square test
F: F test (ANOVA)

Presenting signs and symptoms:

Table (2) figure (3) showed that right hypochondrial pain and upper GIT bleeding were a common complain among HCC patients **Group III** (66.7% and 71.4% respectively). Fatigue was a common complaint in studied **Group I, II and III** (76.0%, 52.0% and 85.7% respectively). Also, a statistically significant difference was reported between different studied groups as regards these presenting manifestations. Jaundice was found in **Groups I, II** and **III** in almost close values (28%, 32% and 33.3% respectively). Moreover, hepatic encephalopathy was reported in **Group II** and **III** (14.3% and 20% respectively).

Table (2): Comparison between the different studied groups according to presenting signs and symptoms.

	HCV (I) (n = 25)		Cirrhosis (II) (n = 25)		HCC (III) (n = 21)		χ^2	p
	No.	%	No.	%	No.	%		
Rt. Hypochondrial pain	10	40.0	6	24.0	14	66.7	8.595*	0.014*
Upper GIT bleeding	0	0.0	9	36.0	15	71.4	26.106*	<0.001*
Fatigue	19	76.0	13	52.0	18	85.7	6.804*	0.033*
Jaundice	7	28.0	8	32.0	7	33.3	0.170	0.918
Encephalopathy	-	-	3	14.3	5	20	50.282*	<0.001*

χ^2: Chi square test
*: Statistically significant at $p \leq 0.05$

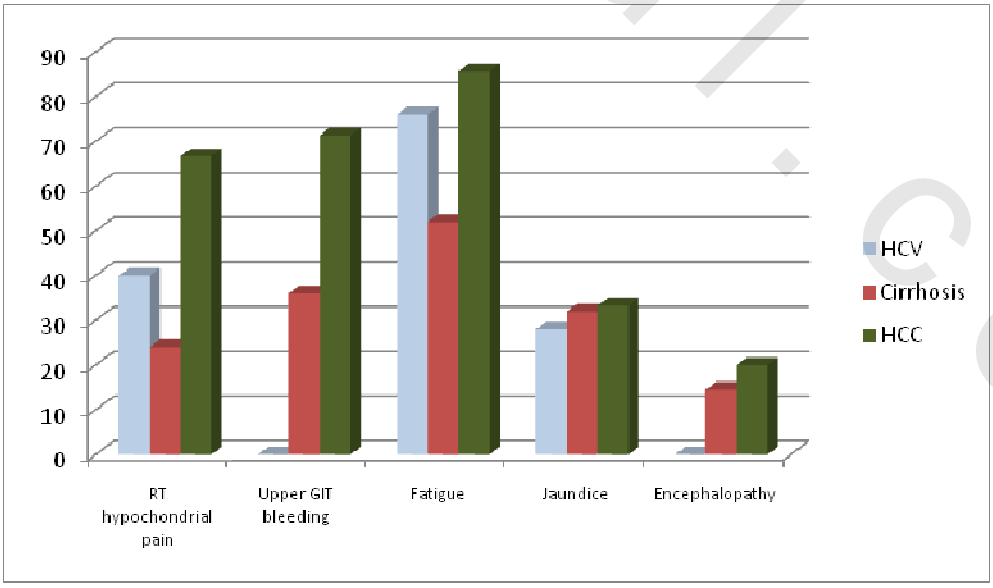

Figure (3): Comparison between the different studied groups according to presenting signs and symptoms.

Clinical examination:

Table (3) Figure (4) showed that hepatomegaly was found in 52.0% of **Group I**, 4.0% of **Group II** and 9.5% of **Group III**; with a highly significant difference between different studied groups. Splenomegaly was a common finding in **Group II** and **III** (56.0%, 66.7% respectively), while in **Group I** it was found in only 8% with a significant difference between different studied groups. As regards ascites, it was a very common finding among HCC and cirrhotic patients **Group III** and **Group II** (85.7% and 52.0% respectively). Also, lower limb edema was a very common finding in the same groups (85.7% and 60% respectively). A statistical significant difference was reported between different studied groups as regards both findings (ascites and lower limb edema). Moreover, spider angioma and palmer erythema were common findings in HCC patients **Group III** (47.6% and 57.1% respectively).

Table (3): Comparison between the different studied groups according to clinical examination parameters.

	HCV (I) (n = 25)		Cirrhosis (II) (n = 25)		HCC (III) (n = 21)		χ^2	p
	No.	%	No.	%	No.	%		
Hepatomegaly	13	52.0	1	4.0	2	9.5	19.390*	<0.001*
Splenomegaly	2	8.0	14	56.0	14	66.7	19.087*	<0.001*
Ascitis	-	-	13	52	18	85.7	36.517*	<0.001*
Lower limb edema	1	4.0	15	60.0	18	85.7	32.806*	<0.001*
Spider angioma	-	-	9	36.0	10	47.6	0.636	0.425
Palmer erythyma	-	-	12	48.0	12	57.1	0.382	0.536

χ^2: Chi square test
*: Statistically significant at $p \leq 0.05$

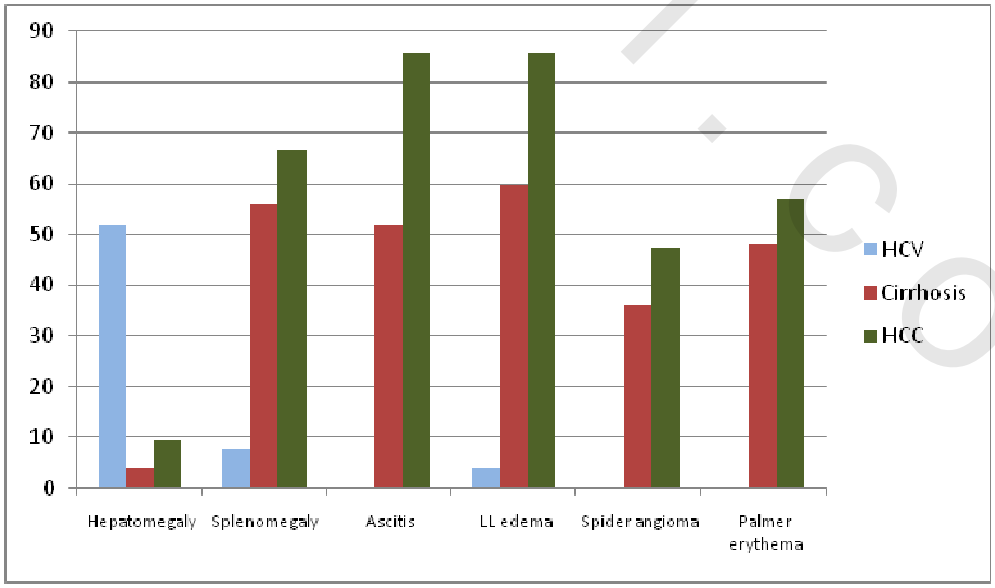

Figure (4): Comparison between the different studied groups according to clinical examination parameters.

CBC findings:

Table (4) showed that anemia was found in the first 3 groups in comparison to the control group, with a statistically significant difference between **Group II** and **Group IV**.

A significant decrease in platelets count was found in **Group I, II** and **III** compared to the control **Group IV**. On the other hand, there was no significant difference in WBCs count in the different studied groups.

Table (4): Comparison between the different studied groups according to CBC findings.

	HCV (I) (n = 25)	Cirrhosis (II) (n = 25)	HCC (III) (n = 21)	Control (IV) (n = 10)	Test of sig.	p
HB (g/dl)						
Min. – Max.	10.0 – 14.50	6.20 – 15.0	3.60 – 13.20	12.20 – 14.20		
Mean ± SD	11.55 ± 1.37	10.04 ± 2.35	10.26 ± 2.94	12.56 ± 1.03	$F=4.667^*$	0.005^*
Median	11.40	10.10	11.0	12.48		
Sig. bet grps	$p_3 = 0.005^*$					
Platelet ($\times10^3$/cmm)						
Min. – Max.	151.0 - 250.0	49.0 - 428.0	36.0 - 655.0	150.0 – 400.0		
Mean ± SD	190.1 ± 29.29	155.7 ± 123.2	157.7 ± 140.6	237.8 ± 97.7	$^{KW}\chi^2=15.839^*$	0.001^*
Median	195.0	120.0	130.0	280.0		
Sig. bet grps	$p_1 = 0.487$ $p_2 = 0.003^*$ $p_3 = 0.005^*$					
WBC ($\times10^3$/cmm)						
Min. – Max.	4.10 – 17.0	1.53 – 17.10	2.60 – 22.0	4.70 – 11.0		
Mean ± SD	7.58 ± 3.06	7.70 ± 4.44	9.41 ± 5.93	8.40 ± 2.12	$^{KW}\chi^2=1.853$	0.604
Median	6.80	7.40	8.50	8.70		

F: F test (ANOVA)
Sig. bet. grps was done using Post Hoc Test (Scheffe)
$^{KW}\chi^2$: Chi square for Kruskal Wallis test
Sig. bet. grps was done using Z for Mann Whitney test
p_1: Comparison between control with HCV
p_2: Comparison between control with cirrhosis
p_3: Comparison between control with HCC
*: Statistically significant at $p \leq 0.05$

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Liver function tests:

Table (5) showed a significant increase in the serum levels of ALT and AST in **Group III** HCC patients compared to other studied groups.

As regards prothrombin activity and albumin level, they were significantly lower in **Group II** (cirrhotic patients) and **Group III** (HCC patients) compared to **(Group I** and **Group IV)**.

Serum bilirubin showed no significant difference between the four studied groups, while alkaline phosphatase was significantly increased in all studied groups, compared to control.

Table (5): Comparison between the different studied groups according to liver function.

	HCV (I) (n = 25)	Cirrhosis (II) (n = 25)	HCC (III) (n = 21)	Control (IV) (n = 10)	$^{KW}\chi^2$	p
ALT (U/L)						
Min. – Max.	20.0 – 760.0	12.0 – 164.0	28.0 – 95.0	12.0 – 36.0		
Mean ± SD	106.4±149.8	55.9 ± 50.6	63.1 ± 22.12	25.90 ± 7.87	19.083*	<0.001*
Median	43.0	37.0	64.0	28.0		
AST (U/L)						
Min. – Max.	21.0 – 780.0	19.0 – 230.0	20.0 – 121.0	20.0 – 41.0		
Mean ± SD	96.60 ± 150.4	62.60 ± 58.77	55.14 ± 32.57	31.15 ± 6.75	8.327*	0.040*
Median	41.0	38.0	43.0	28.50		
Prothrombin activity %						
Min. – Max.	70.0 – 100.0	26.0 – 88.0	34.0 – 86.0	89.0 – 100.0		
Mean ± SD	90.16 ± 9.10	64.84 ± 18.79	66.29 ± 17.87	95.30 ± 4.35	11.097*	<0.001*
Median	90.0	70.0	67.0	95.50		
Albumin(g/dl)						
Min. – Max.	2.90 – 5.80	1.50 – 5.10	1.20 – 4.10	3.60 – 5.10		
Mean ± SD	4.12 ± 0.80	3.07 ± 1.21	2.90 ± 0.74	4.0 ± 0.61	46.187*	<0.001*
Median	4.0	3.10	2.90	3.85		
Bilirubin (mg/dl)						
Min. – Max.	0.50 – 7.80	0.50 – 15.90	0.56 – 16.70	0.60 – 0.97		
Mean ± SD	2.39 ± 1.86	2.96 ± 3.83	4.46 ± 5.95	0.87 ± 0.19	2.210	0.094
Median	1.90	1.50	1.90	0.90		
Alkaline phosphatase (U/L)						
Min. – Max.	44.0 – 98.0	55.07 – 399.0	27.0 – 500.0	13.0 – 121.0		
Mean ±SD	77.04±14.38	107.80±77.80	138.10±96.14	61.70±35.27	26.261*	<0.001*
Median	77.0	90.0	107.0	69.50		

$^{KW}\chi^2$: Chi square for Kruskal Wallis test
Sig. bet. grps was done using Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$
: Statistically significant at $p \leq 0.01$ *: Statistically significant at $p \leq 0.001$

Child-pugh scoring system:

The severity of liver disease was measured using child-pugh classification.

Table (6) Figure (5) showed that in **Group I**, 92.0% of cases were child A, 8% were child B**,** with a median of 5.0. In **Group II**, 40.0% were child A, 40.0% were child B and 20.0% were child C, with a median of 9.0. On the other hand, in **Group III** 28.6% were child A, 42.9% were child B and 28.6% were child C, with a median of 8.0. A statistical significant difference was observed between **Group I** and both **Group II** and **III**.

Table (6): Comparison between different studied groups according to severity of liver disease (child-pugh classification).

	HCV (I) (n = 25)		Cirrhosis (II) (n = 25)		HCC (III) (n = 21)		$^{KW}\chi^2$	p
	No.	%	No.	%	No.	%		
Child score								
A	23	92.0	10	40.0	6	28.6		
B	2	8.0	10	40.0	9	42.9	22.550	<0.001*
C	0	0.0	5	20.0	6	28.6		
Min. – Max.	5.0 – 8.0		5.0 – 14.0		5.0 – 12.0			
Mean ± SD	5.64 ± 0.86		8.24 ± 2.47		8.24 ± 2.39		23.684*	<0.001*
Median	5.0		9.0		8.0			
Sig. bet grps	I-II***,I-III***							

$^{KW}\chi^2$: Chi square for Kruskal Wallis test
Sig. bet. grps was done using Z for Mann Whitney test
p_1: Comparison between HCV with Cirrhosis
p_2: Comparison between HCV with HCC
p_3: Comparison between Cirrhosis with HCC
*: Statistically significant at $p \leq 0.05$

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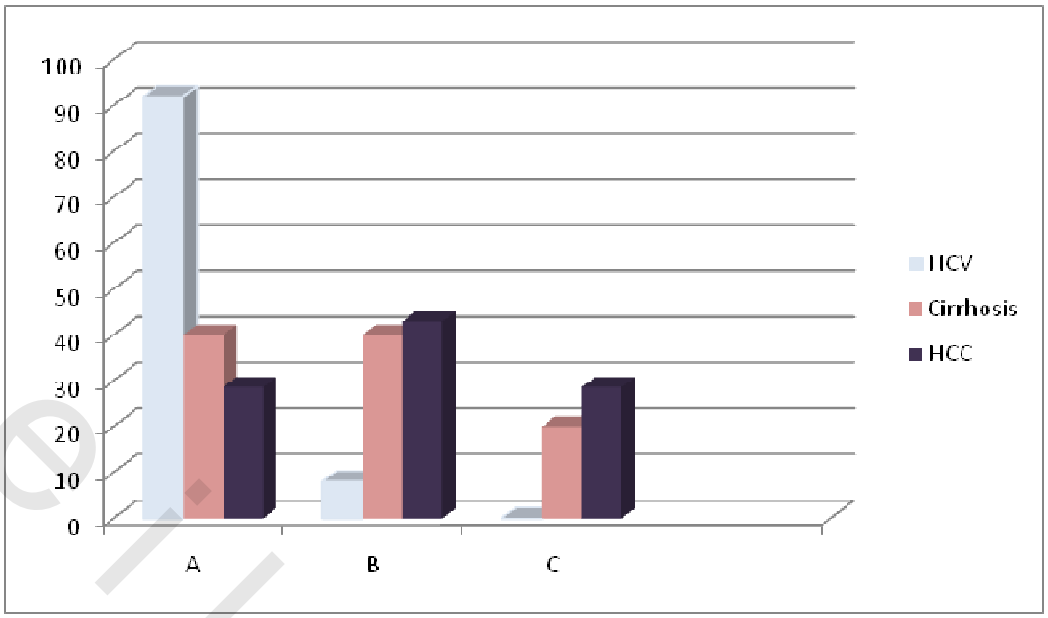

Figure (5): Comparison between different studied groups according to severity of liver disease (child-pugh classification).

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BCLC staging system and its criteria in Group III HCC patients:

According to BCLC staging classification, 47.6% of HCC patients were BCLC stage A, 4.8% were BCLC stage B, 19.0% were BCLC stage C and 28.6% were BCLC stage D. Tumor size of <5 cm and number of nodules <3 were common in these patients as they were found in 66.7% of HCC patients for each of them, while 52.4% of HCC patients had capsular infiltration. Also, portal vein invasion and lymph node metastasis were found in 19.0% of HCC patients. As regards the performance status score, 52.4% of patients had score zero, 28.6% had score 3, while scores 1 and 2 had the same value of 9.5% for each. **Table (7)**

Table (7): Distribution of BCLC staging system in HCC patients (n = 21).

	No.	%
BCLC		
A	10	47.6
B	1	4.8
C	4	19.0
D	6	28.6
Tumor size		
<5 cm	14	66.7
>5 cm	7	33.3
Number of nodules		
<3	14	66.7
>3	7	33.3
Capsular infiltration	11	52.4
Portal vein invasion	4	19.0
L.N metastasis	4	19.0
Performance Status		
0	11	52.4
1	2	9.5
2	2	9.5
3	6	28.6

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Alpha-feto protein (AFP) levels:

Table (8) Figure (6) showed that the mean value of serum AFP levels was 8.75 ± 7.27 ng/ml in **Group I** and 11.85 ± 9.90 ng/ml in **Group II**, while HCC patients **(Group III)** had a higher values of 154.3 ± 191.7 ng/ml and the least values were seen in control group **(Group IV)** (3.06 ± 3.67 ng/ml). Thus, a statistically significant difference was found between different studied groups.

Table (8): Comparison between the different studied groups according to alpha-feto protein (AFP)

Case No.	HCV (I) (n = 25)	Cirrhosis (II) (n = 25)	HCC (III) (n = 21)	Control (IV) (n = 10)
1	6.8	21.1	20	1.2
2	21	19	350	1.9
3	2.1	1.5	6.85	13
4	1.5	2.3	546	3.85
5	19	11	300	1.3
6	21	1.6	7.8	1.1
7	6.7	20	350	1.6
8	14	18	1.8	4.2
9	5.85	1.2	6.9	1.2
10	25	2.6	21.25	1.2
11	29	3.8	290	
12	3.1	14	105	
13	1.2	28.1	6.7	
14	0.9	16	12.1	
15	1.2	1.4	20	
16	1.6	4.8	10.2	
17	4.8	6.8	350	
18	5.1	19.1	210	
19	6.2	22	580	
20	3.7	21.1	38	
21	7.8	25.7	6.85	
22	4.8	30		
23	14	1.2		
24	1.5	2.1		
25	11	1.8		
AFP (ng/ml)				
Min. – Max.	0.90 – 29.0	1.20 – 30.0	1.80 – 580.0	1.10 – 13.0
Mean ± SD	8.75 ± 7.27	11.85 ± 9.90	154.3 ±191.7	3.06 ± 3.67
Median	5.85	11.0	21.25	1.45
$^{KW}\chi^2$ (P)	25.788* (<0.001*)			
Sig. bet grps@	p_1 = 0.014*, p_2= 0.005*, p_3 <0.001*			

$^{KW}\chi^2$: Chi square for Kruskal Wallis test
@Sig. bet. grps was done using Z for Mann Whitney test
p_1: Comparison between control with HCV
p_2: Comparison between control with cirrhosis
p_3: Comparison between control with HCC
*: Statistically significant at $p \leq 0.05$

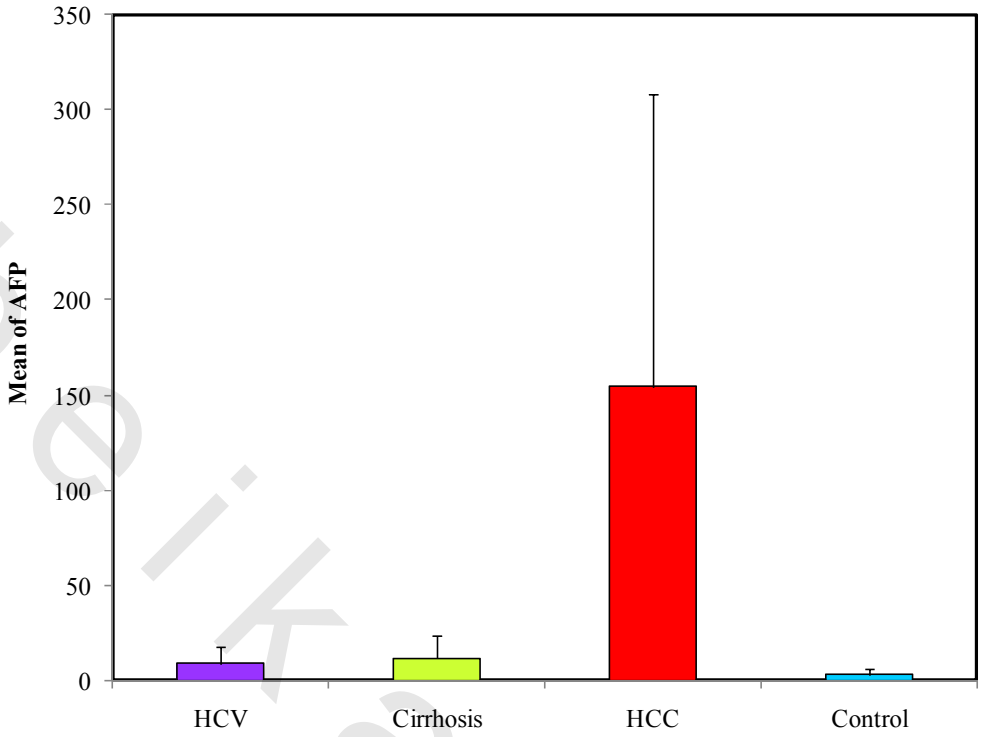

Figure (6): Mean values of serum AFP in the different studied groups.

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Serum clusterin (CLU) levels:

Table (9) Figure (7) showed that the mean value of serum clusterin levels in **Group I** and **Group II** was 143.72 ± 49.44 ng/ml and 154.84 ± 40.03 ng/ml respectively. Highest levels were found in HCC patients **(Group III)**, as their mean value of serum clusterin levels was 217.62 ± 32.89 ng/ml. While the least values were found in healthy subjects **(Group IV)**, as their mean value of serum clusterin levels was 94.10 ± 22.22 ng/ml. Thus a statistically significant difference was found between different studied groups.

Table (9): Comparison between the different studied groups according to clusterin levels.

Case No.	HCV (I) (n = 25)	Cirrhosis (II) (n = 25)	HCC (III) (n = 21)	Control (IV) (n = 10)
1	210	106	266	103
2	159	148	192	81
3	122	185	172	127
4	187	169	206	114
5	160	184	188	106
6	194	160	200	99
7	124	169	266	100
8	72	169	212	83
9	138	117	254	81
10	118	134	180	47
11	188	47	235	
12	116	200	188	
13	121	169	198	
14	165	139	262	
15	210	193	233	
16	210	90	202	
17	184	140	262	
18	139	179	182	
19	50	136	188	
20	104	172	262	
21	175	200	222	
22	42	188		
23	198	175		
24	85	210		
25	122	92		
Clusterin (ng/ml)				
Min. – Max.	42.0 – 210.0	47.0 – 210.0	172.0 – 266.0	47.0 – 127.0
Mean ± SD	143.72 ± 49.44	154.84 ± 40.03	217.62 ± 32.89	94.10 ± 22.22
Median	139.0	169.0	206.0	99.50
F (p)	25.090* (<0.001*)			
Sig. bet grps#	$p_1 = 0.016^*$, $p_2 = 0.002^*$, $p_3 < 0.001^*$			

F: F test (ANOVA)
#Sig. bet. grps was done using Post Hoc Test (Scheffe)
p_1: Comparison between control with HCV
p_2: Comparison between control with cirrhosis
p_3: Comparison between control with HCC *: Statistically significant at $p \leq 0.05$

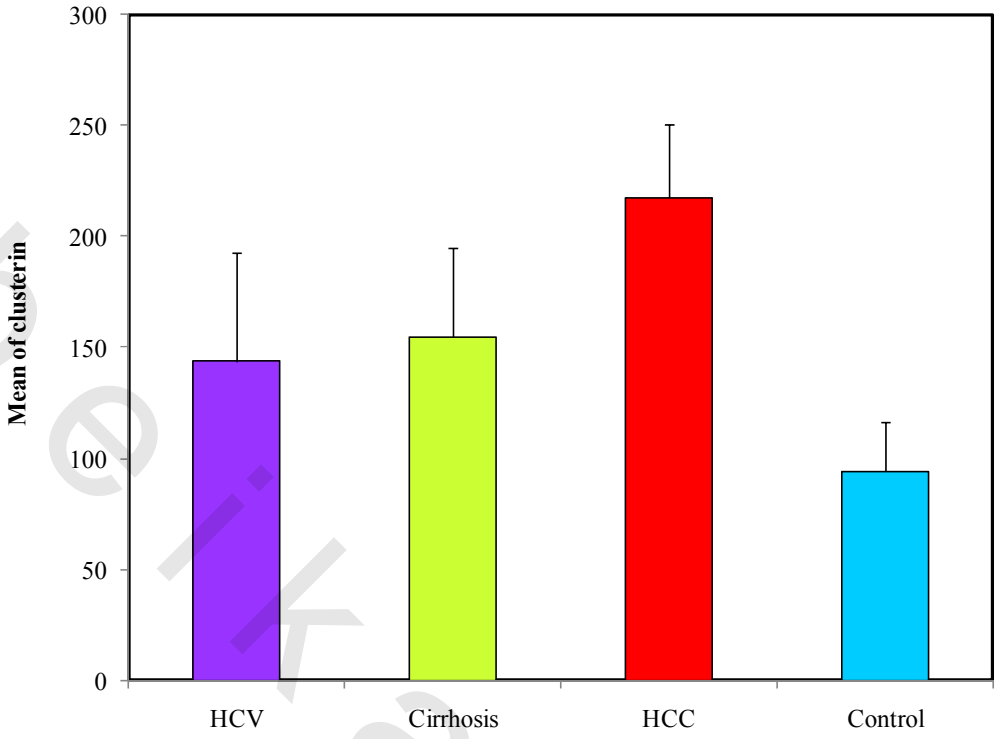

Figure (7): Mean values of serum clusterin levels in different studied groups.

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Diagnostic performance and ROC curve:

The receiver operating characteristics (ROC) analysis was used to study the diagnostic ability of both markers (AFP and clusterin). In HCC patients clusterin at the best cut-off value of 179 ng/ml had a sensitivity of 95.24% , specificity of 70%, positive predictive value (PPV) of 57.14%, and negative predictive value (NPV) of 97.22%, with accuracy 77.46%, while AFP showes much lower values at a cut-off value of 11 ng/ml, the sensitivity was 66.67%, specificity was 62.0%, PPV was 42.42%, NPV was 80.58%, and accuracy of 63.38%. As regards the area under curve (AUC), clusterin had a greater area under the curve than that of AFP as AUC of them was 0.989 and 0.793 respectivly. (These results were observed on comparing HCC patients with HCV postive patients either with or without cirrhosis). **Table (10) Figure (8)**

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Table (10): Diagnostic performance of AFP and clusterin in HCC group against HCV positive patients with and without cirrhosis.

	Cut-off value	HCV+ Cirrhosis Group I+II	HCC Group III	Sensitivity	Specificity	PPV	NPV	Accuracy
AFP	**≤11**	31	7	66.67	62.0	42.42	80.58	63.38
	>11	19	14					
Clusterin	**≤179**	35	1	95.24	70.0	57.14	97.22	77.46
	>179	15	20					

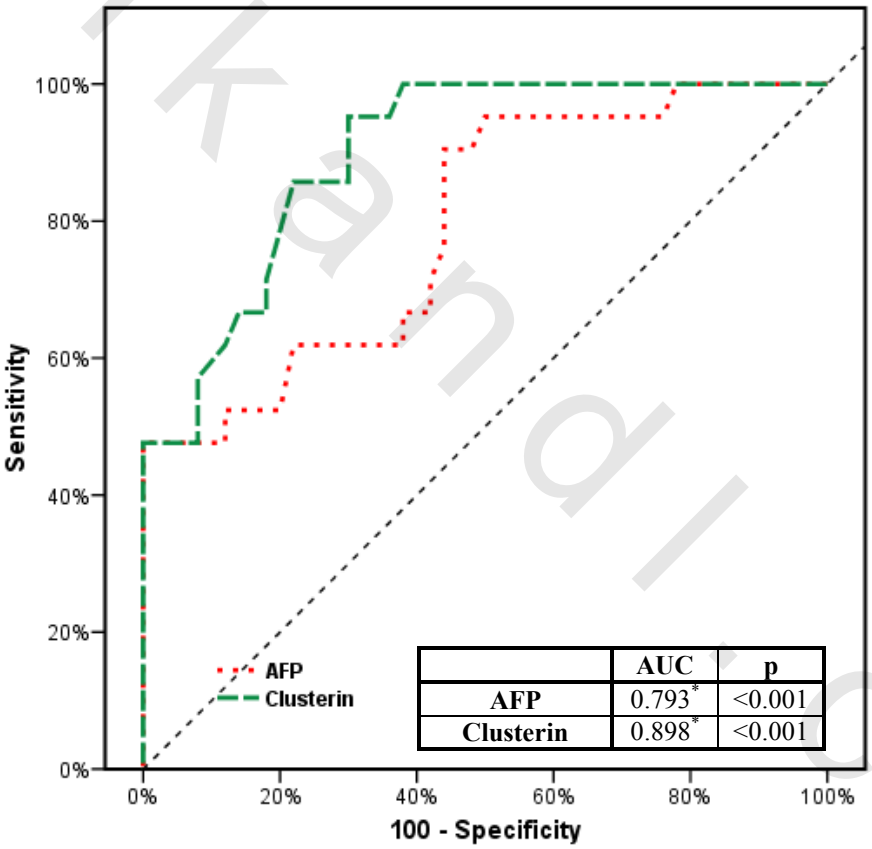

Figure (8): ROC curve for the diagnostic performance of AFP and clusterin to diagnose HCC cases.

On studying both markers in combination using cirrhotic group against HCC group, specificity, PPV, accuracy and AUC were improved as their values were 100.0%, 100.0%, 86.96%, and 0.949 respectively. While, sensitivity and NPV were decreased giving the values of 71.43% and 80.65% respectively. **Table (11) Figure (9)**

Table (11): Diagnostic performance of AFP alone, clusterin alone and AFP and clusterin in combination in HCC group against HCV positive patients with cirrhosis.

	Cut-off value	Cirrhosis Group II	HCC Group III	Sensitivity	Specificity	PPV	NPV	Accuracy
AFP	**≤11**	13	7	66.67	52.0	53.85	65.0	58.70
	>11	12	14					
Clusterin	**≤179**	18	1	95.24	72.0	74.07	94.74	82.61
	>179	7	20					
AFP +Clusterin		25	6	71.43	100.0	100.0	80.65	86.96
		0	15					

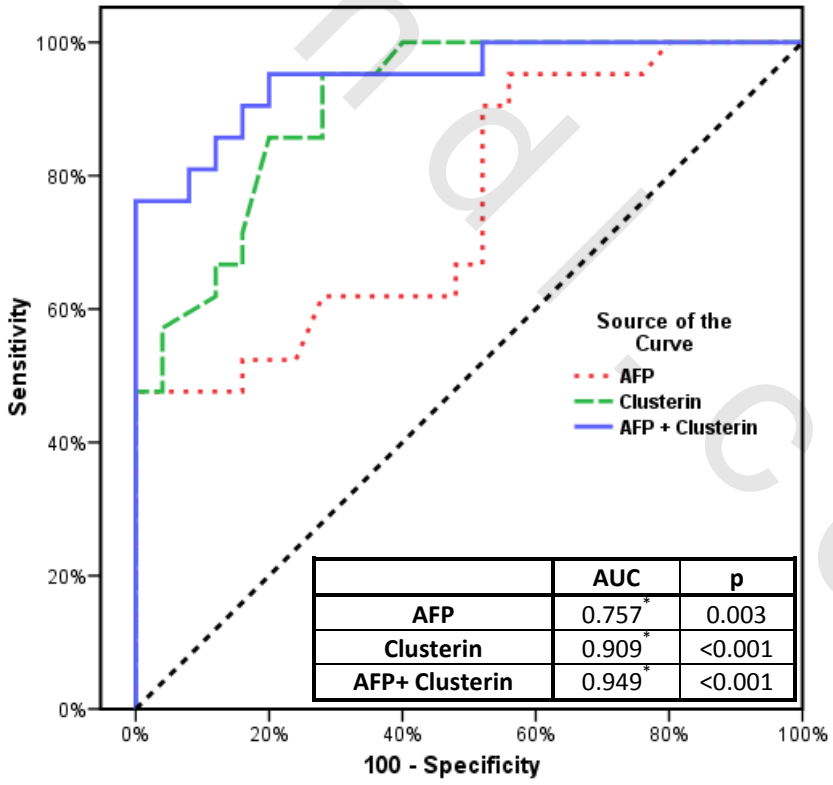

Figure (9): ROC for the diagnostic performance of AFP alone, clusterin alone and AFP and clusterin in combination in HCC group against HCV positive patients with cirrhosis.

On studying HCC group against HCV positive patients with and without cirrhosis, specificity, PPV, accuracy, and AUC of both markers in combination were improved as there values were 100.0%, 100.0%, 91.55% and 0.946. While sensitivity and NPV dropped to 71.0% and 89.29%. **Table (12) Figure (10)**

Table (12): Diagnostic performance of AFP alone, clusterin alone and AFP and clusterin in combination in HCC group against HCV positive patients with and without cirrhosis.

	Cut off value	HCV+ Cirrhosis (Group I+II)	HCC (Group III)	Sensitivity	Specificity	PPV	NPV	Accuracy
AFP	**≤11**	31	7	66.67	62.0	42.42	80.58	63.38
	>11	19	14					
Clusterin	**≤179**	35	1	95.24	70.0	57.14	97.22	77.46
	>179	15	20					
AFP + Clusterin		50	6	71.0	100.0	100.0	89.29	91.55
		0	15					

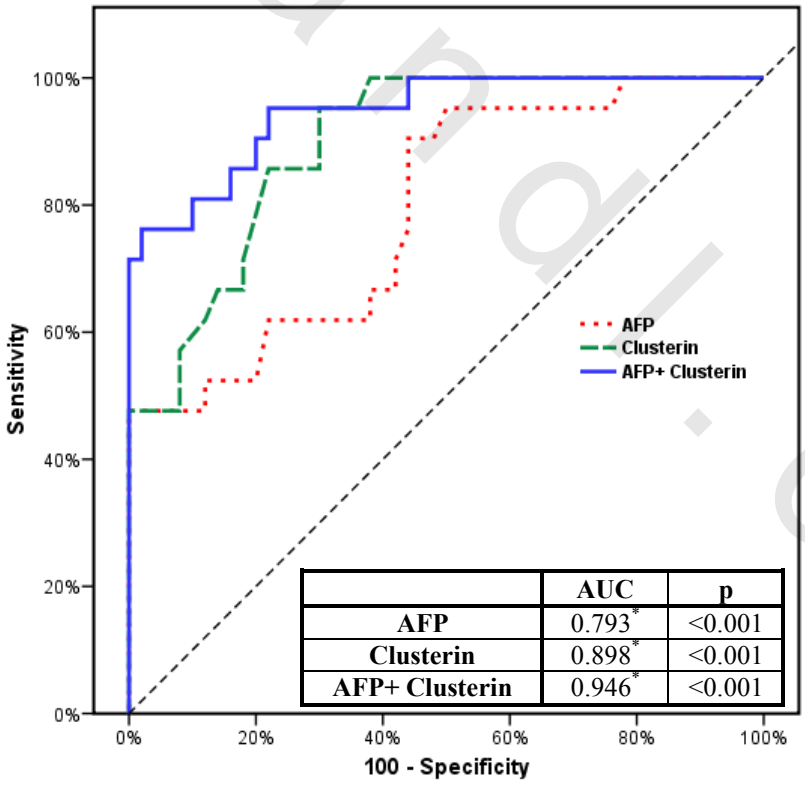

Figure (10): ROC curve for the diagnostic performance of AFP alone, clusterin alone and in combination in HCC against HCV positive patients with or without cirrhosis.

Correlation studies:

Correlation studies showed that there was no significant correlation between serum clusterin levels on one hand and AFP, child score or BCLC stages on the other hand. **Table (13)**

Table (13): Correlation between serum clusterin level on one hand and AFP, child score, BCLC staging on the other hand.

		Clusterin		
		HCV	Cirrhosis	HCC
AFP	r	0.156	0.183	0.136
	p	0.456	0.381	0.556
Child score	r_s	0.130	0.088	0.033
	p	0.535	0.677	0.887
BCLC	r_s	-	-	0.355
	p	-	-	0.115

r: Pearson coefficient
r_s: Spearman coefficient

There was a significant correlation between serum clusterin levels and fatigue, spider angioma and palmer erythema. While there was no significant correlation between serum clusterin levels and the rest of presenting signs and symptoms. **Table (14)**

Table (14): Correlation between serum clusterin level on one hand and presenting signs, symptoms and clinical examination in HCC patients (Group III) on the other hand.

	Clusterin	
	r_s	p
Rt. Hypochondrial pain	0.385	0.085
Upper GIT bleeding	0.192	0.404
Fatigue	0.541*	0.011
Jaundice	-0.167	0.469
Ascitis	0.090	0.699
Encephalopathy	-0.866	0.333
Hepatomegaly	0.081	0.728
Splenomegaly	0.159	0.491
Lower Limb edema	0.135	0.559
Spider angioma	0.616*	0.003
Palmer erythyma	0.494*	0.023

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

In this study there was no significant correlation between serum clusterin levels and any of the laboratory findings. **Table (15)**

Table (15): Correlation between serum clusterin level on one hand and laboratory findings in HCC patients (Group III) on the other hand.

	Clusterin	
	r_s	p
HB	-0.335	0.138
Platelet	-0.005	0.983
WBC	-0.109	0.640
ALT	0.136	0.557
AST	0.002	0.992
Prothrombin activity %	-0.244	0.287
Albumin(g/dl)	-0.035	0.881
Bilirubin (mg/dl)	-0.031	0.894
Alkaline phosphatase (U/L)	0.181	0.432

r_s: Spearman coefficient

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Serum clusterin levels were not affected by tumor size, number of nodules or capsular infiltration in HCC patients. However, according to BCLC staging system, serum clusterin level was significantly increased in stage C than stage A patients, provided that stage B was excluded from comparison as it carries only one patient. Also, serum clusterin levels were significantly increased in HCC patients with portal vein invasion and LN metastasis. **Table (16)**

Table (16): Relation between serum clusterin level on one hand and BCLC staging system with its criteria in HCC patients (Group III) on the other hand.

Criteria	N	Clusterin		Test of sig.	p
		Min. – Max.	Mean ± SD.		
Tumor Size					
<5	**14**	172.0 – 266.0	219.07 ± 35.56	t=0.280	0.783
>5	**7**	180.0 – 266.0	214.71 ± 29.17		
Number of nodules					
<3	**14**	172.0 – 266.0	216.86 ± 34.60	t=0.146	0.885
>3	**7**	180.0 – 262.0	219.14 ± 31.75		
BCLC					
A	**10**	172.0 – 262.0	201.3 ± 27.27	F=4.376*	0.029*
#B	**1**	222.0			
C	**4**	212.0 – 266.0	251.50[a] ± 26.40		
D	**6**	180.0 – 262.0	221.50 ± 33.12		
Capsular infiltration					
Negative	**10**	180.0 – 262.0	204.60 ± 29.33	t=1.828	0.083
Positive	**11**	172.0 – 266.0	229.45 ± 32.64		
Portal vein invasion					
Negative	**17**	172.0 – 266.0	208.44 ± 30.26	t=2.596*	0.018*
Positive	**4**	212.0 – 266.0	247.0 ± 23.62		
L.N metastasis					
Negative	**17**	172.0 – 262.0	207.18 ± 27.16	t=7.648*	<0.001*
Positive	**4**	254.0 – 266.0	262.0 ± 5.66		

t: Student t-test
F: F test (ANOVA)
Sig. bet. grps was done using Post Hoc Test (Scheffe)
a: significant with BCLC (A)
*: Statistically significant at $p \leq 0.05$
excluded from comparison

As regards serum AFP levels, they were not affected neither by tumor size, nor by number of nodules. Also, there were no significant increase in AFP levels in presence of capsular infiltration, portal vein invasion or LN metastasis. But, serum AFP levels according to BCLC stages showed a significant difference, as they were lower in BCLC stage A patients compared to BCLC stage C and D patients. Also, provided that stage B was excluded from comparison as it carries only one patient. **Table (17)**

Table (17): Relation between serum AFP level on one hand and BCLC staging with its criteria in HCC patients (Group III) on the other hand.

	N	AFP			Test of sig.	p
		Min. – Max.	Mean ± SD.	Median		
Tumor Size						
<5	**14**	6.70 – 580.0	240.46 ± 180.20	215.0	Z=1.495	0.135
>5	**7**	10.20 – 300.0	114.10 ± 113.18	68.50		
Number of nodules						
<3	**14**	6.70 – 580.0	196.10 ± 167.64	195.0	Z=0.075	0.940
>3	**7**	10.20 – 546.0	202.81 ± 186.18	138.0		
BCLC						
A	**10**	6.70 – 220.0	77.30 ± 103.10	15.10		
#B	**1**		68.50			
C	**4**	20.0 – 350.0	172.0 ± 136.64	159.0	$^{KW}\chi^2$=6.480*	0.039*
D	**6**	105.0 [a] – 580.0	294.48 [a] ±171.40	295.0		
Capsular infiltration						
Negative	**10**	6.70 – 580.0	241.16 ± 203.33	205.0	Z=0.635	0.526
Positive	**11**	10.20 – 350.0	159.41 ± 129.0	138.0		
Portal vein invasion						
Negative	**17**	6.70 – 580.0	224.19 ± 181.62	205.0	Z=0.910	0.363
Positive	**4**	20.0 – 220.0	115.60 ± 91.96	138.0		
L.N metastasis						
Negative	**17**	6.70 – 580.0	209.89 ± 173.09	180.0	Z=0.493	0.622
Positive	**4**	6.90 – 350.0	149.23 ± 165.61	120.0		

Z: Z for Mann Whitney test
$^{KW}\chi^2$: Chi square for Kruskal Wallis test
Sig. bet. grps was done using Mann Whitney test
a: significant with BCLC (A)
*: Statistically significant at $p \leq 0.05$
excluded from comparison