BioBacta

Journal of Bioscience and Applied Research
www.jbaar.org

MiRNA-122 association with TNF-α in some liver diseases of Egyptian patients

Ahmed Abdelhalim Yameny[1], Sabah Farouk Alabd[1], and Magda Ahmed M. Mansor[2]

[1]Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt

[2]Department of Histology, Faculty of Medicine, Menoufia University, Egypt

Corresponding author: Ahmed A. Yameny. Email: dr.ahmedyameny@yahoo.com

Tel: (002)01002112248, **ORCID number: 0000-0002-0194-9010**

DOI: 10.21608/jbaar.2023.329927

Abstract:

Background: Due to the high frequency of HCC, ongoing research is needed to find precise, non-invasive biomarkers for early identification and follow-up that will improve prognostic results. **Patients and methods:** this study was conducted on 90 patients with liver diseases and 25 healthy control G1, patients divided into 4 groups, (G2) 25 patients with HCV infection, (G3) 25 HCC+HCV infection, (G4) 25 patients with HBV infection, (G5) 15 patients with HCC + HBV. **Results:** Serum miR-122 and TNF-α levels were increased in HCV and HBV infection significantly with p-value <0.001* compared to the control group, and their levels decreased when developed into HCC but still higher than the healthy subjects significantly with p-value <0.001. For discriminating HCV from HCV+HCC the cut-off for miR-122 was >7.1 at sensitivity 100%, specificity 100%, and the AUC was 1.0 (Excellent) P-value <0.001, also the sensitivity and specificity for TNF-α 72%, and 60% respectively with cut off >12.1 and AUC of 0.745 (Good) p-value 0.003. For discriminating HBV from HBV+HCC the cut-off for miR-122 was ≤6.4 at a sensitivity of 86.67% and specificity of 96%, and the AUC of miR-122 was 0.99 (Excellent) P-value <0.001, also the sensitivity and specificity for TNF-α 93.33%, and 48.0% respectively with cut-off ≤15.73, TNF-α has AUC of 0.527 (fair) it was not significant p-value 0.780.

Keywords: miRNA-122, Tumor necrosis factor alpha (TNF-α), HBV, HCV, HCC, Liver diseases.

1. Introduction:

It has been demonstrated that the liver can regenerate significant amounts of tissue after being removed. However, some diseases can overstimulate this ability, leading to excessive cellular matrix and collagen formation. Cirrhosis, an accumulation of fibrotic ECM that impairs the liver's capacity to effectively exchange fluid, results from the decompensation of hepatic fibrosis **(1).**

Two million people die from liver disorders each year globally (3.5% of all fatalities); of these, 50% are due to complications of cirrhosis, and 50% are due to hepatocellular carcinoma (HCC) and viral hepatitis infections **(2).**

Received: August 13, 2023. Accepted: October 10, 2023. Published: December 10, 2023

Liver cancer is one of the most prevalent malignancies in the world, with significant rates of morbidity and death. The most prevalent primary liver cancer, hepatocellular carcinoma (HCC), accounts for 75–85% of all liver cancer cases **(**3**).** The most frequent causes of HCC are chronic liver infection from hepatitis B or C virus (HBV or HCV, respectively) and alcohol addiction **(4),**

It has been evident that hepatitis B is a common condition and that more than 2 billion individuals worldwide have been exposed to HBV. According to estimates from the World Health Organization (WHO), 296 million persons worldwide had chronic HBV infection in 2019.
(https://www.who.int/news-room/fact-sheets/detail/hepatitis-b).

Hepatitis C Virus (HCV) infection is one of the major causes of morbidity and mortality globally and in developing countries HCV is a major contributor to chronic liver disease, HCC, and liver transplantation **(5)**.
With 58 million chronically infected individuals and 1.5 million new infections annually, HCV has a large worldwide impact, WHO estimated that approximately 290,000 people died from hepatitis C in 2019, mostly from cirrhosis and hepatocellular carcinoma. WHO set ambitious goals to eradicate viral hepatitis B and C as a public health threat by 2030 **(WHO June 2022 Report) (6)**.
To reduce the prevalence of HCC, HBV, and HCV, the two main risk factors, must be prevented and treated **(7).**
For the early diagnosis of HCC, alpha-fetoprotein (AFP) screening and ultrasonography are frequently employed. However, there are certain limitations with AFP and ultrasonography in HCC early detection **(8).**

The majority of patients with primary HCC are detected at late stages, which is linked to a poor prognosis and a low survival rate of the illness. Currently, there are no viable biomarkers for early identification of primary HCC. Therefore, it is crucial to find more accurate and reliable markers for the early diagnosis of primary HCC, and many efforts have been undertaken in this direction over the past few decades. The discovery of short noncoding regulatory RNAs called microRNAs (miRNAs) is an evolutionarily conserved gene class **(9).** Noncoding RNAs of the family known as microRNAs (miRNAs) range in length from 17 to 22 nucleotides and play a crucial role in posttranscriptional gene regulation. These master regulators are also sensitive to post-transcriptional and transcriptional control **(10).** The most prevalent liver-specific miRNA, MicroRNA-122 (miR-122), constitutes around 70% of the overall miRNA population in the adult liver **(11).**

According to Boutz DR and colleagues (2011), miRNA-122 controls several genes in the liver that modify the cell cycle, differentiation, proliferation, and apoptosis. This suggests that miRNA -122 can be a reliable and predictive blood marker for alcohol, viral, and chemical-induced liver injury because the change in miRNA-122 levels in the blood is a well-known indicator of liver disease and is prominent early before the increase in liver aminotransferase activity **(12).**

In all phases of HCC development, miRNA-122 is markedly down-regulated and exhibits tumor-suppressive properties (**13**). miRNA 122 is involved in the control of TNFα expression **(14).**
A significant inflammatory cytokine in the progression of liver disease is tumor necrosis factor (TNF-α). This cytokine has the potential to damage the liver, induce cirrhosis, and ultimately lead to hepatocellular cancer **(15)**

High production of TNF is associated with the increase of pro-inflammatory cytokine secretion, the activation of proto-oncogenes, and several genes related to cell growth, invasion, and metastasis of cancer cells **(16).**

This study aims to detect the unclear association between miRNA-122 and TNF-α in liver diseases of Egyptian patients, and the validity of miRNA-122 and TNF-α in early diagnosis of HCC associated with HCV and HBV infection.

SUBJECTS AND METHODS

2.1. Subjects

This study was carried out on 90 Egyptian hepatic patients and twenty-five healthy subjects. They matched in gender, social standard, and residency, all participants were selected from the National Liver Institute, Menofia University, Egypt, at the time from October 2019 to December 2021 (During the COVID-19 pandemic).

All subjects were classified into five groups as follows:

Group (1): This group consisted of 25 healthy subjects with matched age and gender. The subjects of this group were with no evidence of liver disease and were negative for HBV and HCV infection, which served as a control group.

Group (2): This group consisted of 25 patients with HCV infection.

Group (3): This group consisted of 25 patients with hepatocellular carcinoma (HCC) associated with HCV infection.

Group (4): This group consisted of 25 patients with HBV infection.

Group (5): This group consisted of 15 patients with hepatocellular carcinoma (HCC) associated with HBV infection.

2.2. Methods

2.2.1. Blood sample collection and preparation

Five milliliters of venous blood were taken from each patient in all five groups using a vacutainer system and septic venipuncture. The blood sample was separated into two milliliters mixed with sodium citrate for PT-INR and three milliliters for biochemical and molecular analyses.

Routine laboratory investigations

Liver function tests: ASL, ALT, Albumin, total protein, total bilirubin, Direct bilirubin. It was performed on automated Dimension Xpand Plus (Siemens Healthcare, USA).

Prothrombin time PT-INR using Stago-STA Compact, France.

Tumor marker Alpha-Feto protein (AFP)

Quantification determination of AFP by Enzyme-Linked Immunosorbent Assay (ELIZA) analyzed using (Chemux Bioscience, Inc. USA) Lot No: 319091802, according to the manufacturer's guidelines.

Quantification determination of TNF-α by Enzyme-Linked Immunosorbent Assay (ELIZA)

TNF-α was determined using (Wuhan EIAab Science Co., Ltd, China)

Catalog No: E0133h, according to the manufacturer's guidelines

Quantitative polymerase chain reaction (PCR) for HBV and HCV By Real-Time PCR

The viral load of hepatitis C virus RNA and hepatitis B virus DNA was determined by real-time PCR technique, it was performed on automated QIAcube (Extract and purify DNA, RNA, and proteins and QIAGEN's real-time PCR cycler (The Rotor-Gene German).

Table (1): primers of miR-122 and U6 snRNA

	miR-122	U6 snRNA
Forward	5'-GACAAGCCTGGCTACTGTGTT-3'	5′-CTCGCTTCGGCAGCACA-3′
Reverse	5'- GTGGCCCATCTTGTCCTTC-3'	5′-AACGCTTCA CGAATTTGCGT-3′.

Statistical analysis of the data

Data was fed to the computer and analyzed using IBM SPSS software package version 20.0**.** **(**Armonk, NY: IBM Corp**)**. Qualitative data were described using numbers and percentages. The Shapiro-Wilk test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation, median, and interquartile range (IQR). The significance of the obtained results was judged at the 5% level.

3. RESULTS

This study was conducted on 115 subjects (80 male, 35 female) classified into five groups (Control group, HCV, HCV+HCC, HBV, HBV+HCC Groups).

3.1. Demographic data of the studied groups

3.1.1. Gender

The control group included 19 males (76%) and 6 females (24%), the HCV patients group included 18 males (72%) and 7 females (28%), while group 3(HCV+HCC) included 20 males (80%) and 5 females (20%), group 4 HBV patients included 13 males (52%) and 12 females ((48%), and group 5 HBV+HCC patients included 10 males (66.7%) and 5 females (33.3%). No statistically significant difference was detected between the five groups as regards gender (p-value 0.236). (Table 2).

3.1.2. Age

The mean age of the control group was 27.32 ± 5.37 years, while the mean age of the group 2 HCV patients was 52.36 ± 7.83, the mean age of group 3 **HCV+HCC patients was** 60.72 ± 6.17 years, while the mean age of group 4 HBV patients was 39.60 ± 8.26 years, and the mean age of group 5 **HBV+HCC was** 58.20 ± 11.01 years, there was statistically a significant difference between the five groups p-value of 0.001*, and comparing to the control group all the four patients groups were have a statistically significant difference with the same p-value 0.001*for the four groups compared with the control group as shown in table (2).

3.2. Liver function tests:

ALT: There is a significant difference between the five groups, when compared to the control group the four patient groups have a significant elevation (0.001) as shown in Table (3) and Figure (1).

AST: The four patient groups have a statistically significant elevation with the same p-value of 0.001*for the four groups compared with the control group as shown in Table (3) and Figure (2).

Albumin: compared to the control group all four patient groups have a statistically significant decrease with the same p-value of 0.001*for the three groups and 0.003 for the HBV group compared with the control group as shown in Table (3) and Figure (3).

Total Protein: Compared to the control group all four patient groups have a statistically significant decrease with the same p-value of 0.001*for the four groups compared with the control group as shown in Table (3) and Figure (4).

Total bilirubin: compared to the control group all four patient groups have a statistically significant elevation with the same p-value of 0.001*for the three groups and 0.025 for the HBV group compared with the control group as shown in Table (3) and Figure (5).

Direct bilirubin: compared to the control group the three patient groups have a statistically significant elevation with the same p-value of 0.001*for the three groups and 0.846 for HBV group not significant compared with the control group as shown in Table (3) and Figure (6).

Table (2): Comparison between the different studied groups according to demographic data

	Control (n = 25)		**HCV (n = 25)**		**HCV+HCC (n = 25)**		**HBV (n = 25)**		**HBV+HCC (n = 15)**		**Test of Sig.**	**p**
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**		
Gender												
Male	19	76.0	18	72.0	20	80.0	13	52.0	10	66.7	χ^2= 5.547	0.236
Female	6	24.0	7	28.0	5	20.0	12	48.0	5	33.3		
Age (years)												
Min. – Max.	20.0 – 40.0		38.0 – 62.0		46.0 – 71.0		30.0 – 62.0		34.0 – 68.0			
Mean ± SD.	27.32 ± 5.37		52.36 ± 7.83		60.72 ± 6.17		39.60 ± 8.26		58.20 ± 11.01		F= 78.710*	<0.001*
p_1			<0.001*		<0.001*		<0.001*		<0.001*			
Sig. bet. Grps.			p_2=0.002*,p_3<0.001*,p_4=0.140,p_5<0.001*,p_6=0.850,p_7<0.001*									

IQR: **Inter quartile range** SD: **Standard deviation** χ^2: **Chi-square test.**

F: F for One-way ANOVA test, Pairwise comparison bet. each 2 groups were done using **a Post Hoc Test (Tukey)**

p: p-value for comparing the different studied groups.

p_1: p-value for comparing between **Control** and **each other groups.**

p_2: p-value for comparing between **HCV** and **HCV+HCC.**

p_3: p-value for comparing between **HCV** and **HBV**

p_4: p-value for comparing between **HCV** and **HBV+HCC.**

p_5: p-value for comparing between **HCV+HCC** and **HBV**

p_6: p-value for comparing between **HCV+HCC** and **HBV+HCC.**

p_7: p-value for comparing between **HBV** and **HBV+HCC** *: Statistically significant at $p \leq 0.05$.

Table (3): Comparison between the different studied groups according to Liver function

Liver function	Control (n = 25)	HCV (n = 25)	HCV+HCC (n = 25)	HBV (n = 25)	HBV+HCC (n = 15)	Test of Sig.	p
ALT							
Min. – Max.	7.0 – 21.0	26.0 – 86.0	27.0 – 122.0	27.0 – 63.0	48.0 – 1182.0		
Mean ± SD.	13.83 ± 5.64	46.48 ± 15.95	76.56 ± 30.01	43.48 ± 10.33	168.2 ± 286.4	H= 79.591*	<0.001*
Median (IQR)	15.0 (8.0 – 20.0)	42.0 (34.0 – 55.0)	71.0 (52.0 – 98.0)	41.0 (39.0 – 51.0)	82.0 (60.0 – 106.0)		
p_1		<0.001*	<0.001*	<0.001*	<0.001*		
Sig. bet. Grps.		p_2=0.004*,p_3=0.715,p_4=0.001*,p_5=0.001*,p_6=0.438,p_7<0.001*					
AST							
Min. – Max.	7.60 – 23.0	38.0 – 96.0	36.0 – 147.0	31.0 – 69.0	39.0 – 2157.0		
Mean ± SD.	13.75 ± 3.84	54.24 ± 15.98	72.68 ± 35.22	46.60 ± 11.09	242.7 ± 534.5	H= 69.122*	<0.001*
Median (IQR)	14.0 (10.60 – 16.0)	49.0 (41.0 – 62.0)	60.0 (48.0 – 97.0)	47.0 (39.0 – 50.0)	100.0 (56.50 –130.0)		
p_1		<0.001*	<0.001*	<0.001*	<0.001*		
Sig. bet. Grps.		p_2=0.209,p_3=0.274,p_4=0.040*,p_5=0.019*,p_6=0.336,p_7=0.003*					
ALB							
Min. – Max.	3.90 – 5.0	2.39 – 4.70	2.10 – 4.80	3.20 – 5.0	1.90 – 3.80		
Mean ± SD.	4.46 ± 0.41	3.38 ± 0.61	3.24 ± 0.64	3.91 ± 0.45	2.97 ± 0.51	F= 27.625*	<0.001*
Median (IQR)	4.50 (4.10 – 4.80)	3.30 (3.10 – 3.80)	3.10 (2.90 – 3.61)	3.80 (3.60 – 4.10)	3.0 (2.90 – 3.10)		
p_1		<0.001*	<0.001*	0.003*	<0.001*		
Sig. bet. Grps.		p_2=0.878,p_3=0.006*,p_4=0.124,p_5<0.001*,p_6=0.515,p_7<0.001*					
TP							
Min. – Max.	6.30 – 7.80	3.40 – 8.0	4.70 – 7.50	5.50 – 7.50	3.80 – 7.10		
Mean ± SD.	7.20 ± 0.34	6.32 ± 0.97	5.80 ± 0.76	6.37 ± 0.55	5.76 ± 0.81	F= 15.206*	<0.001*
Median (IQR)	7.10 (7.0 – 7.50)	6.10 (5.90 – 6.90)	5.90 (5.10 – 6.10)	6.50 (5.90 – 6.70)	5.80 (5.40 – 6.10)		
p_1		<0.001*	<0.001*	0.001*	<0.001*		
Sig. bet. Grps.		p_2=0.081,p_3=0.999,p_4=0.121,p_5=0.046*,p_6=1.000,p_7=0.077					
Bilirubin total							
Min. – Max.	0.20 – 0.90	0.90 – 2.76	0.90 – 3.10	0.25 – 1.50	0.90 –7.20		
Mean ± SD.	0.49 ± 0.23	1.43 ± 0.57	1.55 ± 0.53	0.78 ± 0.31	2.29 ± 1.74	H= 70.596*	<0.001*
Median (IQR)	0.40(0.39–0.70)	1.30(0.95–1.50)	1.40(1.20–1.80)	0.80(0.50–1.0)	1.20(1.05–3.0)		
p_1		<0.001*	<0.001*	0.025*	<0.001*		
Sig. bet. Grps.		p_2=0.491,p_3<0.001*,p_4=0474,p_5<0.001*,p_6=0.905,p_7<0.001*					
Bilirubin Direct							
Min. – Max.	0.01 – 0.20	0.10 – 0.70	0.10 – 1.20	0.01 – 0.60	0.10 – 4.12		
Mean ± SD.	0.09 ± 0.05	0.33 ± 0.16	0.57 ± 0.31	0.11 ± 0.13	1.02 ± 1.22	H= 61.666*	<0.001*
Median (IQR)	0.09(0.08–0.10)	0.24(0.20–0.50)	0.50(0.38–0.70)	0.08(0.03–0.10)	0.10(0.10–1.90)		
p_1		<0.001*	<0.001*	0.846	<0.001*		
Sig. bet. Grps.		p_2=0.150,p_3<0.001*p_4=0.974,p_5<0.001*p_6=0.201,p_7<0.001*					

IQR: **Inter quartile range** SD: **Standard deviation.**

F: F for One-way ANOVA test, Pairwise comparison bet. each 2 groups were done using **a Post Hoc Test (Tukey)**

H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups were done using a **Post Hoc Test (Dunn's for multiple comparisons test)**

p: p-value for comparing the different studied groups.

p_1: p-value for comparing between **Control** and **each other groups.**

p_2: p-value for comparing between **HCV** and **HCV+HCC.**

p_3: p-value for comparing between **HCV** and **HBV**

p_4: p-value for comparing between **HCV** and **HBV+HCC.**

p_5: p-value for comparing between **HCV+HCC** and **HBV**

p_6: p-value for comparing between **HCV+HCC** and **HBV+HCC.**

p_7: p-value for comparing between **HBV** and **HBV+HCC** *: Statistically significant at $p \leq 0.05$.

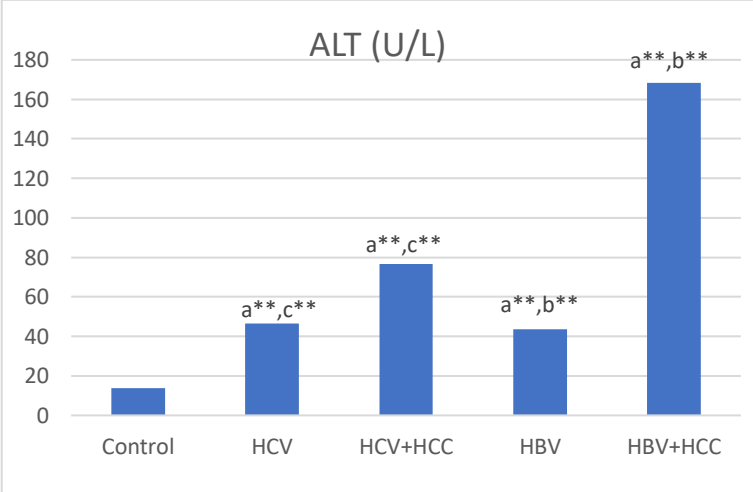

Figure (1): Levels of ALT in all studied five groups.

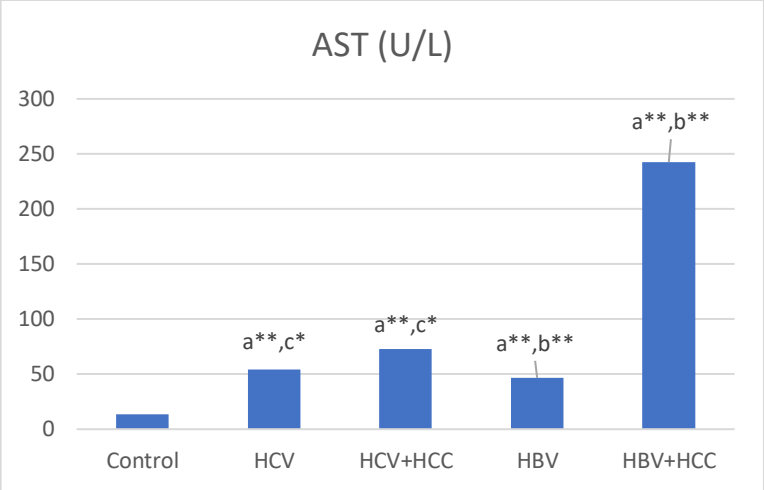

Figure (2): Levels of AST in all studied five groups.

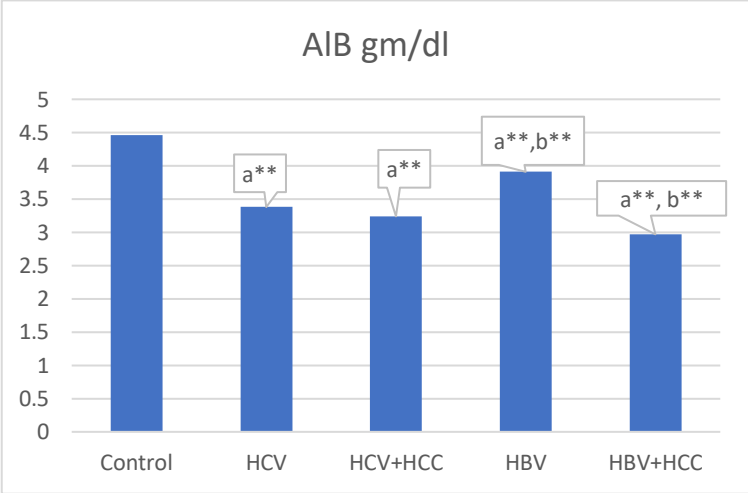

Figure (3): Levels of serum Albumin in all studied five groups.

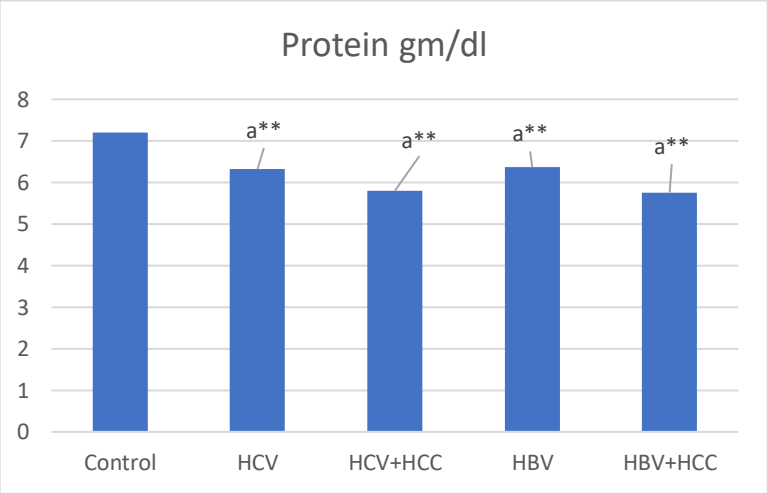

Figure (4): Levels of serum total Protein in all studied five groups.

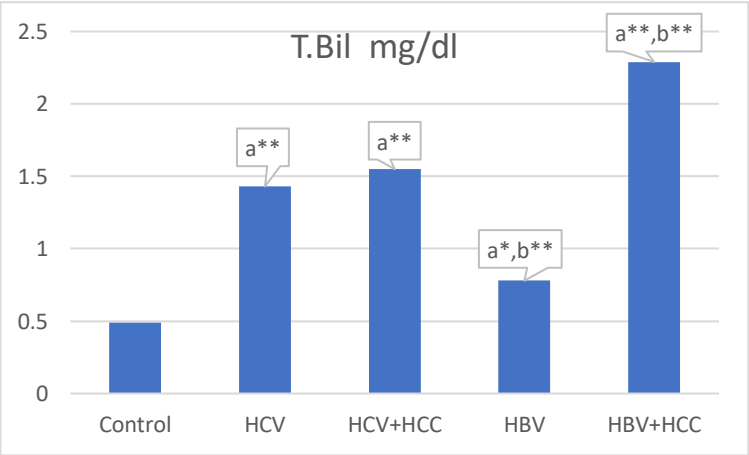

Figure (5): Levels of serum total bilirubin in all studied five groups.

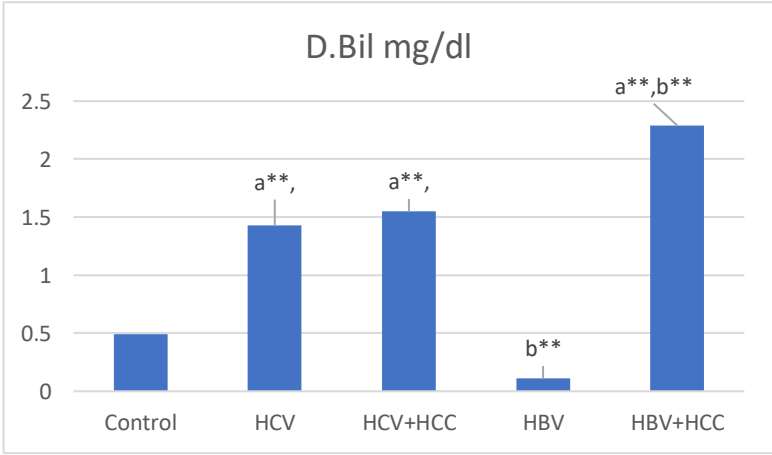

Figure (6): Levels of serum direct bilirubin in all studied five groups.

a: Significant from a control group c: Significant between HCV & HCC+HCV, b: Significant between HBV & HCC+HBV,
*(P<0.05)- **(P<0.01)

3.2.2. Prothrombin time (PT) (INR)

There was a statistically significant difference between the five groups p value <0.001*, while compared to the control group, only two groups elevated the group3 **HCV+HCC, and group 5 HBV+HCC had a** significant statistical difference with p-value <0.001*, but HCV and HBV groups have a p-value 0.116 and 0.797 respectively which have not **a** significant statistically difference compared to the control group as shown in table (4) and figure (7).

Table (4): Comparison between the different studied groups according to INR

	Control (n = 25)	HCV (n = 25)	HCV+HCC (n = 25)	HBV (n = 25)	HBV+HCC (n = 15)	Test of Sig.	p
INR							
Min. – Max.	0.90 – 1.20	1.0 – 1.50	1.01 – 2.20	0.80 – 1.40	1.09 – 1.90		
Mean ± SD.	1.06 ± 0.10	1.22 ± 0.15	1.43 ± 0.33	1.13 ± 0.15	1.58 ± 0.32	F= 18.753*	<0.001*
Median (IQR)	1.10 (1.0 – 1.10)	1.20 (1.10 – 1.30)	1.30 (1.20 – 1.50)	1.20 (1.0 – 1.20)	1.80 (1.25 – 1.85)		
p_1		0.116	<0.001*	0.797	<0.001*		
Sig. bet. Grps.		p_2=0.006*,p_3=0.687,p_4<0.001*,p_5<0.001*,p_6=0.281,p_7<0.001*					

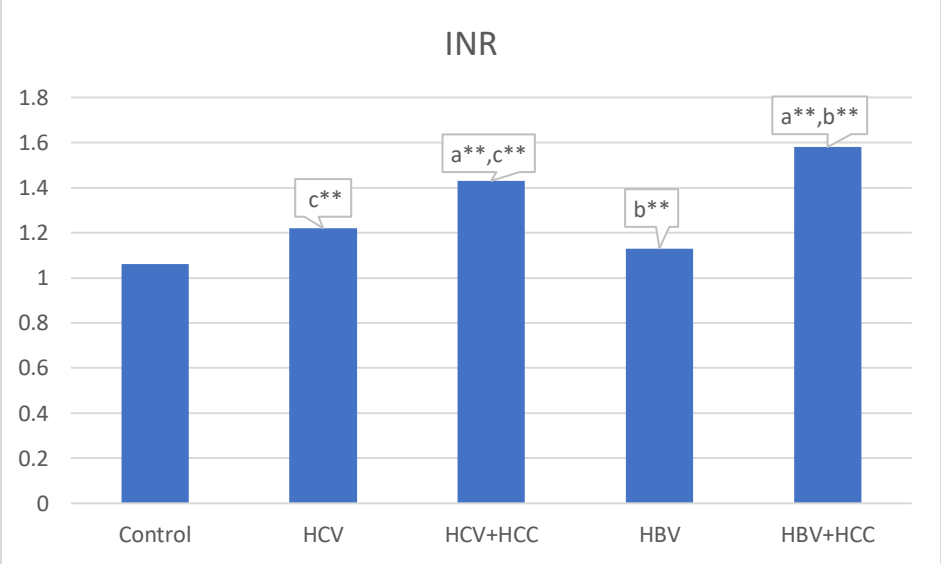

Figure (7): Levels of serum INR in all studied five groups.

a: Significant from a control group c: Significant between HCV & HCC+HCV, b: Significant between HBV & HCC+HBV, *(P<0.05)-**(P<0.01)

3.3. microRNA-122 (miR-122)

miR-122 ranged from 3.80 – 5.50 (Copies/ml) in the control group with a mean of 4.63 ± 0.52 Copies/ml, while in group 2 HCV patients it ranged from 8.30 – 10.50 Copies/ml with a mean of 9.22 ± 0.62 Copies/ml, in group 3 HCV+HCC patients it ranged from 5.0 – 7.10 Copies/ml with a mean of 6.0 ± 0.57 Copies/ml, in group 4 HBV patients it ranged from 6.40 – 8.40 Copies/ml with a mean of 7.40 ± 0.53 (Copies/ml), while in group 5 HBV+HCC patients it ranged from 4.80 – 6.60 (Copies/ml) with mean of 5.76 ± 0.57(Copies/ml), there was a statistically significant difference between the five groups p value <0.001*, compared to control group the four groups G2 G3 G4 G5 have the same a statistically significant difference with the same p-value <0.001*,

Serum miR-122 expression level HCV group (G2) was significantly elevated than **HCV+HCC** (G3) with p-value <0.001*, also Serum miR-122 expression level HBV group (G4) was significantly elevated than **HBV+HCC** (G5) with p-value <0.001*.

Comparing between **HCV** and **HBV** groups Serum miR-122 was highly increased in **HCV** than **HBV** group **(**8.30 – 10.50, 6.40 – 8.40 respectively) with a significant statistical difference(p_3<0.001*).

But there was no significant difference between (the **HCV+HCC)** group and **(the HBV+HCC)** group in the level of Serum miR-122 with p6-value of 0.673 not significant. as shown in Table (5) and figure (8).

Table (5): Comparison between the different studied groups according to miR-122

	Control (n = 25)	**HCV (n = 25)**	**HCV+HCC (n = 25)**	**HBV (n = 25)**	**HBV+HCC (n = 15)**	**Test of Sig.**	**p**
miR-122							
Min. – Max.	3.80 – 5.50	8.30 – 10.50	5.0 – 7.10	6.40 – 8.40	4.80 – 6.60		
Mean ± SD.	4.63 ± 0.52	9.22 ± 0.62	6.0 ± 0.57	7.40 ± 0.53	5.76 ± 0.57	F= 240.44*	<0.001*
Median (IQR)	4.70 (4.30 – 5.10)	9.10 (8.90 – 9.30)	6.0 (5.60 – 6.30)	7.40 (7.0 – 7.80)	5.80 (5.60 – 6.10)		
p_1		<0.001*	<0.001*	<0.001*	<0.001*		
Sig. bet. Grps.		p_2<0.001*,p_3<0.001*,p_4<0.001*,p_5<0.001*,p_6=0.673,p_7<0.001					

IQR: **Inter quartile range** SD: **Standard deviation.**

F: F for One-way ANOVA test, Pairwise comparison bet. Each 2 groups were done using **a Post Hoc Test (Tukey)**

H: H for **Kruskal Wallis test,** Pairwise comparison bet. Each 2 groups were done using a **Post Hoc Test (Dunn's for multiple comparisons test)**

p: p-value for comparing the different studied groups.

p_1: p-value for comparing between **Control** and **each other groups.**

p_2: p-value for comparing between **HCV** and **HCV+HCC.**

p_3: p-value for comparing between **HCV** and **HBV**

p_4: p-value for comparing between **HCV** and **HBV+HCC.**

p_5: p-value for comparing between **HCV+HCC** and **HBV**

p_6: p-value for comparing between **HCV+HCC** and **HBV+HCC.**

p_7: p-value for comparing between **HBV** and **HBV+HCC.**

3.4. Association between miR-122 and TNF-α

Compared with healthy control group, serum miR-122 levels and Tumor necrosis factor-α (TNF-α) were markedly increased in the HCV infection cases group with a significant p-value of<0.001*, and their levels were decreased in the **HCV+HCC group but still higher than** the healthy control group with a significant p-value of<0.001*for both, miR-122 level decreasing in **HCV+HCC group from HCV group with a** significant p-value of <0.001*, and the TNF-α level decreasing in **HCV+HCC group from HCV group with a** significant p-value of 0.010*.

In groups, HBV, and HBV+HCC Compared with the healthy control group, serum miR-122 level, and TNF-α level were markedly increased in the HBV infection cases group with a significant p-value of<0.001*, and their levels were decreased in **HBV+HCC group but still higher** than the healthy control group with a significant p-value of<0.001*for both,

Serum miR-122 and TNF-α levels were increased in HCV and HBV infection, and their levels decreased when the hepatitis viral infection developed into hepatocellular carcinoma HCC but were still higher than the levels in healthy subjects, miR-122 and TNF-α levels have a parallel change as shown in table (6).

Table (6): Comparison between the different studied groups according to miR-122, TNF-α, and AFP

	Control (n = 25)	**HCV (n = 25)**	**HCV+HCC (n = 25)**	**HBV (n = 25)**	**HBV+HCC (n = 15)**	**p**
miR-122	4.63 ± 0.52	9.22 ± 0.62	6.0 ± 0.57	7.40 ± 0.53	5.76 ± 0.57	<0.001*
TNF-α	7.66 ± 0.59	30.07 ± 31.09	12.04 ± 1.65	61.05 ± 199.1	14.36 ± 2.18	<0.001*
AFP	2.80 ± 0.96	15.07 ± 8.08	2496.7± 2417.3	4.07 ± 2.23	11620±19321	<0.001*

p: p-value for comparing the different studied groups.
*: Statistically significant at p ≤ 0.05

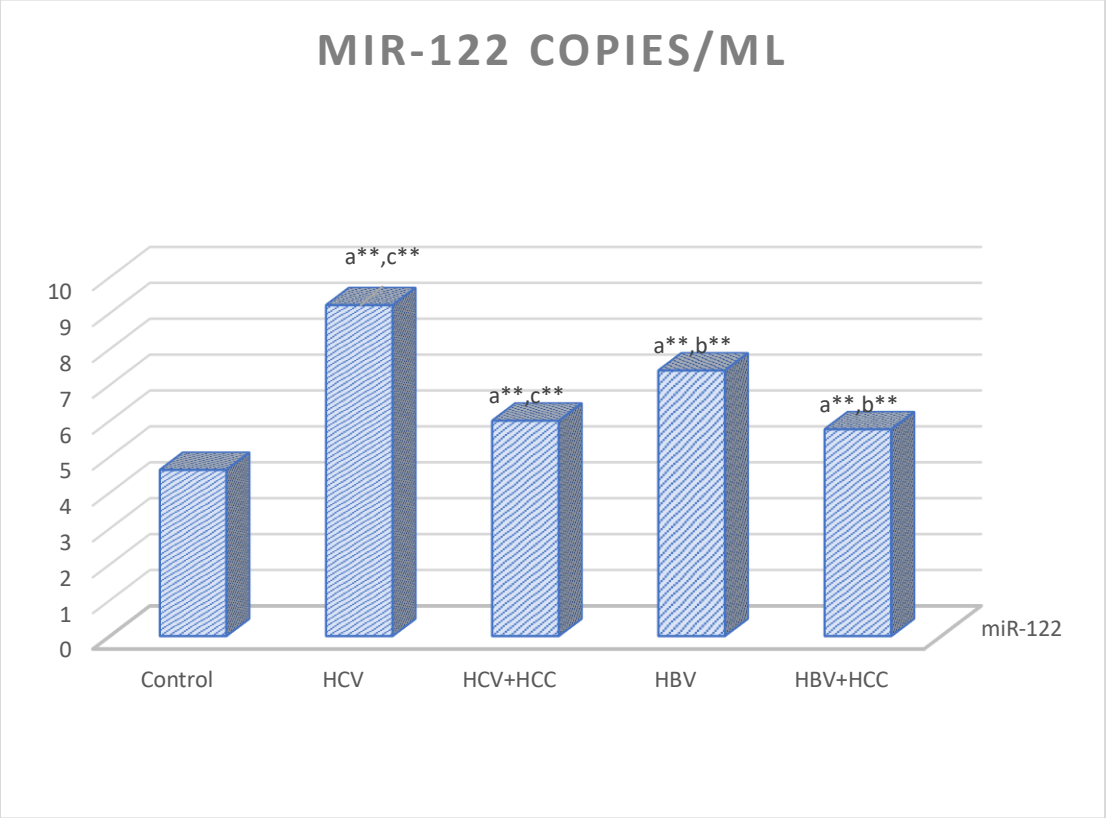

Figure (8): Levels of serum miR-122 in all studied five groups.

a: Significant from a control group c: Significant between HCV & HCC+HCV, b: Significant between HBV & HCC+HBV, *(P<0.05)- **(P<0.01)

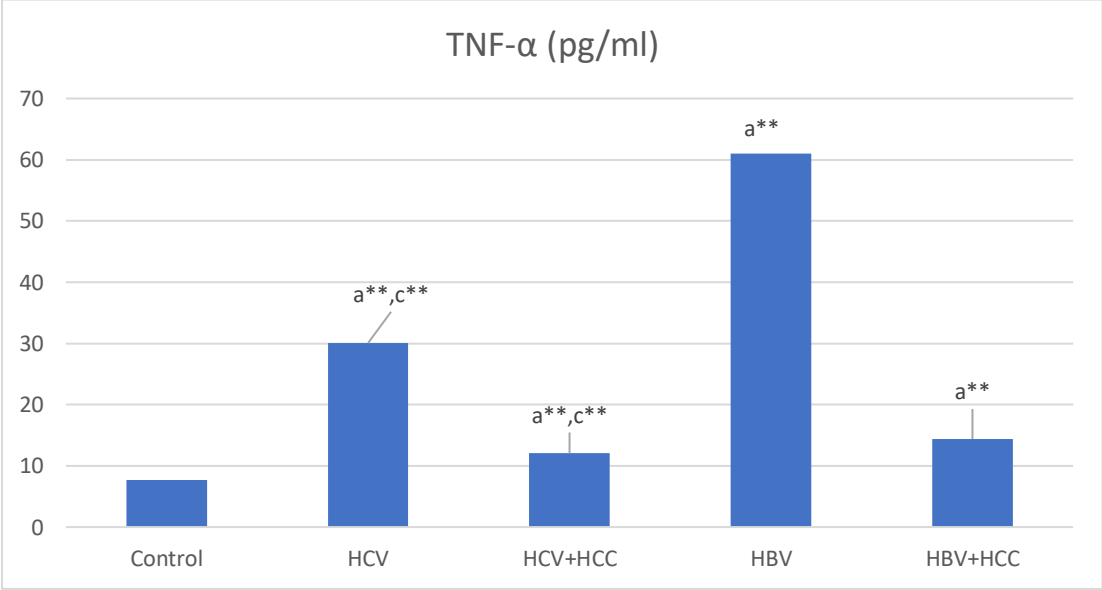

Figure (9): Levels of serum **TNF-α** in all studied five groups.

a: Significant from a control group c: Significant between HCV & HCC+HCV, b: Significant between HBV & HCC+HBV, *(P<0.05)- **(P<0.01)

Prognostic performance for TNF-α, miR-122, and AFP to discriminate HCV from HCV+HCC.

Table (7) the results showed that the cut-off for miR-122 was >7.1 at a sensitivity of 100% and specificity of 100%, but the sensitivity and specificity for TNF-α 72%, and 60% respectively with a cut-off>12.1, while AFP was was had a cut-off ≤27 with sensitivity 96% and specificity 92%, the AUC of miR-122 was 1.0 (Excellent), significant P-value <0.001* with PPV 100% and NPV 100%, TNF-α has AUC of 0.745 (Good) it was significant p-value 0.003*, PPV 64.0 and NPV 68.2, while AFP has AUC 0.93(Excellent) which significant p-value <0.001*, PPV 92.3 and NPV 95.8.

Receiver Operating Characteristics (ROC) Curves analysis:

ROC curve analysis was designed for miR-122, TNF-α, and AFP to discriminate HCV-infected patients against **HCV+HCC** group patients. ROC curve was performed as cut off for disease progression to evaluate the sensitivity and specificity for miR-122 and TNF-α compared with AFP to diagnose HCC from HCV infection. As shown in Figure (10).

Table (7): Prognostic performance for TNF-α, miR-122 and AFP to discriminate HCV (n= 25) from HCV+HCC (n = 25)

	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
miR-122	1.000	<0.001*	1.0 – 1.0	>7.1	100.0	100.0	100.0	100.0
TNF-α (pg/ml)	0.745	0.003*	0.607 – 0.883	>12.1	72.0	60.0	64.3	68.2
AFP	0.930	<0.001*	0.885 – 1.0	≤27	96.0	92.0	92.3	95.8

AUC: Area Under a Curve p-value: Probability value CI: Confidence Intervals
NPV: Negative predictive value PPV: Positive predictive value
*: Statistically significant at p ≤ 0.05

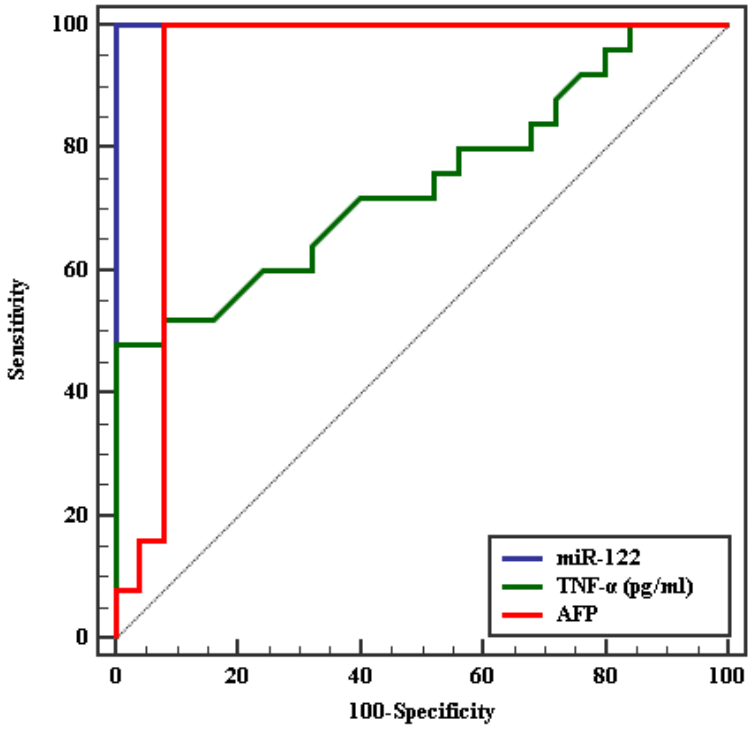

Figure (10): ROC curve for TNF-α, miR-122, and AFP to discriminate HCV (n= 25) from HCV+HCC (n = 25)

Prognostic performance for TNF-α, miR-122, and AFP to discriminate HBV from HBV+HCC.

Table (8) the results showed that the cut-off for miR-122 was ≤6.4 at a sensitivity of 86.67% and specificity of 96%, the sensitivity and specificity for TNF-α 93.33%, 48.0% respectively with cut-off ≤15.73, while AFP has a cut off >9 with sensitivity 100% and specificity100%, the AUC of miR-122 was 0.99 (Excellent) significant P-value <0.001* with PPV 92.9% and NPV 92.3%, TNF-α has AUC of 0.527 (fair) it was not significant p-value 0.780, PPV 51.9 and NPV 92.3, while AFP has AUC 1.0 (Excellent) which significant p-value <0.001*, PPV 100 and NPV 100.

Receiver Operating Characteristics (ROC) Curves analysis:

ROC curve analysis was designed for miR-122, TNF-α, and AFP to discriminate HBV-infected patients against **HBV+HCC** group patients. ROC curve was performed as cut off for disease progression to evaluate the sensitivity and specificity for miR-122 and TNF-α compared with AFP to diagnose HCC from HCV infection. As shown in figure (11).

Table (8): Prognostic performance for TNF-α, miR-122 and AFP to discriminate HBV+HCC (n= 25) from HBV (n = 25)

	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
miR-122	0.991	<0.001*	0.971 – 1.0	≤6.4	86.67	96.0	92.9	92.3
TNF-α (pg/ml)	0.527	0.780	0.342 – 0.712	≤15.73	93.33	48.0	51.9	92.3
AFP	1.000	<0.001*	1.0 – 1.0	>9	100.0	100.0	100.0	100.0

AUC: Area Under a Curve p-value: Probability value CI: Confidence Intervals
NPV: Negative predictive value PPV: Positive predictive value
*: Statistically significant at p ≤ 0.05

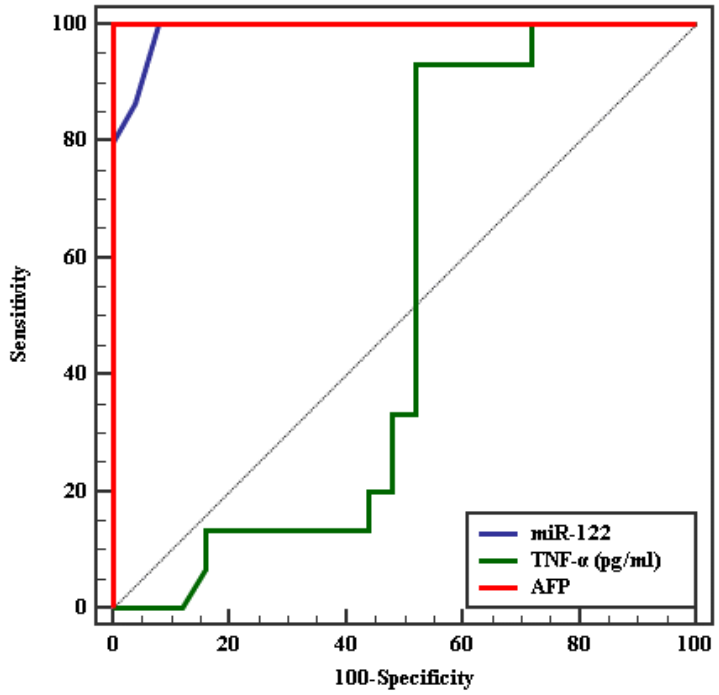

Figure (11): ROC curve for TNF-α, miR-122, and AFP to discriminate HBV+HCC (n= 15) from HBV (n = 25)

4. DISCUSSION

Liver diseases are a major cause of mortality and morbidity worldwide, Cirrhosis, viral hepatitis, and liver cancer account for over 2 million deaths worldwide from liver disease, which accounts for 4% of all deaths (1 out of every 25 deaths); 1 in 3 deaths from liver disease are female. According to this estimate, liver cancer causes 600,000 to 900,000 fatalities **(2).** By 2030, the WHO signatories planned to eliminate viral hepatitis no longer a danger to public health. In comparison to the baseline year of 2015, WHO defined elimination as a 65% decrease in mortality and a 90% decrease in incidence **(WHO. Global Health Sector Strategy on Viral Hepatitis, 2016–2021) (17).** The report concentrates on hepatitis B and C since they account for 96% of all hepatitis-related deaths.

The major method of HCC surveillance recommended by the European Association for the Study of the Liver (EASL) is semi-annual abdominal ultrasonography, with or without alpha-fetoprotein (AFP) as the primary strategy **(EASL Guidelines 2018) (18).**

Recent findings, however, have emphasized the limits of ultrasound-based monitoring, including its operator dependence and significant center-to-center performance variability, poor sensitivity to identify HCC at an early stage if used alone without AFP, and risk of screening-related complications **(19).** If ultrasound liver visibility is judged to be insufficient, an MRI or CT scan should be considered instead. Ultrasound liver visualization may benefit from systematic documentation and scoring **(20).**

Serum AFP is highly significantly elevated in HCC patients associated with HCV or HBV infection in some Egyptian patients **(21),** While AFP has been utilized as a marker for HCC diagnosis and prognosis, only 60% of HCC patients show positive AFP, according to **(22).**

Compared to historical cohorts where many patients had active viremia and were prone to frequent false positive AFP findings, AFP may have an even larger additional benefit to ultrasonography. Notably, in actual practice, physical harms of AFP, i.e., extra diagnostic testing because of false positive or inconclusive results, are frequently avoided when physicians follow biomarker patterns when interpreting AFP readings rather than rigidly following a single-measurement threshold of 20 ng/mL **(23).**

Tayob and colleagues have shown that biomarker performance is greatly improved when longitudinal biomarker data are used rather than a single threshold evaluation **(24).**

Alpha-fetoprotein (AFP) is the most widely used biomarker for the detection of HCC, however, its diagnostic relevance is progressively being questioned because of its limited sensitivity, especially for early HCC, according to **(Debes JD et al., 2021).** 20% of HCC patients can often have readings over 400 ng/mL confirm the diagnosis.

Due to the high frequency of HCC, ongoing research is needed to find precise, non-invasive biomarkers for early identification and follow-up that will improve prognostic results. Although there has been a lot of research in this area, it still requires substantial work to confirm regularly examined biomarkers.

Therefore, we have decided to look for trustworthy biomarkers to diagnose HCC, HCV, and HBV. This research may add to earlier investigations that sought to improve patient therapy outcomes.

Small non-coding RNAs called microRNAs (miRNAs) mediate post-transcriptional gene silencing by accelerating the degradation of mRNA and suppressing translation **(26)**,

One of the most highly expressed miRNAs in any tissue, miR-122 is a liver-specific microRNA that is widely expressed in hepatocytes [about 660,000

copies per cell]. It accounts for over 72% of the total miRNA pool in the liver **(27,28).**

Recently, the development of the anti-miR122 therapeutic miravirsen. Miravirsen has demonstrated in vitro antiviral activity against all HCV genotypes, Miravirsen interferes with the functions of miR-122 both in viral proliferation and in cholesterol homeostasis, miravirsen has demonstrated broad antiviral activity and a relatively high genetic barrier to resistance (29).

The results of this study showed that serum **miR-122** levels compared to the control group the two groups HCV and HCV+HCC have the same statistically significant difference with the same p-value <0.001*, Serum miR-122 expression level HCV group (G2) was significantly elevated than **HCV+HCC** (G3) with p-value <0.001*,

This agrees with the result data obtained by Gaber DA et al., 2022 they reported that: A significant difference between the HCC group and the HCV patients in terms of miR-122 expression (p = 0.004) suggests that miR-122 has a prognostic function as a predictor of HCC in individuals with chronic HCV **(30).**

Also, the above results are supported by Enas M et al. Their study demonstrated that patients' serum had higher levels of miRNA 122 expression than the control group. A substantial increase in miR-122 was also seen in the HCV group (3.2± 2) compared to the HCC group (1.2± 0.46), and the control group (1.09 ±0.0) (P ≤0.0001). Furthermore, compared to the control group, the expression level of miRNA 122 was higher in the HCC **(31).**

These results are in agreement with those of El-Garem H **et al., 2014 (32)** who reported that serum from patients with HCV had higher levels of miRNA-122 compared with serum from healthy controls.

TNF-α is primarily generated by activated monocytes, macrophages, endothelial cells, and lymphocytes and has biological action through binding to soluble TNF-binding receptors (TNFR1 and TNFR2) **(33).** TNF-α has been associated with a poor prognosis in patients with severe acute respiratory syndrome (SARS)و, but the serum TNF-α level is not a significant biomarker for diagnosis or prognosis of mild COVID-19 patients **(34).**

TNF-α, a host factor, is hypothesized to play a significant role in hepatocarcinogenesis by inducing necroinflammation and fibrogenic factors **(35).**

Serum TNF-α can be used as a biomarker to differentiate between healthy and infected patients with HCV or HBV and the development of HCC **(36).** TNF-α is a powerful pro-inflammatory cytokine in and of itself **(37).** Necroinflammation in hepatocytes causes mutagenesis and oncogene activation from proto-oncogenes in host cells, resulting in HCC **(35).** TNF-α is also known to generate HCC via the chronic inflammatory route by activating and differentiating hepatic progenitor cells **(38).**

Concerning the association between miR-122 and TNF-α in liver diseases of Egyptian patients this study results showed that compared with healthy control cases, serum miR-122 levels and Tumor necrosis factor-α (TNF-α) were markedly increased in the HCV infection cases group with a significant p-value of<0.001*, and their levels were decreased in **HCV+HCC** group but still higher than the healthy control group with a significant p-value of<0.001*for both, miR-122 level decreasing in **HCV+HCC** group from **HCV** group with a significant p-value of <0.001*, and the TNF-α level decreasing in **HCV+HCC** group from **HCV** group with a significant p-value of 0.010*, then serum miR-122 and TNF-α levels together can differentiate between HCV infected patients and healthy subjects significantly, also can differentiate HCC+HCV patients from healthy control and HCV infected patients significantly.

In the case of HBV infection, our study results showed in groups HBV and HBV+HCC Compared with healthy control cases, serum miR-122 level and TNF-α level were markedly increased in the HBV infection cases group with a significant p-value of<0.001*, and their levels were decreased in **HBV+HCC** group but still higher than the healthy control group with a significant p-value of<0.001* for both.

Serum miR-122 and TNF-α levels were increased in HCV and HBV infection, and their levels decreased when the hepatitis viral infection developed into hepatocellular carcinoma HCC but were still higher than the levels in healthy subjects, then miR-122 and TNF-α levels have parallel changes.

Conflicts of Interest:

The authors affirm that there are no conflicts of interest.

Funding:

This research received no external funding.

5. REFERENCES

1. Neshat SY, Quiroz VM, Wang Y, Tamayo S, Doloff JC. Liver Disease: Induction, Progression, Immunological Mechanisms, and Therapeutic Interventions. Int J Mol Sci. 2021 Jun 24;22(13):6777. doi: 10.3390/ijms22136777.
2. Asrani SK, Devarbhavi H, Eaton J, Kamath PS. The burden of liver diseases in the world. J Hepatol. 2019 Jan;70(1):151-171. doi: 10.1016/j.jhep.2018.09.014. Epub 2018 Sep 26.
3. Li D., Ji Y. M., Guo J. L., Guo Q. Upregulated expression of *MTFR2* as a novel biomarker predicts poor prognosis in hepatocellular carcinoma by bioinformatics analysis. *Future Oncology*. 2021;**17**(24):3187–3201. doi: 10.2217/fon-2020-1160.
4. Villanueva A. Hepatocellular Carcinoma. N Engl J Med. 2019; **380:1450**–1462.
5. Esmat G. Hepatitis C in the eastern Mediterranean region. East Mediterr Health J. 2013;19(7):587-8.
6. WHO HCV report 2022: https://www.who.int/news-room/fact-sheets/detail/hepatitis-c
7. Sharafi H, Alavian SM. The Rising Threat of Hepatocellular Carcinoma in the Middle East and North Africa Region: Results From Global Burden of Disease Study 2017. Clin Liver Dis (Hoboken). 2020 Jan 29;14(6):219-223. doi: 10.1002/cld.890.
8. Ahn H. R., Baek G. O., Yoon M. G., et al. HMBS is the most suitable reference gene for RT-qPCR in human HCC tissues and blood samples. *Oncology Letters*. 2021;**22**(5): p. 791. doi: 10.3892/ol.2021.13052.
9. Gebert, L. F. R., & MacRae, I. J. (2019). Regulation of microRNA function in animals. Nature Reviews. Molecular Cell Biology, 20(1), 21–37. 10.1038/s41580-018-0045-7.
10. Alkan AH, Akgül B. Endogenous miRNA Sponges. Methods Mol Biol. 2022; 2257:91-104. doi: 10.1007/978-1-0716-1170-8_5. PMID: 34432275.
11. Girard M, Jacquemin E, Munnich A, Lyonnet S, and Henrion-Caude A. 2008. miR-122, a paradigm for the role of microRNAs in the liver. *J. Hepatol.* 48:648–656.
12. Boutz DR, Collins PJ, Suresh U, Lu M, Ramírez CM, Fernández-Hernando C, et al. Two-tiered approach identifies a network of cancer and liver disease-related genes regulated by miR-122. J Biol Chem 2011; 286(20): 18066–18078. 7.
13. Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, Huang Y, Chen HC, Lee CH, Tsai TF. MicroRNA-122 plays a critical role in liver

homeostasis and hepatocarcinogenesis. J Clin Invest. 2012;122: 2884–2897.

14. Sendi H, Mead I, Wan M, Mehrab-Mohseni M, Koch K, Atala A, et al. miR-122 inhibition in a human liver organoid model leads to liver inflammation, necrosis, steatofibrosis and dysregulated insulin signaling. PLoS One. 2018;13: e0200847. doi: 10.1371/journal.pone.0200847.
15. Ma L, Chen S, Mao X, Lu Y, Zhang X, Lao X, Qin X, Li S. The association between TNFR gene polymorphisms and the risk of hepatitis B virus-related liver diseases in Chinese population. Sci Rep. 2018;**8**(1):9240. doi: 10.1038/s41598-018-27623-7.
16. Méndez-García LA, Nava-Castro KE, Ochoa-Mercado TL, Palacios-Arreola MI, Ruiz-Manzano RA, Segovia-Mendoza M, Solleiro-Villavicencio H, Cázarez-Martínez C, Morales-Montor J. Breast Cancer metastasis: are cytokines important players during its development and progression? J Interferon Cytokine Res. 2019;**39**(1):39–55. doi: 10.1089/jir.2018.0024.
17. World Health Organization. *Global Health Sector Strategy on Viral Hepatitis 2016–2021*, 2nd ed.; World Health Organization: Geneva, Switzerland, 2016.
18. European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J Hepatol 2018; 69:182–236.
19. Tzartzeva K, Obi J, Rich NE, Parikh ND, Marrero JA, Yopp A, Waljee AK, et al. Surveillance Imaging and Alpha Fetoprotein for Early Detection of Hepatocellular Carcinoma in Patients with Cirrhosis: A Meta-analysis. Gastroenterology 2018; 154:1706–1718 e1701.
20. Loomba R, Lim JK, Patton H, El-Serag HB. AGA Clinical Practice Update on Screening and Surveillance for Hepatocellular Carcinoma in Patients with Nonalcoholic Fatty Liver Disease: Expert Review. Gastroenterology 2020; 158:1822–1830.
21. Yameny, A., Alabd, S., Mansor, M. Evaluation of AFP for diagnosis of HCC in Egyptian patients. *Journal of Medical and Life Science*, 2023; 5(1): 43-48. doi: 10.21608/jmals.2023.329306
22. Wang G., Lu X., du Q., et al. Diagnostic value of the γ-glutamyltransferase and alanine transaminase ratio, alpha-fetoprotein, and protein induced by vitamin K absence or antagonist II in hepatitis B virus-related hepatocellular carcinoma. *Scientific Reports*. 2020;**10**(1): p. 13519. doi: 10.1038/s41598-020-70241-5.
23. Atiq O, Tiro J, Yopp AC, Muffler A, Marrero JA, Parikh ND, Murphy C, et al. An assessment of benefits and harms of hepatocellular carcinoma surveillance in patients with cirrhosis. Hepatology 2017; 65:1196–1205.
24. Tayob N, Corley DA, Christie I, Almers L, Rahal AK, Richardson P, White DL, et al. Validation of the Updated Hepatocellular Carcinoma Early Detection Screening Algorithm in a Community-Based Cohort of Patients with Cirrhosis of Multiple Etiologies. Clin Gastroenterol Hepatol 2020.
25. Debes JD, Romagnoli PA, Prieto J, et al. Serum biomarkers for the prediction of hepatocellular carcinoma. Cancers. 2021;13(7):1681.

26. Winter J., Jung S., Keller S., Gregory R.I., Diederichs S. Many roads to maturity: MicroRNA biogenesis pathways and their regulation. Nat. Cell Biol. 2009; 11:228–234.
27. Lagos-Quintana M., Rauhut R., Yalcin A., Meyer J., Lendeckel W., Tuschl T. Identification of Tissue-Specific MicroRNAs from Mouse. Curr. Biol. 2002; 12:735–739. doi: 10.1016/S0960-9822(02)00809-6.
28. Zhao X. F., Li N., Lin D. D., Sun L. B. (2020). Circulating MicroRNA-122 for the Diagnosis of Hepatocellular Carcinoma: A Meta-Analysis. Biomed. Res. Int. 2020, 5353695. 10.1155/2020/5353695
29. Yameny, A. miRNA-122 from Laboratory biomarker to the treatment of HCV. *Journal of Bioscience and Applied Research*, 2017; 3(4): 145-151. doi: 10.21608/jbaar.2017.125861
30. Gaber DA, Shaker O, Younis AT, El-Kassas M. LncRNA HULC and miR-122 Expression Pattern in HCC-Related HCV Egyptian Patients. Genes (Basel). 2022 Sep 18;13(9):1669. doi: 10.3390/genes13091669.
31. Enas M. Ghoneima, Aza M. Abed El-Aziza, Tawfik M. Abd El-Mottaleba, Ghada R. El-Hendawyc, Hossam M. El-Ezawyb, Fatma O. Khalila. Profiling of microRNA-122 in chronic hepatitis C. 2016. Menoufia Med J 29:826–834.
32. El-Garem H, Ammer A, Shehab H, Shaker O, Anwer M, El-Akel W, Omar H. Circulating microRNA, miR-122 and miR-221 signature in Egyptian patients with chronic hepatitis C related hepatocellular carcinoma. World J Hepatol 2014; **6**:818–824.
33. Macewan DJ. TNF receptor subtype signaling: Differences and cellular consequences. Cell Signal 2002; 14:477-92.
34. Alabd, S., Yameny, A. The association between Tumor Necrosis Factor-alpha level (TNF-α) and moderate COVID-19 patients in Egypt. *Journal of Bioscience and Applied Research*, 2021; 7(4): 223-228. doi: 10.21608/jbaar.2021.251241
35. Aroucha DCBL, do Carmo RF, Moura P, Silva JLA, Vasconcelos LRS, Cavalcanti MSM, Muniz MTC, Aroucha ML, Siqueira ERF, Cahú GGOM, Pereira LMMB, Coêlho MRCD. High tumor necrosis factor-α/interleukin-10 ratio is associated with hepatocellular carcinoma in patients with chronic hepatitis C. Cytokine. 2013;**62**(3):421–425. doi: 10.1016/j.cyto.2013.03.024.
36. Yameny, A., Alabd, S., Mansor, M. Serum TNF-α levels as a biomarker in some liver diseases of Egyptian patients. *Journal of Medical and Life Science*, 2023; 5(1): 1-8. doi: 10.21608/jmals.2023.329303
37. Barbosa MLC, Fumian MM, Miranda ALP, Barreiro EJ, Lima LM. Therapeutic approaches for tumor necrosis factor inhibition %J Brazilian. J Pharm Sci. 2011; **47:427**–446.
38. Jing Y, Sun K, Liu W, Sheng D, Zhao S, Gao L, Wei L. Tumor necrosis factor-α promotes hepatocellular carcinogenesis through the activation of hepatic progenitor cells. Cancer Lett. 2018; **434:22**–32. doi: 10.1016/j.canlet.2018.07.001.