Egypt. J. Chem. **Vol. 68**, No. 5 pp. 527 - 537 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Innovative Diagnostic Model Based on Beta-Catenin, Tumor Necrosis Factor-Alpha, Interleukin 21 and CA19.9 Biomarkers for Follow-up of HCC Development after DAA Therapy for HCV Infection

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Abstract

Background & Aims: Our goals were to assess the predictive value of β-catenin, TNF-α, IL-21, and CA19-9 biomarkers and the diagnostic performance of a created model for early prediction of HCC after DAAs. **Materials and Methods:** ELISA was used to estimate serum levels of investigated biomarkers in 84 adult patients and 20 healthy individuals. To create an internally validated model predicts HCC development following DAA therapy of HCV patients, we employed linear regression analysis. **Results:** For HCV infected patients compared to the treated group, significant higher expression was detected in β-catenin ($P < 0.0001$) and TNF-α ($P < 0.002$) but a substantial increase was detected only in β-catenin ($P < 0.0001$) in LC. The AUC of β-catenin, TNF-α, IL-21, and CA19-9 for the potential diagnostic values of HCV-infected HCC patients from non-malignant individuals were 0.810, 0.829, 0.719, and 0.743; respectively. A newly developed MSB model based on β-catenin, TNF-α, IL-21, and CA19-9 had an AUC of 0.933 with sensitivity of 89.3%, and specificity of 93.7% at the best cut-off value of 3.09. **Conclusion:** The combination of β-Catenin, TNF-α, IL-21, and CA19-9 biomarkers constitute an outstanding diagnostic model for HCC (MSB model) that performs better pinpointing than each one alone.

Keywords: HCC, HCV, DAA, β-Catenin, Interleukin 21 (IL-21), Tumor Necrosis Factor-alpha (TNF-α), CA19-9.

1. Introduction

One of the most prevalent primary liver cancers, hepatocellular carcinoma (HCC) is rapidly rising in prevalence worldwide and is a leading cause of cancer-related death, particularly in men [1]. The advent of hepatitis C virus (HCV), which is thought to be the major etiology in Egypt, Japan, and the USA and the second most prevalent cause of HCC, is one of the risk factors for developing HCC [2]. The incidence of HCC continues to rise despite the fast-paced period of direct acting antiviral regimens (DAA) in the treatment of HCV [3]. Even when the virus has been successfully eradicated, individuals still experience chronic HCV complications such liver cirrhosis and HCC. Given the high rate of recurrence, postoperative HCC patients' long-term survival is inadequate. Furthermore, there is a pressing need to find novel serological markers with high accuracy and practicality for the early identification of HCC because serum α-Fetoprotein level detection of HCC is hampered by its low sensitivity [4]. Proteantigens, cytokines, enzymes, isoenzymes, and transcripts of associated genes are the different categories into which serological indicators can be separated [4]. The current study set out to examine the function of β-catenin (β-cat), tumor necrosis factor-alpha (TNF-α), and interleukin 21 (IL-21) in the early detection of HCV-associated HCC after DAA therapy. A protein called β-cat (proteantigen) is linked to the intracellular region of E-cadherin, which is a component of the adherents' junction [5]. Stem and progenitor cell self-renewing proliferation, and cell fate regulation are only a few of the developmental processes that are impacted by the Wnt/Wingless (Wg) signaling pathway, of which β-cat is a crucial component [6]. Twenty to forty percent of HCC tumors have a mutation that affects the Wnt/β-catenin pathway, which seems to be the most common occurrence in these tumors [7]. Cytokines as essential elements of the immune system are Intercellular regulators associated with viral control and infection-induced damage [8]. promotion of Angiogenesis, tumor-associated inflamed cells are drawn in, and tumor growth is directly regulated. are all believed to be significantly influenced by cytokine production [9, 10]. Tumor necrosis factor-α (TNF-α) and other inflammatory cytokines are essential components of inflammation in chronic HCV infection. Numerous cell types, such as tumor cells, neutrophils, fibroblasts, keratinocytes, and macrophages, generate TNF-α [11]. HCC promotion, cirrhosis, and liver injury have all been connected to this cytokine [12]. TNF-α initiates intracellular downstream signaling by interacting with its receptor on the cell membrane [13]. Interleukin-21 (IL-21). The newest identified member of the Type-I cytokine superfamily with immune system-regulating properties [14]. Numerous immune cells, including natural killer (NK) cells, activated CD4+ T cells, and the pro-inflammatory T helper 17 subgroup, are the primary producers of interleukin 21 [15]. Additionally, IL-21 has been shown to be essential for managing persistent viral infections in a number of in vivo investigations using animal models [16]. Sialyl-Lewis A antigen, another name for carbohydrate antigen 19-9 (CA19-9), is a cancer indicator frequently used to test for several digestive system tumors in humans [17]. A poor prognosis is linked to elevated serum CA19-9 levels in gastric,

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Received date 25 November 2024; Accepted date 04 February 2025
DOI: 10.21608/ejchem.2025.339164.10880

colorectal, and pancreatic cancers [18]. Lately, a poor prognosis for HCC was correlated with CA19-9 levels more than 37 ng/mL [19]. Therefore, the goal of this investigation was to confirm the function of serum β-catenin (β-cat), interleukin 21 (IL-21), and tumor necrosis factor alpha (TNF-α) in conjunction with CA19-9 as forecasting markers for the development of HCC in liver disorders associated to HCV following the use of direct-acting antivirals.

2. Experimental

2.1. Samples and Study Cases

Between March 2022 and May 2023, 104 participants participated in a case-control study (40 HCV-related LC patients, 44 HCV-related LC evolved to HCC patients and 20 healthy persons served as a control group). The patients who received Sofosobuvir-based DAA treatment follow the Egyptian national treatment guidelines for the treatment of genotype 4 CHC infection. At a 12-week follow-up, undetectable HCV-RNA using a quantitative RT-PCR assay was used to define sustained virological response (SVR12). All participants gave their written agreement, and the study was authorized by the Mansoura University Faculty of Medicine's Investigation and Ethics Committee in compliance with the Declaration of Helsinki. WHO Universal Trial Number: MDP.23.10.135). Five millilitres of venous blood were drawn from each participant, allowed to coagulate, and then centrifuged for ten minutes at 5000 rpm. Before being used, the serum was collected and kept at - 20 ºC. All patients underwent complete laboratory testing, including CBC, ELISA for HCV antibody, PCR for HCV RNA viral load, serum ALT, AST, alkaline phosphatase, T. bilirubin, albumin, INR, creatinine, alpha-Fetoprotein (AFP), CEA, and CA19-9. Fibrosis-4-score (FIB4), AAR, PLR, NLR, and APRI were computed, and each patient was categorized based on the Child-Pugh Score [20].

2.2. Measurement of Serum β-catenin, TNF-α and IL-21

Following the manufacturer's instructions, a commercially available ELISA kit (Bldg., 501 Changsheng S Rd, Nanhu Dist., Jiaxing, Zhejiang, China) was used to test the serum levels of β-catenin, TNF-α, and IL-21. Abdominal ultrasonography, triphasic CT abdomen, serum AFP, and histological confirmation were used to diagnose the HCC group. There was no indication of distant metastases or local invasion in the cirrhotic group. At the time of recruitment, there was no biochemical or clinical indication of liver disease or any known medical conditions in the control group. Patients with HCC who had undergone surgical, interventional, or medicinal treatment, patients with malignancies other than HCC, and patients with other viral infections (HBV infection or co-infection with HCV) were excluded.

2.3. Statistical analysis

Version 24 of the SPSS software program was utilized. The interquartile range (IQR) and median were used to represent continuous variables. The Kruskal Wallis and Mann-Whitney tests were used to evaluate group comparisons. A P-value of less than 0.05 was deemed significant. Spearman's correlation coefficient was used to determine the correlation between the variables. The best cut-off values for the examined markers with sensitivity and specificity were found by plotting receiver operating characteristic (ROC) curves after a model for predictions was constructed using linear regression [21].

3. Results

3.1. Clinical and pathological characteristics of the study subjects

The adult patients with HCV related chronic liver diseases divided into 44 HCC patients on top of LC (20 HCV infected HCC patients and 24 HCV- treated HCC patients) and 40 HCV-related LC patients (20 HCV infected LC patients and 20 HCV-treated LC patients) in addition to 20 healthy subjects were enrolled in the current study. HCV- treated HCC patients showed 12 (50%) patients were Child-Pugh A, 9 (37.4%) patients were Child-Pugh B and 3 (12.5%) patients were Child-Pugh C, while in HCV infected HCC patients, 10 (50%) patients were in Child-Pugh B and 10 (50%) patients were Child-Pugh C with significant difference between two groups (P value < 0.0001). However, no significant difference was detected in platelets count, ALT, AST, AFP and Albumin between HCV infected HCC patients and HCV treated HCC patients, but significant difference was detected in liver cirrhosis activity (P < 0.0001) and Child Pugh score (P < 0.0001) between HCC groups (Table 1 & 2).

3.2. Serum levels of β-Catenin, TNF-α, IL-21 and CA19-9 in all study groups

In HCC patients, increased serum levels of β catenin, TNF-α, IL-21 and CA19-9 were detected in HCV- infected HCC patients compared to HCV- treated HCC with significant difference in β-Catenin (27 vs 11.9 ng/mL, P < 0.0001) and TNF-α (80.5 vs 48.9 ng/L, P = 0.002) but no significant difference was recorded in IL-21(180 vs 99.9 ng/L) or CA19-9 (19.5 vs 12.8 U/mL). The median β-Catenin, TNF-α, IL-21 and CA19-9 serum levels were significantly higher in HCV- infected HCC patients compared to HCV- infected LC and controls group (β-Catenin: 27 vs. 16.5 and 9 ng/mL, P < 0.0001), (TNF-α: 80.5 vs. 49.9 and 34.3 ng/L, P < 0.0001), (IL-21: 180 vs. 141.9 and 67.9 ng/L, P = 0.001) & (CA19-9: 19.5 vs. 6.5 and 2.8 U/mL, P = 0.008). In liver cirrhotic patients, median serum level of β- catenin was significantly decreased in HCV treated LC than in HCV infected LC (11.2 vs. 16.5, P < 0.0001) on contrary to TNF-α, IL-21 and CA19-9 revealed increased serum levels with no significant difference in HCV treated LC compared to HCV infected LC (TNF-α 52.8 vs. 49.9 ng/L), (IL-21 147.9 vs. 141.9 ng/L) and (CA19-9: 15 vs. 6.5 U/mL) (Table 2). Moreover, HCV- treated LC showed significant increase of TNF-α, IL-21 and CA19-9 compared with HCV-treated HCC and controls (TNF-α: 52.8 vs 48.9 and 34.3 ng/L, P = 0.005), (IL-21: 147.9 vs 99.9 and 67.9 ng/L, P < 0.0001) & (CA19-9: 15 vs 12.8 and 2.8 U/mL, P = 0.003) but β-Catenin was higher in HCC samples compared with LC and controls (β-Catenin: 11.9 vs. 11.2 and 9 ng/mL, P = 0.05), Summarized data for all markers are shown in (Table 3 & Figure 1).

3.3. Correlation between Serum Expressions of β-Catenin, TNF-α, IL-21 and CA19-9 with the Clinicopathological Parameters of HCC

The correlations between serum levels of β-Catenin, TNF-α, IL-21 and CA19-9 with the clinicopathological features of HCC patients are summarized in (Tables 4). There was no significant correlation between β-Catenin, TNF-α, IL-21, CA19-9 levels

and sex, age, tumor size, histologic grade and ascites. Significant correlation was recorded between β-Catenin and pathological diagnosis (r = -0.477, p = 0.002) & Child Pugh score (r = 0.323, p = 0.042), Significant correlations were shown between TNF-α and tumor site (r = 0.357, p = 0.024), Activity of Liver cirrhosis (r = 0.340, p = 0.032), Child Pugh score (r = 0.436, p = 0.005), AFP (r = 0.362, p = 0.022), AST (r = 0.315, p = 0.048), Total bilirubin (r = -0.433, p = 0.005) & INR (r = 0.500, p = 0.001). Significant correlations were shown between IL-21and pathological diagnosis (r = -0.332, p = 0.037), Child Pugh score (r = 0.348, p = 0.028), AST (r = 0.321, p = 0.043), & AAR (r = 0.479, p = 0.002). Significant correlations were shown between MSB model and pathological diagnosis (r = 0.631, p = 0.0001), Activity of Liver cirrhosis (r = -0.457, p = 0.003) & Child Pugh score (r = -0.424, p = 0.006).

3.4. Diagnostic performances of β catenin, TNF-α, IL-21, CA19-9 and MSB model

The potential diagnostic role of these markers in HCC was analyzed by receiver operating characteristic (ROC) curve and the area under curve (AUC). The ROC curve demonstrated optimal sensitivity, specificity and accuracy of β-catenin, TNF-α, IL-21 and CA19-9 for discriminating HCV- infected HCC patients from nonmalignant individuals were (68.8%, 75% & 72.7%), (75%, 78.6% & 77.3%), (50%, 75%& 65.9%) & (75%, 71.4% & 72.7%) respectively, with AUC (0.810, 0.829, 0.719, and 0.743) respectively. The cut-off values were 16.73 ng/ml for β-catenin, 58.42 ng/L for TNF-α,152.56 ng/L for IL-21 and 11.7 U/mL for CA19-9. The developed MSB model from combining the four biomarkers (β catenin, TNF-α, IL-21 and CA19-9) made by linear regression with cut off value 3.09 showed sensitivity 89.3%, specificity 93.7% and accuracy 90.9% for discriminating HCV infected HCC patients from nonmalignant individuals with AUC 0.933 (Figure 2).

MSB model = 6.295 + [β catenin × (- 0.087)] + [IL21 × 0.004] + [TNF-α × - 0.019] + [CA19-9 × 0.064].

ROC curve analysis of β-catenin, TNF-α, IL-21 & CA19-9 was not discriminate HCV-treated HCC patients from nonmalignant individuals. MSB model had sensitivity of 62.5%, specificity of 70.8% and accuracy 66.1% at a cut off value 4.1523 with AUC 0.680 for discriminating HCV- treated HCC patients from nonmalignant individuals (Figure 2). ROC curve analysis was then performed to determine the optimal cutoff levels of β catenin, TNF-α, IL-21 and MSB model for discriminating HCV infected HCC from HCV treated HCC. The optimal cut-off level of β catenin was 14.73 (sensitivity 75%, specificity 87.5%, AUC 0.840), TNF-α cut off at 56.1 (sensitivity 81.3%, specificity 70.8%, AUC 0.792), IL-21 was 99.88 (sensitivity 50%, specificity 50%, AUC 0.638), while the optimal cut-off level of (MSB) model was 3.0330 (sensitivity 83.3%, specificity 81.2%, AUC 0.904), Table (5).

Table 1: Demographic and clinical data of study patients with chronic liver disease (CLD) with or without HCC in comparison with healthy individuals

Characteristics[#]	Healthy individuals (n = 20)	Group I (CLD without HCC) N = 40		Group II (CLD with HCC) N = 44		P value*
		Subgroup Ia HCV-LC patients (n = 20)	*Subgroup Ib HCV treated -LC patients (n = 20)*	*Subgroup IIa HCV - HCC patients (n = 20)*	*Subgroup IIb HCV- treated HCC patients (n = 24)*	
Biochemical markers:						
GGT (U/L)	19.5 (15-34.8)	25 (14.3-86.5)	28 (22-51.8)	50 (24.3-70)	46.5 (27-68.3)	0.013
CRP (mg/L)	2.05 (1-3.8)	4 (3-10.5)	4 (3-7.3)	14.5 (5.8-18)	6.75 (3.63-14)	0.0001
AST (U/ml)	21 (21-25.5)	35 (23.8-52.5)	39 (29.3-63.8)	49 (29.5-57)	44 (33.3-106)	0.0001
ALT (U/ml)	20 (20-31.5)	22 (21-27.3)	24 (20.3-39.5)	34 (21.5-49.5)	37.5 (24.3-61)	0.007
Albumin (g/dL)	4.95 (4.3-5)	2.8 (2.4-3.6)	3.25 (2.9-3.5)	2.85 (2.6-3.1)	3.3 (2.8-3.8)	0.0001
T. Bil. (mg/dL)	0.55 (0.5-0.6)	1.85 (1.4-2.7)	1.4 (0.8-1.9)	3.1 (2.7-3.2)	1.3 (0.7-2.1)	0.0001
Alk. Phos. (U/L)	5 (5-5)	8 (5-9.75)	5 (5-7)	6 (5-7)	6 (5-8)	0.026
Hematological markers:						
WBCs	5.8 (5-7.4)	3.2 (2.2-4.05)	2.5 (1.6-5.04)	3.5 (2.6-5.2)	3.3 (2.6-5.5)	0.002
Lymphcyte	2.6 (1.95-3.14)	0.7 (0.41-0.98)	0.5 (0.38-0.68)	1.1 (0.62-1.5)	0.8 (0.57-1.36)	0.0001
Neutrophil	2.87 (2.2-3.59)	1.66 (1.1-2.5)	1.42 (0.93-3.1)	2.36 (1.3-3.2)	2.2 (1.13-3.73)	0.113
Hb (g/dL)	14.3 (13.7-15)	9.7 (7.9-11.2)	9.6 (8.2-10.9)	11.3 (10-13)	11.3 (8.2-12.5)	0.0001
Plt Count (10^9/L)	246 (223.4-275.4	51 (44.4-77.3)	59.4 (34.5-86.4)	67 (47-80)	86.5 (46.3-116.8)	0.0001
INR	1 (1-1.1)	1.4 (1.3-1.8)	1.5 (1.3-1.6)	1.45 (1.15-1.7)	1.3 (1.2-1.5)	0.0001
Tumor markers:						
AFP (ng/mL)	1.35 (1.1-2.34)	3.1 (1.5-5.4)	4 (2.3-5.9)	45 (20.8-92)	15 (6.5-49.3)	0.0001
CEA (ng/mL)	1.25 (1-2.3)	3.1 (1.8-6.5)	3.85(1.8-4.3)	4.3 (2.5-4.4)	2.3 (1.9-3.2)	0.034
Demographic data:						
Age (yr)	29.5 (24-41)	53 (41-58)	53.5 (35.5-61)	55 (49.8-58)	57 (54-59)	0.0001

Sex	**Male**	**13 (65%)**	**13 (65%)**	**14 (70%)**	**12 (60%)**	**16 (66.7%)**	**0.969**
	Female	**7 (35%)**	**7 (35%)**	**6 (30%)**	**8 (40%)**	**8 (33.3%)**	
Clinical presentation:							
Ascites	**Absent**	**0 (0%)**	**3 (15%)**	**7 (35%)**	**7 (35%)**	**12 (41.4%)**	**0.023**
	Mild	**0 (0%)**	**4 (20%)**	**8 (40%)**	**5 (25%)**	**5 (25%)**	
	Moderate	**0 (0%)**	**4 (20%)**	**2 (10%)**	**2 (10%)**	**3 (33.3%)**	
	Marked	**0 (0%)**	**9 (45%)**	**3 (15%)**	**6 (30%)**	**4 (20%)**	
Activity of LC	**Mild**	**0 (0%)**	**8 (40%)**	**12 (60%)**	**5 (25%)**	**19 (47.5%)**	**0.001**
	Moderate	**0 (0%)**	**12 (60%)**	**8 (40%)**	**15 (75%)**	**5 (13.9%)**	
Child-Pugh Score	**Class A**	**0 (0%)**	**4 (20%)**	**9 (45%)**	**0 (0%)**	**12 (50%)**	**0.001**
	Class B	**0 (0%)**	**11 (55%)**	**7 (35%)**	**10 (50%)**	**9 (37.5%)**	
	Class C	**0 (0%)**	**5 (25%)**	**4 (20%)**	**10 (50%)**	**3 (12.5%)**	

Abbreviations: GGT, gama glutamyl transeferase; CRP, C creative protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALB, Albumin; T.BIL, total bilirubin; Alk. Ph, Alkaine phosphatase; WBCs, white blood cells; Hb, haemoglobin; Plt; platelets, INR, international normalized ratio; AFP, alpha fetoprotein; CEA, carcinoembryonic antigen.
[#]Biochemical markers, Hematological markers, Tumor markers are represented as Median and interquartile range (25-75%); Kruskal Wallis tests were used for data analysis. Sex and clinical presentation are represented as frequency and percent.
* P > 0.05 is considered not significant; P < 0.05 considered significant; P < 0.001 considered very significant; P < 0.0001 is considered extremely significant.

Table 2: Fibrosis and inflammatory indexes of study patients with chronic liver disease (CLD) with or without HCC in comparison with healthy individuals

Indexes [#]	**Healthy individuals (n = 20)**	**Group I (CLD without HCC) (n = 40)**		**Group II (CLD with HCC) (n = 44)**		**P value***
		Subgroup Ia HCV-Non Treated LC patients (n = 20)	**Subgroup Ib HCV-Treated LC patients (n = 20)**	**Subgroup IIa HCV-Non treated HCC patients (n = 20)**	**Subgroup IIb HCV- Treated HCC patients (n = 24)**	
PLR	**93.9 (77-126)**	**82.2 (56.8-121)**	**115 (71-255)**	**61.6 (44-85)**	**82.3(59.4-179)**	**0.017**
NLR	**1 (0.79-1.5)**	**2.6 (1.2-3.9)**	**2.8 (2.1-5.6)**	**1.9 (0.85-3.2)**	**2.9 (1.5-4.1)**	**0.001**
AAR	**1.1 (0.78-1.1)**	**1.5 (1.1-1.77)**	**1.44 (1.1-1.6)**	**1.48 (1-2.25)**	**1.4 (0.87-1.8)**	**0.021**
APRI	**0.23 (0.19-0.26)**	**1.57 (1.1-2.24)**	**1.36 (0.97-2.8)**	**1.7 (0.77-2.7)**	**1.4 (1-2.4)**	**0.0001**
FIB4	**0.55 (0.41-0.74)**	**6.4 (4.29-10)**	**6.45 (3.37-8.79)**	**5.3 (3.65-11.3)**	**4.95 (3.59-11)**	**0.0001**

Abbreviations: PLR, Platelet to Lymphocyte Ratio; NLR, Neutrophil to Lymphocyte ratio; AAR, AST/ALT ratio; APRI Index, AST to platelet ratio index; FIB4, Fibrosis index based-4 biomarkers. Data are represented as Median and interquartile range (25-75%); Kruskal Wallis tests were used for data analysis.
* P > 0.05 is considered not significant; P < 0.05 considered significant; P < 0.001 considered very significant; P < 0.0001 is considered extremely significant.

Table 3: Levels of β Catenin, TNF-α, IL-21, CA19-9 & MSB model in study groups before and after HCV treatment using DAA.

Marker [#]	**Group I (CLD without HCC), n = 40**		**P value***	**Group II (CLD with HCC), n = 44**		**P value****
	Subgroup Ia (HCV-non treated LC patients)	***Subgroup Ib (HCV-treated LC patients)***		***Subgroup IIa (HCV-non treated HCC patients)***	***Subgroup IIb (HCV-treated HCC patients)***	
β Catenin	**11.2 (9.6-13.9)**	**16.5 (15.3-18.9)**	**< 0.0001**	**27 (13.2-29.3)**	**11.9 (9.8-13.8)**	**< 0.0001**
TNF-α	**52.8 (39.2-62)**	**49.9 (44-69.9)**	**0.962**	**80.5 (57.6-144)**	**48.9 (40.7-60.7)**	**0.002**
IL-21	**147.9 (109-166)**	**141.9 (115-182)**	**0.863**	**180 (79.5-347)**	**99.9 (75-117)**	**0.149**
CA19-9	**15 (5.9-20.6)**	**6.5 (4.2-15)**	**0.149**	**19.5 (11.8-30)**	**12.8 (6.8-17.5)**	**0.141**
MSB	**4.1 (3.4-4.4)**	**3.9 (3.1-4.3)**	**0.276**	**3.9 (3.2-4.3)**	**2.1 (1.7-2.7)**	**< 0.0001**

 # Data are presented as Median and Interquartile Range (IQR).
** P > 0.05 is considered not significant and p value < 0.05 is considered significant. The difference between HCV- treated LC & HCV-LC was calculated using Mann-Whitney Test.*
*** P > 0.05 is considered not significant and p value < 0.05 is considered significant. The difference between HCV- treated HCC & HCV-HCC was calculated using Mann-Whitney Test.*

Table 4: Relation between serum levels of investigated biomarkers and clinicopathological features of 44 patients with HCC.

Clinicopathologic data*	β Catenin (ng/mL)	TNF-α (ng/mL)	IL-21 (ng/mL)	CA19-9 (ng/mL)	MSB model
Gender					
Male	**12.7 (10.7-16.2)**	**53.2 (40.4-76.6)**	**97.3 (81.9-130.2)**	**14.6 (10.5-26)**	**3.3 (2.1-4.1)**
Female	**13.7 (10.9-28)**	**60.4 (46.4-108)**	**107.6 (75.7-205.9)**	**10.5 (2.15-17.7)**	**2.7 (2 -3.9)**
Age					
≥ 50	**14.5 (10.2-27)**	**54.9 (39-67.1)**	**89 (83.9-98.7)**	**14.4 (7-21)**	**3.2 (2- 4.1)**
< 50	**12.8 (10.8-26)**	**56.9 (42-92.3)**	**104.6 (75.6-204.3)**	**18 (8.9-35)**	**2.4(2.2-4.1)**
Tumor size (cm)					
≤ 5	**13.9 (10.8-27)**	**55.4 (42-92.3)**	**104.6 (77-227.8)**	**14.4 (7.7-22.5)**	**3 (2.1) [2 - 4.1)**
> 5	**11.3 (9.2-12.8)**	**60.7 (36.9-67.1)**	**89 (74.9-104.6)**	**14.4 (7.5-24)**	**3.9 (1) [3 – 4)**
Tumor site					
Rt liver	**13.1 (11.2-25.10**	**53.2 (39.1-67.1)**	**89.5 (76.5-125.9)**	**14.4 (8.3-24)**	**3.1 (2.2-4.2)**
Lt liver	**10.6 (7.9-13.9)**	**55.2 (47-82.5)**	**126.9(101.2-172)**	**15.6 (10.2-38.8)**	**3.5 (2.4-4.1)**
Rt and Lt	**18.2 (9.9-27.8)**	**108.6(48.6-165)**	**188.2 (74.8-285.5)**	**11.8 (2-17.2)**	**2.5 (1.6-3.9)**
Histologic grade					
I	**12.6 (10.8-23)**	**55.4 (39.2-69.3)**	**89.9 (77-126.9)**	**14.4 (10.5-32.5)**	**3.1 (2.1-3.9)**
II	**12.8 (10.2-28)**	**60 (43.4-138.2)**	**110.7 (83.9-271.7)**	**11 (2.2-17.6)**	**3.2 (2.2-4.1)**
III	**13.1 (10.2-22.3)**	**55.7(21.9-126)**	**81.8 (53.1-350)**	**19.2(11.1-32.3)**	**3.1 (1.7-4.2)**
Child Pugh Score**					
Class A	**11.9 (10.9-13.1)**	**46.9 (37-64.3)**	**104.6 (74.8-114.3)**	**12.8 (6.8-14.6)**	**3.8 (3.2-4.5)**
Class B	**13.9 (12-19.2)**	**54.9 (40.7-60.7)**	**89 (76-128.8)**	**14.6 (10.5-24)**	**3.1 (2.2-4.1)**
Class C	**27 (10.6-28.9)**	**97.8(62.9-148)**	**282 (91.5-346.1)**	**17.2 (2-32)**	**2 (1.7-3.1)**
LC activity, #**					
Mild	**12.6 (10.7-14.2)**	**99.9 (74.9-117)**	**99.9 (74.9-117)**	**14.2 (9.7-18)**	**3.8 (2.9-4.2)**
Moderate	**17.2 (10-7-28.4)**	**100.7 (87-286.7)**	**100.7 (87-286.7)**	**14.4 (4.6-37.3)**	**2.2 (1.7-3.2)**
DAA, #**					
Non treated	**27 (12.4-29.7)**	**95.4 (65.3-148.5)**	**276.9 (86.2-370.2)**	**17.7 (8.8-32.8)**	**2 (1.6-2.9)**
Treated	**12.4 (10 -14.2)**	**46.9 (39 -60.7)**	**97.3 (75.2-117)**	**13.8 (7.3-18)**	**3.9 (2.9-4.2)**
Ascites					
Absent	**12.4 (11.-17.7)**	**57.7 (38.5-67.3)**	**96.8 (77.5-117)**	**16.1 (11.8-24)**	**3 (2.2-3.8)**
Mild	**13.9 (11.6-24.9)**	**50.5 (40.7-102.6)**	**101.1(74.8-448.1)**	**10.5 (8.3-23.2)**	**3.9 (2.4-4.2)**
Mod	**13.5 (9.7-18)**	**49.7 (27.9-56.4)**	**92.9 (79.3-161.9)**	**11.7 (7.1-16.5)**	**4.1 (3.6-4.2)**
Marked	**27 (9.9-28.2)**	**93 (53.2-162)**	**271.7 (81.2-315.4)**	**14.6 (2-31)**	**2.2 (1.7-3.6)**

**Data are presented as Median and Interquartile Range (IQR).*

** r1, r2, r3 and r5 indicate for spearman correlation coefficient for significant correlations of β catenin, TNF-α, IL-21 & MSB model.

p1 < 0.05, p2 < 0.05, p3 < 0.05 & p5 < 0.05 is considered significant for the data difference of β catenin, TNF-α, IL-21 & MSB model respectively**.**

Table 5: The diagnostic value of serum β Catenin, TNF-α, and IL-21 markers & MSB model for distinguishing study groups.

Markers	Comparison Groups	AUC	*P* Value	Cut-off	Sensitivity %	Specificity %
β Catenin	**Healthy & LC**	**0.943**	**0.0001**	**11.59**	**93.8**	**83.3**
	Healthy & LC (ttt)	**0.690**	**0.05**	**9.82**	**70**	**75**
	HCC & (Healthy + LC)	**0.810**	**0.001**	**16.73**	**68.8**	**75**
	HCC (ttt) & (Healthy + LC (ttt))	**0.629**	**0.075**	**11.99**	**50**	**71.9**
	HCC & HCC (ttt)	**0.840**	**0.0001**	**14.73**	**75**	**87.5**
	HCC & LC	**0.713**	**0.04**	**19.16**	**68.8**	**81.2**
TNF-α	**Healthy & LC**	**0.821**	**0.001**	**40.4**	**87.5**	**83.3**
	Healthy & LC (ttt)	**0.819**	**0.003**	**40.93**	**75**	**83.3**
	HCC & (Healthy + LC)	**0.829**	**0.001**	**58.42**	**75**	**78.6**
	HCC (ttt) & (Healthy + LC (ttt))	**0.585**	**0.078**	**45.94**	**58.3**	**59.4**
	HCC & HCC (ttt)	**0.792**	**0.002**	**56.1**	**81.3**	**70.8**
	HCC & LC	**0.744**	**0.018**	**63.5**	**68.8**	**75**
IL-21	**Healthy & LC**	**0.839**	**0.003**	**99.29**	**81.3**	**100**
	Healthy & LC (ttt)	**0.958**	**0.0001**	**88.67**	**90**	**100**
	HCC & (Healthy + LC)	**0.719**	**0.017**	**152.56**	**50**	**75**
	HCC (ttt) & (Healthy + LC (ttt))	**0.461**	**0.079**	**99.88**	**50**	**46.9**
	HCC & HCC (ttt)	**0.638**	**0.143**	**99.88**	**50**	**50**
	HCC & LC	**0.582**	**0.429**	**181.37**	**50**	**75**
CA19-9	**Healthy & LC**	**0.690**	**0.90**	**5.2**	**62.5**	**66.6**
	Healthy & LC (ttt)	**0.838**	**0.002**	**8.7**	**70**	**83.3**
	HCC & (Healthy + LC)	**0.743**	**0.008**	**11.7**	**75**	**71.4**
	HCC & HCC (ttt)	**0.639**	**0.140**	**17.8**	**56.3**	**79.2**
	HCC (ttt) & (Healthy + LC (ttt))	**0.587**	**0.077**	**10.35**	**62.5**	**53.1**
	HCC & LC	**0.676**	**0.09**	**13.7**	**75**	**75**
MSB model	**Healthy & LC**	**0.969**	**0.0001**	**4.3124**	**100**	**87.5**
	Healthy & LC (ttt)	**0.894**	**0.0001**	**4.3859**	**91.7**	**80**
	HCC & (Healthy + LC)	**0.933**	**0.0001**	**3.0862**	**89.3**	**93.7**
	HCC (ttt) & (Healthy + LC (ttt))	**0.680**	**0.022**	**4.1523**	**62.5**	**70.8**
	HCC & HCC (ttt)	**0.904**	**0.0001**	**3.0330**	**83.3**	**81.2**
	HCC & LC	**0.883**	**0.0001**	**3.0862**	**81.3**	**93.7**

Abbraviations: LC, HCV infected-liver cirrhosis patients; LC (ttt), HCV-treated liver cirrhosis patients; HCC; HCV-infected HCC patients, HCC (ttt); HCV-treated HCC patients.

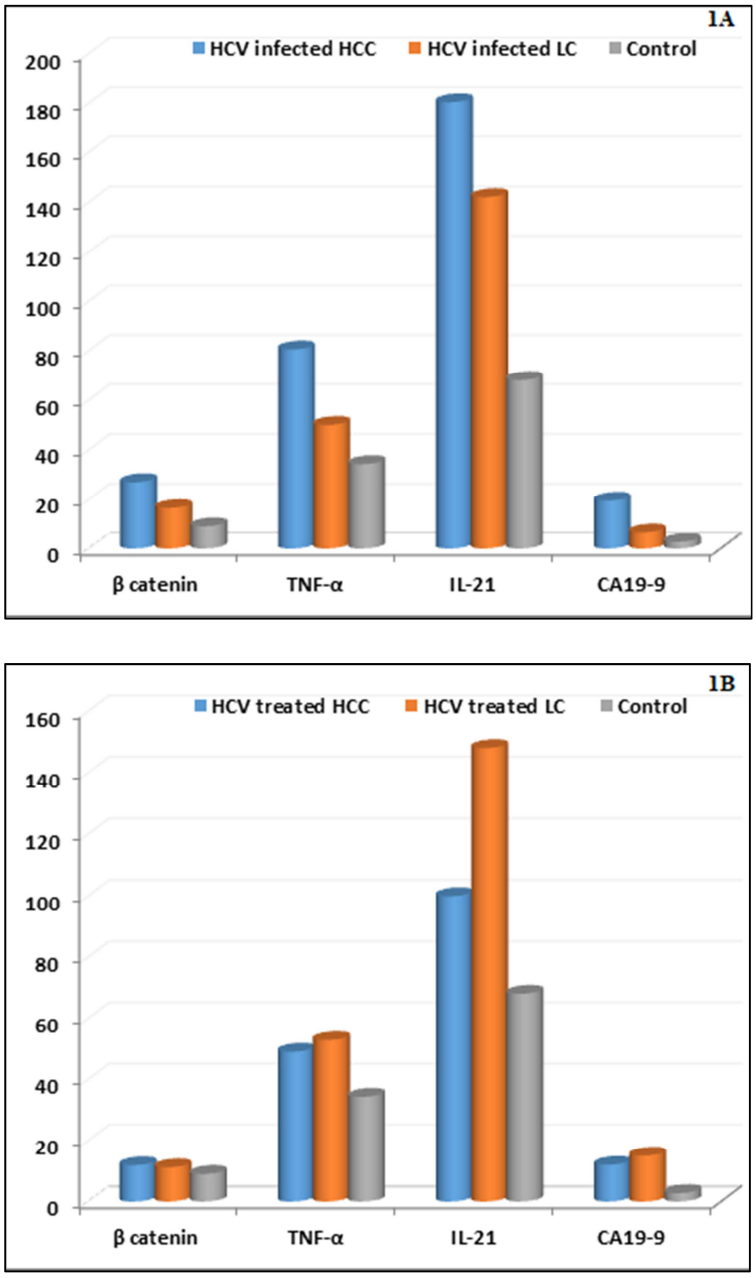

Figure 1: Serum levels of investigated biomarkers in HCC, LC and healthy control groups before and after HCV treatment using DAA. **A.** Before HCV treatment: the four markers showed significant difference between groups with β catenin ($P < 0.0001$), TNF-α, ($P < 0.0001$) IL-21 ($P = 0.001$) and CA19-9 (P value = 0.008). **B**. After HCV treatment: there are significant differences between groups with IL-2 ($P > 0.0001$), TNF-α ($P < 0.05$), and CA19-9 ($P < 0.05$) but the difference with β Catenin levels was not significant ($P > 0.05$).

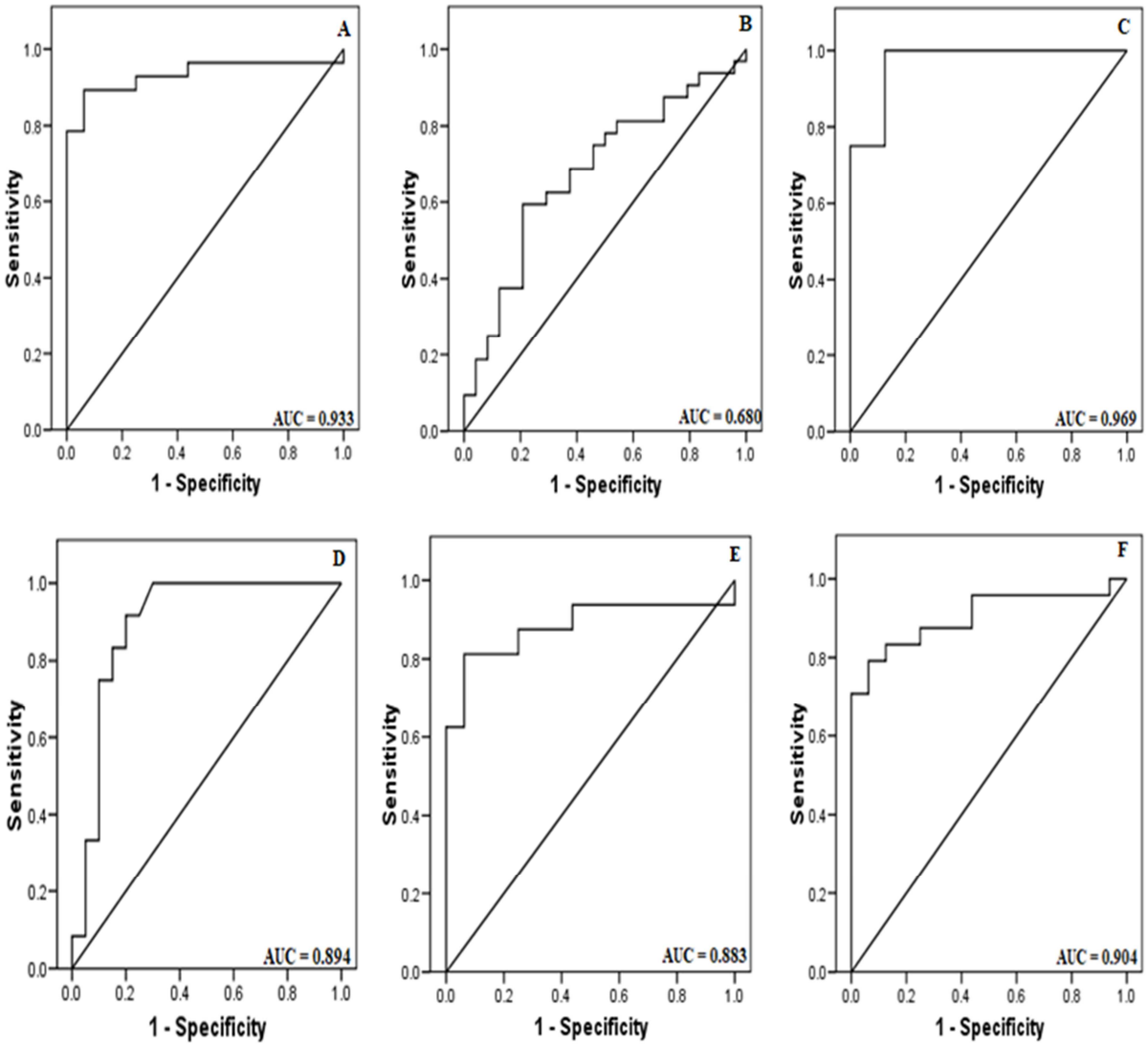

Figure 2: The ROC curves and AUC for MSB model to differentiate between: **A.** HCV-infected HCC patients and Nonmalignant individuals (HCV-infected LC patients + Healthy controls); P < 0.0001. **B.** HCV-treated HCC patients & Nonmalignant individuals (HCV treated LC patients + Healthy individuals), P < 0.05. **C.** HCV-infected LC patients and healthy individuals (P value < 0.0001), **D.** HCV-treated LC patients and healthy individuals (P < 0.0001), **E.** HCV-infected HCC patients and HCV-infected LC patients (P < 0.0001) & **F.** HCV-infected HCC patients & HCV-treated HCC patients (P < 0.0001).

4. Discussion

Hepatocellular carcinoma (HCC), the 5th most prevalent cancer in males and the 7th most common in women, is a widespread issue in the world [22]. The high prevalence of HCV, which is believed to be over 14% in the general population, may be the cause of this increased incidence of HCC [23]. Finding new, suitable single or panel serum indicators that could be employed for early identification of HCC in high-risk patients is necessary because the prognosis of HCC is extremely challenging [24-26]. In the current investigation, patients with HCV-infected HCC had a considerably greater serum concentration of AFP than patients in other groups. These findings are consistent with a prior study that found that patients with HCC had considerably greater serum levels of AFP than both healthy controls and individuals with chronic hepatitis C [24]. Additionally, AST was considerably higher in HCC patients than in LC patients, which is consistent with a prior study that found liver function test values were significantly higher in HCC patients than in CLD patients [27]. In the current investigation, serum levels of TNF-α, β catenin, IL-21, and CA19-9 were assessed in patients with LC and HCC. Following HCV clearance with DAA treatment, serum levels of β catenin were much lower, although they were

remained higher than those of healthy people. In HCV-related LC patients, Serum β catenin levels were significantly decreased in HCV treated cirrhotic patients. The development of HCC is significantly influenced by the dysregulation of Wnt/b-catenin signaling, which is initiated by binding directly to the HCV-NS5A protein and stimulates angiogenesis, proliferating, and the epithelial–mesenchymal transition [28]. Because chronic HCV infection inhibits GSK-3β action through serine 9 phosphorylation (p-ser9-GSK-3β), it activates the Wnt/β-catenin signaling pathway, resulting in a persistent non-phosphorylated β-catenin [29]. In persistent HCV infection, p-ser9-GSK-3β was phosphorylated in a PKA-dependent manner. Unexpectedly, following HCV removal by DAA, Wnt/β-catenin signaling stayed active in long-term HCV-infected cells. Considering that Wnt/β-catenin signaling is crucial for tumor development and that DAA was unable to restore it even after HCV was abolished [30]. Therefore, the development of therapeutic medicines to eradicate HCV and lower β-catenin levels in individuals infected with HCV is urgently needed. According to Zekri et al. [24], serum levels of β-catenin had a sensitivity and specificity of 70% and 68.6%, respectively, at a cut-off value of 8.75 ng/ml with an AUC of 0.729 between HCC and LC groups. These results are consistent with the ability of serum levels of β-catenin to distinguish between patients with HCV-infected HCC and those with liver cirrhosis, with a sensitivity and specificity of 68.8% and 75%, respectively, at a cut-off value of 16.73 ng/ml with an AUC of 0.810. In patients with HCV, TNF-α was one of the best host hereditary indicators of HCC [31]. In this study, TNF-α was able to distinguish between HCC and LC as well as between HCC and nonmalignant patients. These results implied that prior to antiviral treatment, the immunological background was impacted. TNF-α levels decreased in HCC patients and slightly increased in liver cirrhotic patients following effective antiviral medication, but these changes did not significantly alter the risk of HCC. This suggests that antiviral medication may alter the immunological milieu, which could lead to the development of HCC. Tissues are successfully shielded from the invasion of cancerous cells by immune system stimulation. In immunological surveillance, NK cells and cytotoxic T lymphocytes are both powerful effectors. By fostering an environment that encourages immunogenic activation rather than repression, TNF-α mediates the immunological response against tumor cells [32]. Tumor cells can avoid being attacked by cytotoxic T lymphocytes and reduce in vivo antitumor responses when TNF signaling is suppressed [33]. It has been demonstrated that the pleiotropic cytokine IL-21 significantly influences the CD8 T cell response to both acute and long-term viral infections. However, it is still unclear how IL-21 signaling affects the CD4 T cell response to viral infection [34]. In recent years, there has been increasing evidence suggesting the biological functions of IL-21 contribute to the pathophysiology of autoimmune and chronic inflammatory illnesses [35]. Furthermore, it has been demonstrated that IL-21 plays a significant role as a helper cytokine during acute HCV infection [36]. We demonstrated that patients who acquired new-onset HCC had significantly elevated serum levels of IL-21 before to receiving DAAs therapy, which is in contrast to a previous study that comparable patients who generated HCC with those who did not develop HCC after DAAs therapy. In our study, IL-21 was reduced in HCC patients following viral therapy, but it was still high when compared to healthy individuals. Patients treated with DAAs who went on to develop HCC had elevated serum levels of nine of these genes (MIG, IL22, TRAIL, APRIL, VEGF, IL3, TWEAK, SCF, and IL21), indicating that alterations in inflammatory cytokine levels during DAAs treatment may also have an impact on the development of hepatocellular cancer linked to HCV [33]. Antitumor surveillance that contributes to the development of HCC may be impacted by changes in the balance between inflammatory and anti-inflammatory responses. These immunological status changes and the resulting cytokine and chemokine profiles before and after DAA treatment may serve as a signal to help identify patients who are at risk of developing HCC [24]. Patients with HCC have a poor prognosis because CA19-9 has been linked to severe cirrhosis and liver inflammation. Serum levels are high in about 30% of HCC patients [37]. In Comparison with healthy individuals, CA19-9 was higher in patients with HCC and liver cirrhosis in the current study. This is consistent with According to earlier reports, individuals with HCC and HCV (the majority of whom had cirrhosis) had significantly higher levels of CA19-9 than controls [38]. According to a prior study, serum CA19-9 was found to be a unique serum indicator for HCC patients' forecasting of survival [39]. This finding supports the ability of serum CA19-9 to distinguish HCC patients from nonmalignant ones. Patients with a single small HCC receiving RFA, AR, or NAR can have their recurrence reliably predicted by TMS that combines AFP, CEA, and CA19-9. The findings influence the choices made in day-to-day clinical practice [40]. In a UK54 investigation using cirrhotic and cured HCV patients in the Scottish database and the STOP-HCV group researchers tested six HCC forecasting models in two different groups. They discovered that the best sensitivity and precision were exhibited by the prediction model that incorporated age, gender (male), albumin, bilirubin, and platelet count (aMAP). It was found that the prevalence of HCC was not associated with variants of PNPLA3 and WNT3A-WNT9A. Age, male sex, diabetes, platelet count, GGT level, albuminemia, and GRS were all found to be independent risk variables by multivariate modeling. When the GRS

was added, the clinical model's performance for 5-year HCC prediction improved somewhat (C-Index 0.786 and 0.783), but it was still comparable to the aMAP score (C-Index 0.769). Furthermore, the researchers came to the conclusion that cirrhosis patients can be categorized into various HCC risk classes based on variations that impact lipid metabolism and the Wnt–β-catenin signaling pathway. They also found that adding this genetic data to the clinical scoring system could somewhat enhance its ability to stratify HCC risk [41]. Similar to other models in earlier research, we found that the MSB model, which combines the detection of β-Catenin, TNF-α, IL-21, and CA19-9 markers, distinguished efficiently between HCC patients and nonmalignant individuals before and after HCV treatment with high sensitivity (89.3%) and specificity (93.7%), with an AUC of 0.933. This suggests that the MSB model may be a potentially novel model for prediction of HCC.

5. Conclusions

The risk of HCC cannot be eliminated by effective antiviral therapy. The predictive values of β-catenin, TNF-α, IL-21, and CA19-9 biomarkers and a newly developed model based on these four biomarkers were investigated for early prediction of HCC after DAAs. The newly developed model (MSB model) showed high degrees of sensitivity (89.3%) and specificity (93.7%). However, the improved prediction values of BSM-model for HCC patients should be validated by a larger-scale study.

6. Conflicts of interest

The authors declare that there is no conflict of interest.

7. Formatting of funding sources

The current work received no fund from any funding authority.

8. Acknowledgments

The authors would like to thank all staff members of Gastrointestinal Surgery Center, Mansoura University for their kind help during the study.

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