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# PhD THESIS

## DIAGNOSIS AND PROGNOSIS IN HEPATOCELLULAR CARCINOMA - A MULTIFACETED APPROACH

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## GENERAL PART

Liver cancer poses a significant health challenge globally, ranking as the second leading cause of cancer-related deaths worldwide. While China shoulders the most considerable burden with over 50% of reported cases, East Asia exhibits the highest incidence rate per 100,000 population. Romania and the Republic of Moldova have the highest incidence rates in Europe. The correlation between mortality and incidence underscores the severity of hepatocellular carcinoma (HCC).

Multiple factors contribute to HCC, with cirrhosis standing out as the primary risk factor. Chronic viral hepatitis, notably HBV and HCV infections, significantly elevate the risk, alongside chronic alcohol consumption. Metabolic disorders like obesity and diabetes, as well as genetic conditions such as hemochromatosis, also increase the risk of HCC. Prevention strategies encompass universal HBV vaccination, antiviral therapies for HBV and HCV infections, and lifestyle adjustments like reducing alcohol intake and managing metabolic disorders. Additionally, medications such as aspirin, statins, and metformin exhibit potential in HCC prevention, though further research is necessary for conclusive recommendations.

Recent advancements in chronic HBV and HCV infection treatments have yielded favorable viral clearance and suppression outcomes, reducing HCC risk. However, the risk of HCC development persists in these populations, underscoring the importance of ongoing surveillance, particularly among patients with liver cirrhosis. Screening recommendations are tailored to individual risk factors, liver disease severity, and comorbidities.

Screening methods encompass serological and imaging tests, with abdominal ultrasonography as the primary tool. Although its effectiveness varies with operator expertise and patient characteristics, combining ultrasound with serological tests like AFP enhances detection rates. The optimal surveillance interval remains to be determined, with recommendations ranging from 3 to 12 months. Biannual surveillance appears optimal, especially for early-stage HCC detection and improving patient outcomes.

Diagnosis relies on imaging methods, playing a crucial role in HCC identification. Contrast-enhanced ultrasound (CEUS) boasts reasonable specificity (Sp) and positive predictive value (PPV), albeit with slightly lower sensitivity (Se), particularly for smaller nodules. Computed tomography (CT) and contrast-enhanced magnetic resonance imaging (MRI), due to specific criteria like arterial phase hyperenhancement (APHE) and late-phase washout, are indispensable for HCC diagnosis. Pathologic diagnosis via biopsy is recommended for lesions lacking a precise imaging diagnosis, with CEUS also aiding in characterizing portal vein thrombosis (PVT) with good Sp and Se.

Biomarkers such as AFP, des-gamma-carboxy prothrombin (DCP), and glypican-3 (GPC3) contribute to HCC diagnosis but possess limitations, necessitating a combined approach for enhanced diagnostic accuracy. Emerging scores like the GALAD score exhibit promising diagnostic performance, affirming the superiority of algorithms over single biomarker utilization.

HCC staging integrates tumor size and liver function, impacting prognosis. The Barcelona Clinic Liver Cancer (BCLC) classification system is widely adopted, categorizing patients into prognostic stages to guide optimal therapeutic choices. Complications associated with HCC are manifold, including portal thrombosis and paraneoplastic syndromes (PNS).

PNS should be considered and assessed during HCC diagnosis and staging due to their potential impact on patient survival. These syndromes include erythrocytosis, thrombocytosis, hypercholesterolemia, or hypoglycemia. Treatment is influenced by BCLC staging, with early diagnosis correlating with a broader therapeutic spectrum.

## Special Part

The thesis is divided into three chapters, each with a specific objective related to studying HCC. The first chapter aims to assess the efficacy of five biomarkers, both independently and in combination, using a statistical model to predict the probability of HCC. The second chapter examines the performance of CEUS and AFP in characterizing PVT. The third chapter explores the prevalence, clinical characteristics, and survival outcomes of patients with HCC who have PNS, assessing the impact of each PNS on patient survival.

## GENERAL OBJECTIVES

(1) To evaluate and contrast the diagnostic capabilities of five biomarkers (AFP, AFP-L3, DCP, CK19, and GPC3) in predicting the presence of HCC in a Western Romanian patient cohort.

(2) To investigate the possible integration of biomarkers in a statistical model for forecasting HCC likelihood and compare it to the GALAD score.

(3) To assess the Se and Sp of CEUS in distinguishing between benign and malignant PVT.

(4) To examine the relationship between AFP levels and the nature of PVT and possible association with CEUS in a diagnostic score.

(5) To investigate the epidemiology of Paraneoplastic Syndromes in our patients diagnosed with HCC.

(6) To assess the impact of Paraneoplastic Syndromes on the survival rates of patients with HCC.

## 1. MATERIALS AND METHODS

### Diagnosis of liver cirrhosis and HCC

The diagnosis of liver cirrhosis and HCC relied on a combination of clinical observations, liver elastography using the FibroScan® device, and various biological assessments such as FibroTest-ActiTest and FibroMax. The criteria for diagnosing HCC followed EASL guidelines, involving the detection of liver lesions with specific blood flow patterns observed through imaging techniques like Contrast-Enhanced Ultrasound (CEUS), Contrast-Enhance Computed Tomography (CE-CT), or Contrast-Enhance Imaging (CE-MRI), with staging done according to the BCLC system.

### Assessment of biomarkers

Clinical assessments, medical history collection, and liver function tests were conducted, and biomarkers like AFP, AFP-L3, DCP, GPC3, and CK19 were analyzed. The GALAD score was calculated using a specific formula. Blood samples were collected, centrifuged, and stored for biomarker analysis using enzyme-linked immunosorbent assay (ELISA) kits. DCP and AFP-L3 levels were measured using kits from Cusabio®, AFP and GPC3 with R&D Systems®, and CK19 with kits from ABclonal®. Threshold values for biomarkers were based on previous research, with specific cutoffs for AFP, AFP-L3, DCP, CK19, and GPC3 established to optimize diagnostic accuracy.

### Diagnosis of PVT and quantification of AFP

AFP levels were measured from venous blood samples obtained from fasting patients using a vacutainer system without anticoagulants. The serum was extracted within four hours of collection, with a minimum volume of 0.5 mL required for analysis. The AFP measurement was conducted using VITROS® XT 7600 or VITROS® XT 3600 analyzers. These analyzers have a sensitivity threshold of 0.24 ng/mL, meaning they can detect AFP levels as low as 0.24 ng/mL. Among the 101 participants, 81 had documented serum AFP levels, evaluated for association with PVT type, utilizing AFP thresholds of 20 ng/mL and 200 ng/mL as per EASL guidelines.

PVT diagnosis initially arose during ultrasound examinations utilizing high-resolution equipment such as GE Healthcare Logic E9 and Philips Epiq7. Suspicion was based on increased echogenicity within the portal vein lumen, a sign of potential thrombosis. CEUS was used to distinguish between benign and malignant PVT types. This technique follows the European

Federation of Societies for Ultrasound in Medicine and Biology guidelines, ensuring standardized and accurate diagnosis.

CE-CT or CE-MRI was employed as the diagnostic reference method, with contrast agents administered intravenously. The findings of the thrombus extension classification, which included four levels, ranging from partial blockage of the PV to complete obstruction extending into the superior mesenteric vein (SMV), were of paramount importance. Expert radiologists analyzed images to determine PVT nature and extent, leveraging CE-CT and CE-MRI techniques.

**Diagnosis of PNS**

Without universally recognized diagnostic guidelines for PNS in HCC patients, we applied criteria derived from the existing body of research. Erythrocytosis was determined by a hemoglobin concentration above 16.5 g/dL or a Red Blood Cell (RBC)count over 5.5 × 10^12/L in males. In females, erythrocytosis was indicated by hemoglobin levels exceeding 15 g/dL or an RBC count above 5.0 × 10^12/L. Crucially, we excluded other conditions like polycythemia vera and myeloproliferative disorders for both sexes. Hypoglycemia was defined by plasma glucose levels falling below 64.98 mg/dL, hypercholesterolemia was identified with serum cholesterol levels surpassing 220.42 mg/dL, and thrombocytosis was recognized by a platelet count over 400 × 10^9/L.

**Statistical Analysis and Institutional Review Board Statement**

The study, a testament to its unwavering commitment to ethical research, strictly adhered to the guidelines outlined in the World Medical Association Declaration of Helsinki, with approval from local research ethics committees and university and hospital review boards. Statistical analysis, conducted with the highest standards, utilized MedCalc Version 19.4 and Microsoft Office Excel 2019, generating descriptive statistics for patient demographics, anthropometrics, and clinical data. The normality of data distribution was assessed using the Kolmogorov-Smirnov test, with appropriate statistical tests applied accordingly. Area under the curve (AUC) analysis determined discriminative cutoff values, optimizing sensitivity and specificity. Univariate and multivariate regression analyses identified factors associated with HCC presence, employing the Akaike Information Criterion for variable selection. Kaplan-Meier survival analysis estimated survival probabilities and median survival times. The statistical significance for all tests was maintained at $p < 0.05$, ensuring robust findings and interpretations.

## 2. RESULTS

### 2.1 DIAGNOSIS AND PREDICTION ROLE OF HCC BIOMARKERS

#### 2.1.1 Diagnostic role of AFP, AFP-F3, DCP, Glypican, and CK 19 levels

The study involved 154 participants who had already been identified with liver cirrhosis. Of these, 95 participants (61.7%) were further found to have HCC, as determined through clinical assessments, biological tests, and radiological imaging. These individuals were categorized into two groups based on HCC: one group comprised 95 individuals with both liver cirrhosis and HCC, and a control group consisting of 59 individuals with liver cirrhosis but did not develop HCC.

An in-depth examination focused on the levels of GPC3 differentiated by sex revealed that among patients with HCC, the median GPC3 levels between men and women did not show a significant disparity (with median values for men at 424.24, ranging from 150.45 to 6422.11, and for women at 373.66, ranging from 129.48 to 10032.18, p=0.6770). On the other hand, for patients without HCC, there was a significant distinction, as women exhibited considerably higher median GPC3 levels than men (364.57 [170.93-611.17] for females vs. 219.56 [29.75-699.96] for males, p=0.0027).

For the entire group of participants (n=154), the median concentrations for AFP, AFP-L3, DCP, GPC3, and CK19 were reported as 31.06 (ranging from 0.25 to 1423), 2.68 (from 0.09 to 98.1), 1.49 (from 0.1 to 1061), 319.15 (from 29.75 to 10032.18), and 1.31 (from 0.13 to 41.91), respectively. There were notable differences in these biomarkers among participants diagnosed with HCC compared to those who were not, with all showing significant variance ($p<0.0001$) except for CK19 ($p=0.0763$).

Applying specific threshold values for diagnosing HCC in 95 affected subjects—20 ng/dL for AFP, 7% for AFP-L3, 40 ng/mL for DCP, 0.0414 ng/mL for GPC3, and 6.25 ng/mL for CK19—it was found that 80 out of 95 patients (84.2%) presented elevated levels of AFP, 66 (69.5%) had increased AFP-L3 levels, and 94 (98.5%) showed elevated DCP levels. In 52 patients (54.7%), all three indicators were above the normal range. Further investigation into the 15 HCC patients with AFP levels below the 20 ng/dL threshold showed that 12 had DCP levels exceeding 40 ng/mL. This adjustment raised the accuracy of HCC detection from 84.2% to 96.8%. Utilizing CK-19 and GPC3 values concurrently improved the identification accuracy of HCC patients from 84.2% to 92.6%. DCP was more effective than GPC3 and CK19 ($p<0.0001$). There was no statistically significant difference in performance between AFP and AFP-L3; however, both were more predictive of HCC presence than GPC3 ($p<0.0001$) and CK19 ($p<0.0001$). Moreover, GPC3 was more accurate in identifying HCC than CK19 ($p=0.0067$).

### 2.1.2 GALAD and GALKA scores for predicting the presence of HCC

In our research, the GALAD score was determined for all participants, showing a median of -1.34, ranging between -5.08 and 7.26. The most effective threshold value for the GALAD score to forecast the occurrence of HCC was identified as more significant than 0.19. This threshold demonstrated a high accuracy (AUC: 0.98) with a 95% CI from 0.949 to 0.997. It also showed a Se of 93.7%, Sp of 91.5%, an NPV of 90.1%, and a PPV of 94.7%.

Univariate and multivariate regression analyses were utilized to establish a novel prognostic score, focusing on the previously discussed biomarkers. The univariate analysis revealed marked differences between individuals with and without HCC in several factors: age, ALT, AFP, AFP-L3, DCP, GPC3, CK19, serum bilirubin, and serum albumin levels, all showing significant variations ($p<0.001$ for each, except for serum bilirubin and albumin levels where $p=0.001$). For the multivariate regression analysis, a significance threshold of 0.05 was applied to discern variables significantly associated with the presence of HCC. This process utilized three different regression models.

The construction of the regression model followed a stepwise forward selection approach, utilizing the AIC to determine the most fitting model. The model that incorporated AFP, AFP-L3, GPC3, CK19, and serum albumin levels emerged as the superior predictor for the occurrence of HCC. This model showed significant effectiveness with AFP ($p=0.0001$, beta coefficient = 0.00069 ± 0.00016, OR=0.985 with a CI of 0.973-0.997), AFP-L3 ($p=0.0031$, beta coefficient = 0.005 ± 0.0016, OR=1.084 with a CI of 1.008-1.166), GPC3 ($p=0.0013$, beta coefficient = 0.000066 ± 0.000020, OR=0.924 with a CI of 0.913-0.961), CK19 ($p=0.038$, beta coefficient = 0.01 ± 0.005, OR=0.942 with a CI of 0.924-0.976), and serum albumin ($p<0.0001$, beta coefficient = 0.235 ± 0.041). Based on these predictors, the GALKA score was created as follows: 0.005 * AFP-L3 + 0.00069 * AFP + 0.000066 * GPC3 + 0.01 * CK19 + 0.235 * Serum Albumin - 0.277. This score was computed for all participants, determining an optimal threshold for predicting HCC to be greater than 0.32. This threshold achieved an AUC curve of 0.98, with a Se of 96.8%, Sp of 93%, PPV of 95.8%, and NPV of 94.8%.

The predictive capability of the newly proposed GALKA score was compared with that of the established GALAD score, revealing no significant difference in their performance ($p=0.792$). These scores were evaluated for their effectiveness in predicting the occurrence of PVT. The analysis revealed no significant discrepancy in their predictive capabilities, with the GALAD score showing an AUC of 0.67 (95% CI: 0.593 to 0.746) and the GALKA score exhibiting an AUC of 0.63 (95% CI: 0.553 to 0.710), p-value at 0.4918. However, the GALAD score demonstrated superior performance in predicting larger HCC sizes (greater than 5 cm), achieving an AUC of 0.78 (95% CI: 0.716 to 0.850) compared to the GALKA score, which had an AUC of 0.69 (95% CI: 0.619 to 0.770), with a significant p-value of 0.0069.

## 2.2 USEFULNESS OF CEUS AND AFP FOR THE CHARACTERIZATION OF PVT IN HCC

The research encompassed 101 participants diagnosed with liver cirrhosis and PTV, comprising 73 males and 28 females. The average age of the subjects was 62.7 years, with a standard deviation of 9.2 years. Within the study group of individuals with cirrhosis but not HCC, 29.2% (7

out of 24) were categorized as Child-Pugh A, 41.6% (10 out of 24) as Child-Pugh B, and the remaining 29.2% (7 out of 24) as Child-Pugh C. Among those with both cirrhosis and HCC, 19.5% (15 out of 77) were classified as Child-Pugh A, 49.3% (38 out of 77) as Child-Pugh B, and 31.2% (24 out of 77) as Child-Pugh C. The average MELD score was 15.9 (with a standard deviation of 7.1) for subjects with cirrhosis without HCC and 15.8 (with a standard deviation of 7.1) for those with both cirrhosis and HCC, indicating no significant difference between the two groups (p=0.9616).

In the study, the average length of the spleen for participants without HCC (n=24) was 16 cm with a standard deviation of 3.3 cm, and 83.3% (20 out of 24) exhibited splenomegaly (spleen size greater than 12 cm). For those diagnosed with HCC (n=77), the mean spleen diameter was measured at 14.2 cm with a standard deviation of 2.7 cm, with 76.6% (59 out of 77) having splenomegaly (p=0.0085). Participants with a tumor in a vein (TIV) (n=75) had an average spleen length of 13.9 cm with a standard deviation of 3.1 cm. In contrast, individuals with benign PVT (n=26) showed an average length of 16.2 cm with a standard deviation of 3 cm (p=0.0015).

### 2.2.1 AFP serum levels for PVT characterization

Serum levels of AFP were measured in 81 participants (80.2%), including 20 without HCC (24.7%) and 61 with HCC (75.3%). Among these, AFP levels were below 20 ng/dL in 33.3% (27/81) of individuals, between 20 and 199 ng/dL in 18.5% (15/81), and 200 ng/dL or higher in 48.2% (39/81). Patients with HCC displayed significantly elevated mean AFP levels compared to those without HCC ($p < 0.0001$). When considering CT/MRI findings, among the group with AFP levels under 20 ng/dL (n=27), PVT was classified as benign in 77.8% (21/27) of cases, and TIV was identified in 22.2% (6/27). In the group with AFP levels between 20 and 199 ng/dL (n=15), TIV was found in 73.3% (11/15) and benign PVT in 26.7% (4/15). For participants with AFP levels 200 ng/dL or above (n=39), all (100%) had TIV.

Using AFP levels below 20 ng/dL to exclude HCC for classifying PVT, 78% (21/27) were accurately identified as benign PVT. Conversely, using an AFP threshold of 200 ng/dL or more as a criterion for HCC, all participants (39/39) were correctly identified as having TIV. There was a statistically significant association between AFP concentrations and the identification of PVT using CEUS (correlation coefficient = 0.28, $p = 0.0098$) and CT/MRI (correlation coefficient = 0.3, $p = 0.0057$). However, no significant relationship was observed between AFP levels and the extent of PVT ($p = 0.4458$).

### 2.2.2 The Performance of CEUS for the Characterization of PVT

Based on findings from CEUS, TIV was identified in 74.3% (75 out of 101) of the participants. Meanwhile, when assessed using reference imaging techniques such as contrast-enhanced CT/MRI, 72.3% were found to have TIV. A notable association was identified between the size of HCC lesions and the extent of PVT (correlation coefficient = 0.24, $p = 0.0318$), as well as between the extent of PVT and the HCC morphology (whether it was single, multicentric, or diffuse) (correlation coefficient = 0.33, $p = 0.003$). Within the group having benign PVT, 40% (2 out of 5) presented with multicentric HCC. Meanwhile, among those with TIV, 20.8% (15 out of 72) had a single tumor smaller than 50 mm, 6.9% (5 out of 72) had a single tumor more significant than 50 mm, 55.6% (40 out of 72) exhibited multicentric HCC, and 16.7% (12 out of 72) showed diffuse HCC.

The efficacy of CEUS in identifying the nature of PVT was exceptionally high, showing a Se) of 98.6% (95% CI 92.6–100%) and a Sp of 89.3% (95% CI 71.8–97.7%). An impressive 97% (98 out of 101) of participants were accurately diagnosed by CEUS as having either benign PVT or TIV, with CE-CT/MRI as the benchmark. The comparison between those diagnosed with HCC and those without HCC showed no significant variance in the precision of classifications: 97.4% (75 out of 77) versus 95.8% (23 out of 24), $p = 0.7757$.

### 2.2.3 The Performance of the Combined Use of AFP Serum Levels and CEUS for the Characterization of PVT

A univariate regression analysis explored the relationship between PVT identification using CEUS and AFP serum concentrations (ng/dL) against the contrast-enhanced CT/MRI benchmark.

The findings demonstrated significant correlations for PVT identification by CEUS and AFP levels with the standard reference ($p < 0.001$ and $p = 0.006$, respectively). Further analysis through multivariate logistic regression, incorporating both CEUS-derived PVT data and AFP serum concentrations, affirmed their association with PVT identification via CT/MRI, showing significance for PVT by CEUS ($\beta = 0.88 \pm 0.05$, $p < 0.001$) and AFP levels ($\beta = 0.16 * 10^{-4} \pm 0.0000026$, $p < 0.03$). Employing these variables in a composite regression model created a scoring system for PVT classification: 0.88 * (1 for CEUS-classified malignant PVT, 0 for benign) + 0.16 * 10^−4 * AFP levels.

This model's threshold for accurately predicting malignant PVT was established at >0.92, demonstrating exceptional diagnostic performance (AUC = 0.99), with a Seof 98.21% (95% Confidence Interval, CI, 90.4–100%), Sp at 100% (CI 86.3–100%), a PPV of 100%, an NPV of 96.2%, a positive likelihood ratio (+LR) of 8.18, and a negative likelihood ratio (−LR) of 0.018. Comparing the AUC values, the PVT scoring system performed better in distinguishing PVT from CEUS (AUC of 0.99 versus 0.93, $p = 0.025$) and AFP serum concentrations (AUC of 0.99 versus 0.96, $p = 0.047$).

## 2.3 PARANEOPLASTIC SYNDROMES IN HEPATOCELLULAR CARCINOMA

### 2.3.1 Frequency of PNS in individuals with HCC

This study involved 378 individuals diagnosed with HCC, whose ages ranged between 32 and 93 years, with an average age of 64.86 ± 8.85 years. The population comprised 70.6% men (257) and 29.4% women (111). Participants were categorized into two groups based on the occurrence of PNS: those with PNS (n = 97) and those without PNS (n = 281). The group with PNS accounted for 25.7% of the entire study population, showing various syndromes such as hypoglycemia (6.9%), erythrocytosis (4.5%), hypercholesterolemia (10.9%), and thrombocytosis (3.4%). The average age of patients in the PNS-positive group was 64.2±8.35 years, with a significantly higher male ratio (82.5%) compared to the general male percentage in the HCC cohort (70.6%) (p=0.0226).

According to the Child-Pugh classification, an assessment of the participants showed that the PNS-positive group had a higher incidence of Child-Pugh C stage (31.8%), suggesting a link between PNS and a more severe disease outcome in HCC. Furthermore, it was observed that 90.7% of the HCC patients with PNS had underlying cirrhosis, and vascular invasion was noted in 41.2% of these patients, which was more frequent compared to 30.6% in those without PNS (p=0.0731).

The group with PNS displayed alpha-fetoprotein levels that were, on average, fivefold higher than those without PNS, a statistically significant difference ($p < 0.0001$). Hypoglycemia was found in 27.3% of patients and was linked to a higher incidence of adverse outcomes within the Child-Pugh C category. Conversely, erythrocytosis and hypercholesterolemia, present in 19.3% and 43.2% of patients, respectively, indicated a more favorable prognosis among patients classified within the Child-Pugh A-B stages.

Among the patients with PNS, the conditions identified included hypoglycemia in 26.8%, erythrocytosis in 17.5%, hypercholesterolemia in 42.3%, and thrombocytosis in 13.4%. From an etiological perspective, the occurrence of PNS was most frequently associated with hepatitis C virus infection (33%), whereas MASLD had the lowest association rate at 13.4%. The multivariate analysis underscored the importance of various factors, including albumin levels, total bilirubin, AFP, DM, and a tumor size smaller than 5 cm, particularly in the group with positive PNS compared to those without PNS.

### 2.3.2 Correlation between patients' survival and PNS

The median survival period for patients affected by PNS was 145 days, ranging from 1 to 2819 days. This contrasts with a median survival of 208 days, within the same range, for individuals not experiencing these syndromes, indicating no significant statistical difference (p=0.3286).

In an analysis that matched 97 subjects from the non-PNS group based on factors including age, sex, biological tests, Child-Pugh score, and BCLC stage, significant variations were observed. Specifically, individuals with erythrocytosis displayed notable differences in tumor size, albumin, and AFP levels when compared to those without paraneoplastic syndrome, with p-values less than 0.001. Nonetheless, no statistically significant difference was found in the median survival times

between patients with erythrocytosis and those without PNS (480 vs. 204 days, p=0.2755). This observation remained consistent in the subgroup analysis of matched pairs.

Patients exhibiting hypoglycemia showed significant differences in age and tumor size in contrast to those free from PNS (p<0.0001). Despite being younger, they had larger tumors, and their median survival time was significantly shorter (130 vs. 220 days, p=0.0428). The matched comparison, however, did not reveal any significant disparity.

Before matching, the survival analysis of individuals with thrombocytosis did not show a significant difference in median survival time compared to those without PNS (53 vs. 220 days, p=0.0737). A similar pattern of results was observed in the matched comparison (61 vs. 233 days, p=0.0831).

## 3. DISCUSSIONS

### 3.1 DIAGNOSIS AND PREDICTION ROLE OF HCC BIOMARKERS

HCC poses a significant public health challenge, attributed to its high fatality rates. Early detection is paramount in combating this malignancy; a consistent screening strategy can facilitate it. Guidelines by the EASL and the AASLD advocate for bi-annual ultrasound scans, optionally accompanied by AFP testing, as a screening method for HCC. In contrast, guidelines prevalent in Asia favor the employment of biomarkers for this purpose. The effectiveness of blood biomarkers in the surveillance of individuals at risk for HCC is debated among researchers and healthcare professionals due to variability in Se and Sp across studies, the diversity of participant groups, and differing threshold values. We examine the efficacy of five specific biomarkers, analyzed from the same cohort, in diagnosing HCC.

In this research, AFP concentrations were markedly elevated in patients with HCC compared to those with cirrhosis (p< 0.0001). This aligns with findings from other studies. Nonetheless, the chosen cutoff value influences the diagnostic Se and Sp. Employing a 20 ng/mL threshold for diagnosing HCC in our analysis resulted in a Seof of 74.7% and a Sp of 100% for AFP. Furthermore, AFP demonstrated the highest AUC at 0.94 among the tested biomarkers. Marrero et al. found a comparable Se of 59% and Sp of 90% with the same AFP cutoff, mirroring our results. A meta-analysis published recently calculated AFP's overall Se and Sp at 61% and 87%, respectively. Opting for a higher cutoff value was linked to reduced Se, dropping to 22% for a 200 ng/mL cutoff and 18% for 400 ng/mL. AFP's high Sp could be attributed to the observation that more than half of HCC cases involve a lesion larger than 5 cm or multiple lesions.

Nonetheless, patients with low AFP levels may still develop sizable HCCs, suggesting that factors other than AFP contribute to carcinoma size. Pang et al. have recommended integrating AFP with additional biomarkers to enhance diagnostic accuracy. In our investigation, incorporating DCP to assess individuals with normal or low AFP levels enhanced the accuracy of correct classifications to 96.8%. Furthermore, including CK-19 levels and Glypican improved the rate of accurate classifications to 92.6%.

Continuous research is dedicated to uncovering new blood biomarkers for HCC, leading to the discovery of numerous candidates over recent years. Despite this progress, only a few biomarkers have been integrated into clinical routines, favored for their non-invasive nature, ease of reproducibility, and reliable outcomes. A comprehensive analysis involving six studies and 2447 patients revealed that AFP-L3's diagnostic performance for early-stage HCC includes a Sp of 92% and a Se of 34%, with an AUC of 0.75. Our findings showed AFP-L3's Sp at 91.5% and Se at 75.8%, with an AUC of 0.91, indicating improved Sein detecting later stages and larger HCC sizes. Despite AFP-L3's lower Se in early HCC stages, its high Sp surpasses total AFP, offering the advantage of differentiating HCC from non-malignant liver diseases in patients with elevated serum AFP levels. Furthermore, AFP-L3 has been closely linked to severe HCC complications, including PVT and intrahepatic spread.

DCP, another serum biomarker evaluated for HCC diagnosis, ranked third in our study with an AUC of 0.82, trailing behind AFP and AFP-L3. Marrero and colleagues also reported a similar AUC value for DCP at 0.72, highlighting AFP's superior Se compared to DCP and AFP-L3. Nonetheless, DCP's effectiveness in detecting early-stage HCC is limited, with a Se of just 26.3% reported. Our analysis, however, indicates a higher Se, which we attribute to the advanced stages and

larger sizes of the HCC cases studied. DCP's diagnostic performance improves when used alongside other biomarkers, a finding supported by a phase II study and corroborated by our research.

In our research, GPC3 surpassed CK-19 in performance but lagged behind AFP in HCC diagnosis accuracy, with AUCs of 0.72 versus 0.94, respectively. This contrasted with earlier reports where GPC3 outperformed AFP, boasting Seand Sp rates of 84–85% and 92–95% against AFP's 50–79% and 80–90%. The discrepancy could stem from GPC3's superior early-stage liver cancer detection and lack of correlation with tumor size. The study observed high Sp for GPC3, yet its Se varied significantly from other research, possibly due to differing measurement methods and sample sizes. Further investigation is necessary to confirm these results and to examine different factors influencing GPC3's effectiveness as a liver cancer biomarker. The combined use of AFP and GPC3 appears promising for heightened diagnostic precision.

CK19 is recognized as a marker for HCC stem cells, playing a role in the cancer's development, spread, and recurrence. Our investigation found that CK19 levels did not significantly differ between individuals with and without HCC, rendering it less practical for HCC diagnosis than other biomarkers assessed in our study. This observation aligns with findings from Raziky et al., who reported a Se and Sp of 63.4% and 55% for CK19 and suggested that its combined use with AFP enhances diagnostic Se. However, our study noted improved Sp but reduced Se with this biomarker combination.

Emerging research indicates that a multi-biomarker approach could enhance HCC detection significantly. The GALAD scoring system, which integrates serum biomarkers (AFP, AFP-L3, and DCP) with demographic data (gender and age), exemplifies this approach. In evaluating GALAD's efficacy within our cohort, we observed high diagnostic accuracy for HCC, reflected by an AUC of 0.98. Building on GALAD's foundation, we developed the GALKA score, incorporating AFP, AFP-L3, Glypican, CK19, and serum albumin levels—a marker of liver synthetic function and an independent predictor for HCC development. This novel model demonstrated superior predictive capability for HCC presence ($p < 0.001$).

Our GALKA score showcased an outstanding AUC of 0.98, surpassing the diagnostic performance of other biomarkers reviewed in our study. Although the AUC matched GALAD's, GALKA exhibited heightened Se and Sp at 96.8% and 93%, respectively, compared to GALAD's at 93.7% and 91.5%. Including four specific biomarkers for HCC and a protein indicative of liver function in the GALKA score synergistically enhances its diagnostic accuracy. Additionally, excluding DCP from our model avoids potential complications with anticoagulant medications, making the GALKA score a practical option for real-world application.

In their research, Li et al. validated the GALAD score, comparing it against various other scoring systems and combinations of biomarkers, revealing GALAD's superiority with AUC values of 0.925 and 0.945. Additionally, a systematic review by Guan et al. endorsed the substantial effectiveness of GALAD as a tool for screening or diagnosing HCC. Conversely, a phase 3 biomarker study in the United States reported that GALAD's efficacy was moderate, showing no significant advantage over AFP-L3 alone or the carcinoma early detection screening method. It was noted that GALAD's performance varied depending on the underlying cause of chronic liver disease in high-risk patients, with reduced effectiveness in those with HBV etiology. At the same time, higher Se and AUC values were observed in patients with HCV and non-viral liver diseases. The decline in HCV-related cirrhosis cases due to DAAs contrasts with the rise in MASLD, where GALAD still demonstrates a commendable AUC of 0.91 for detecting MASLD-associated HCC.

The Se and AUC of GALAD are correlated with the BCLC stage. The high AUC and Se of GALAD in our study might be attributed to approximately half of our cohort being outside the Milan criteria, suggesting that GALAD is particularly adept at identifying more advanced stages of HCC. This observation led us to speculate whether the GALKA score could offer distinct performance benefits in a cohort with early-stage HCC. This hypothesis requires further investigation in a larger group of early-stage HCC patients.

### 3.2 USEFULNESS OF CEUS AND AFP FOR THE CHARACTERIZATION OF PVT IN HCC

The presence of malignant PVT elevates an HCC patient to an advanced stage C, according to the BCLC staging system, significantly limiting the range of therapeutic options and ruling out

most curative treatments. The approach to treating HCC patients with PVT depends on various factors, including the patient's liver function, the stage of the liver cancer, and the extent of the PVT. In response, a Chinese consensus has outlined treatment strategies based on the Child-Pugh stage, PVT extent, and grade. These strategies include surgery, hepatic artery infusion chemotherapy, TACE, external and internal radiation therapy, local ablation therapies, and systemic therapy, underscoring the critical need for accurate diagnosis and staging of PVT.

In recent years, CEUS has become an increasingly valuable diagnostic tool, particularly for liver applications like the detection and characterization of focal liver lesions and PVT, improving the diagnostic capabilities of traditional B-mode and Doppler ultrasound. We evaluated the efficacy of CEUS in characterizing PVT, revealing a Se of 98.6% and Sp of 89.3%. These results are consistent with other studies that have assessed CEUS for PVT characterization, reporting similarly high Se and Sp. For instance, an analysis of 50 HCC patients with PVT reported a 100% Se and 83% Sp for CEUS, using CE-CT/MRI as a reference. Tarantino et al. compared the sensitivities of color Doppler US, CEUS, and fine-needle biopsy in detecting malignant thrombi in patients with cirrhosis, HCC, and PVT, concluding that CEUS had the highest Se at 88%. Rossi et al. also found CEUS to have significantly higher Se than CT in detecting and characterizing PVT. A meta-analysis highlighted the pooled Se and Sp of CEUS in PVT characterization as 0.94 (95%CI, 0.89–0.97) and 0.99 (95%CI, 0.80–1.00), respectively.

AFP has been extensively evaluated in various contexts related to HCC, including as a diagnostic tool in monitoring individuals at heightened risk of developing HCC and for follow-up after treatment interventions. Different thresholds have been explored to determine AFP's diagnostic efficacy, especially in detecting small HCCs, where its utility as a surveillance tool is limited due to suboptimal performance. Nevertheless, AFP levels exceeding 20 ng/mL have demonstrated relatively high Se (60%) and Sp (90.6%) for HCC diagnosis. However, at a higher threshold of 200 ng/mL, Sedrops to 22%, albeit with improved Sp of 99.4%, highlighting a trade-off between Seand Sp at different cutoff levels. A study by Tateishi et al. confirmed the effectiveness of a 200 ng/mL cutoff for HCC smaller than 5 cm, showing varied Se, Sp, and likelihood ratios (LR+) when comparing 20 ng/mL versus 200 ng/mL cutoffs: Sefrom 0.49 to 0.71, Sp from 0.49 to 0.86, and LR+ from 1.28 to 4.03, and then from 0.04 to 0.31, 0.76 to 1.0, and 1.13 to 54.25, respectively.

Another aspect of AFP's role in HCC diagnosis and prognosis is its performance in cases where HCC develops with low or normal AFP levels. Carr et al. found that 58% of HCC patients in their study had AFP levels below 100 IU/mL (equivalent to approximately 121 ng/dL), and nearly half of the patients with large HCCs (≥5 cm) also presented with normal or low AFP levels (≤100 IU/mL). Additionally, among patients with PVT, 19% had AFP levels below 20 IU/mL (24.2 ng/dL), and 25% had AFP levels ranging from 20 IU/mL to 100 IU/mL. However, the study did not specify whether the observed PVT cases were benign or tumor-induced thrombosis, leaving a gap in understanding AFP's predictive value in conjunction with PVT characteristics.

AFP has been extensively evaluated in the context of HCC diagnosis, patient surveillance for HCC risk, and post-treatment follow-up. Various cutoff values have been investigated to assess AFP's diagnostic accuracy, particularly for detecting small HCC, which was limited in surveillance applications due to poor performance. Nevertheless, as a diagnostic tool for HCC, AFP levels exceeding 20 ng/mL exhibited relatively high Se at 60% and Sp at 90.6%. In contrast, a cutoff of over 200 ng/mL resulted in a Se drop of 22% but an increase in Sp to 99.4%. Tateishi et al. demonstrated the effectiveness of a 200 ng/mL cutoff for diagnosing smaller HCCs (less than 5 cm), showing significant improvements in Se, Sp, and positive likelihood ratio compared to a 20 ng/mL cutoff.

An essential aspect of AFP's diagnostic and prognostic utility is its variation in patients with HCC, particularly those with low or normal AFP levels. Carr et al. reported that 58% of HCC patients in their study had AFP levels below 100 IU/mL (121 ng/dL), including 49% of patients with large HCCs (≥5.0 cm) who also presented with normal or low AFP levels (≤100 IU/mL). Interestingly, 19% of patients with PVT had AFP levels under 20 IU/mL (24.2 ng/dL), and 25% had AFP levels between 20 IU/mL and 100 IU/mL. However, the study did not specify the nature of PVT as benign or TIV.

Furthermore, few studies have focused on the significance of AFP levels in PVT characterization. Our study assessed the effectiveness of different AFP cutoffs for classifying PVT,

using CT/MRI as a reference. It explored the relationship between AFP levels and the extent of PVT type (benign or TIV). Employing a cutoff of <20 ng/dL for ruling out, 78% of subjects were accurately identified as having benign PVT. In contrast, ≥200 ng/dL cutoff correctly identified 100% of subjects as having TIV. Thus, AFP levels above 20 ng/dL in patients with liver cirrhosis and PVT strongly indicate TIV presence. This finding aligns with recent research showing a significant association between higher AFP levels and TIV in a retrospective study of 819 non-transplant HCC patients.

We also introduced a PVT score combining CEUS and AFP levels for more accurate PVT characterization. This score demonstrated superior predictive capability for malignant TIV with an AUC of 0.99, Se of 98.2%, and Sp of 100%, outperforming the individual use of CEUS or AFP. This is the first study to merge CEUS and AFP for PVT characterization.

Regarding liver disease etiology in our cohort, ALD and HCV were the predominant causes associated with PVT, constituting 37.5% and 25% of cases, respectively. This contrasts with other research identifying ALD and HBV as the most common causes of PVT, highlighting the variability in underlying etiologies across different studies.

The correlation between cirrhosis severity and PVT incidence varies significantly. In contrast, the prevalence of PVT in individuals with compensated cirrhosis is relatively low at about 1%; it increases to 8–25% among LT candidates. This prevalence further escalates to roughly 35% in patients afflicted with both cirrhosis and HCC. In our investigation, the MELD scores between patients with and without HCC were closely matched, 15.9 ± 7.1 versus 15.8 ± 7.1, respectively, showing no significant difference ($p = 0.9616$). Similarly, the severity of portal hypertension did not significantly differ between individuals with benign PVT and those with tumor-induced vascular involvement.

### 3.3 PARANEOPLASTIC SYNDROMES IN HEPATOCELLULAR CARCINOMA

Within our study group, PNS occurrence was noted at 25.7%, which closely mirrors the 21% reported by Ülger et al. in their recent work. This incidence aligns with data primarily from Asian studies, which have documented PNS prevalence rates ranging from 27.8% to 30.9%. Notably, Feng et al. found a lower incidence rate of 18.7%, though their research was limited to patients undergoing hepatic resection. Given the association between PNS, advanced liver disease, and HCC progression, it has indicated that patients suitable for liver resection generally exhibit early-stage HCC and better-preserved liver function.

Hypercholesterolemia emerged as our cohort's most frequently observed PNS, observed in 10.9% of patients. This finding resonates with earlier studies by Chang in 2013 and Qu in 2014, which reported higher incidences of 24.5% and 23.2%, respectively. Conversely, Ülger et al. identified hypercholesterolemia as the least common PNS at a rate of only 2.4%. Following hypercholesterolemia, hypoglycemia and erythrocytosis were identified with prevalences of 6.9% and 4.5%, respectively, with thrombocytosis being the least common at 3.4%.

Our analysis revealed a significant association between PNS and older age, with a notable male predominance of 82.5% ($p=0.0226$), diverging from the general male distribution among HCC patients at 70.6%. This skew towards male patients must be contextualized within the general male-to-female incidence ratio of HCC, which typically ranges from 2 to 2.5 to 1, reflecting a 2 to 1 ratio in our study. Additionally, a greater prevalence of Child-Pugh C stage was observed within the PNS-positive group (31.8%), along with a higher incidence of vascular invasion (41.2%) compared to PNS-negative patients (30.6%). Significantly, PNS-positive patients also demonstrated considerably higher levels of AFP ($p <0.0001$).

Our analysis also revealed a notable disparity in the prevalence of hypercholesterolemia between patients with HBV-associated PNS (51.8%) and those with HCV infection (37.5%). The potential role of genetic predisposition linked to metabolic syndrome in the incidence of hypercholesterolemia among HCC patients warrants further investigation. This theory is somewhat supported by our findings, where DM showed a statistically significant, though not decisive, association with PNS, remaining significant in hypercholesterolemia ($p<0.026$). A significant relationship was also identified between hypercholesterolemia and AFP levels, particularly in cases where tumor sizes exceeded 5 cm, suggesting a closer association between hypercholesterolemia and tumor size.

In our cohort, hypoglycemia stood out as the second most common paraneoplastic syndrome (PNS), affecting 6.9% of patients, with rates elsewhere reported between 5.8% and 13.1%. This condition tends to be more prevalent in China compared to Europe, typically emerging due to clinical symptoms in the disease's terminal stages when the tumor burden is significant.

The association between PNS and survival outcomes in HCC patients has been a focal point of several studies, revealing that PNS often correlates with reduced survival times. Qu et al. observed a median survival period of 15 months for patients with PNS, markedly shorter than the 55 months noted for those without PNS ($p = 0.003$). Chang et al. reported a similar trend, with a median survival difference of 12.4 weeks for PNS patients versus 18.3 weeks for those without ($p = 0.025$). Consistent with these findings, our research indicated that the median survival for PNS patients was 145 days, compared to 208 days for those without PNS ($p = 0.3286$).

Upon implementing propensity score matching to account for variables such as age, gender, serum AFP levels, etiology, and Child-Pugh score, we performed a subgroup analysis specifically on PNS. This detailed analysis revealed significant differences in tumor size, albumin levels, and AFP levels between patients with erythrocytosis and those without. However, no substantial disparity in median survival times was observed ($p = 0.2755$), even after matching. This outcome supports the findings of Qu et al. and Chang et al., who also did not report a significant decrease in average survival time among patients with erythrocytosis. However, Feng et al. identified erythrocytosis as a favorable prognostic marker in patients undergoing liver resection. This suggests a specific context in which erythrocytosis could indicate a more favorable outcome, possibly due to lower pre-operative blood transfusion needs.

Our analysis also highlighted those patients with hypoglycemia, typically younger and with larger tumors, exhibited significantly shorter median survival times than those without PNS ($p = 0.0428$). However, no significant differences were found in the matched analysis. Survival rates for patients experiencing hypercholesterolemia and thrombocytosis also trended lower than those without PNS, aligning with prior research but without reaching statistical significance.

## 4. CONCLUSIONS

(1) Among the biologic markers of HCC (AFP, AFP-L3, GPC3, and CK19), AFP emerged as the most accurate predictor of HCC, achieving an AUC of 0.94 and outperforming all other studied biomarkers.

(2) The introduction of GPC3 and CK19 into the GALKA scoring system yielded a robust performance, equivalent to the GALAD score, with an AUC of 0.98.

(3) CEUS is a highly effective imaging technique for distinguishing benign PVT from malign PVT, showcasing a 98.6% Se and 89.3% Sp.

(4) When combined with AFP levels, CEUS's accuracy in the characterization of PVT is further enhanced, underscoring the utility of integrating AFP into diagnostic evaluations.

(5) Our investigation identified a 25.7% prevalence of PNS within the HCC patient population, with hypercholesterolemia being the most frequently observed PNS.

(6) We found a significant correlation between PNS and factors such as tumor size and AFP levels, suggesting diabetes mellitus is a potential risk factor for developing PNS.

(7) Our findings underscore the importance of evaluating newly diagnosed HCC patients for the presence of PNS, as these syndromes significantly impact patient outcomes.