JCB
J Circ Biomark 2026; 15: 58-68
ISSN 1849-4544 | DOI: 10.33393/jcb.2026.3774
ORIGINAL RESEARCH ARTICLE

Relevance of existing blood-based biomarkers for detection of hepatocellular carcinoma: a prospective case-control study

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ABSTRACT

Introduction: This study evaluated the diagnostic performance of conventional blood-based biomarkers for detecting hepatocellular carcinoma (HCC) in patients with chronic liver disease (CLD).
Methods: In this prospective case-control study, adults with CLD (with and without HCC) underwent blood sampling for alpha-fetoprotein (AFP), des-gamma-carboxy prothrombin (DCP), and cell-free DNA (cfDNA). Diagnostic performance was assessed individually and in combination using regression analyses, with radiological imaging as the reference standard. Pre- and post-treatment biomarker levels were analyzed in a subset of HCC patients.
Results: Among 453 analyzed participants (131 HCC, 322 non-HCC controls), AUROCs for AFP, DCP, and cfDNA were 0.84, 0.76, and 0.64, respectively. At Youden index-derived thresholds, sensitivities were 62% for AFP, 65% for DCP, and 59% for cfDNA, with corresponding specificities of 93%, 74%, and 64%. Combining biomarkers via regression models did not improve diagnostic performance. AFP demonstrated poor sensitivity (32%) for early-stage HCC detection, and performance varied by disease etiology. Among treatment response markers, DCP showed superior potential.
Conclusion: In a real-world CLD cohort, conventional blood-based biomarkers demonstrate limited sensitivity for early-stage HCC detection. These findings highlight the need for molecular and genetic approaches to improve early HCC diagnosis.

Keywords: Hepatocellular Carcinoma (HCC), biomarkers, AFP, DCP/PIVKA-II, cell-free DNA, liquid biopsy

Introduction

Hepatocellular Carcinoma (HCC) primarily occurs in patients with chronic liver disease (CLD). For the screening and surveillance of HCC, the American Association for the Study of Liver Diseases (AASLD) recommends using a combination of ultrasound (US) and alpha-fetoprotein (AFP) testing (1). This recommendation acknowledges that US performance is highly operator-dependent and further compromised in patients with obesity. As a result, US alone has a sensitivity of only 53% for early-stage HCC detection. When combined with AFP, sensitivity improves modestly to 63%, but at the cost of reduced specificity (1).

Although AFP is the most widely used blood-based biomarker for screening and detection, its diagnostic performance for detecting HCC has been suboptimal (2-4). The overall sensitivity of AFP ranges from 32 to 61%, depending upon the selected cut-off, which ranged from 20 to 400 ng/mL, with a specificity of more than 95% for all cases (5). AUROC for AFP ranges has been reported to be approximately 0.8 (6,7). However, for early-stage HCC, further decrease in sensitivity has been reported (8). It is also acknowledged and widely accepted that AFP is not the best diagnostic tool for detection of HCC, even though it is highly specific (9). In search of better blood-based biomarkers for HCC detection, researchers have explored protein induced by vitamin K absence/antagonist-II (PIVKA-II), also known as des-gamma-carboxy prothrombin (DCP). However, clinical studies have shown questionable outcomes regarding its effectiveness as an HCC screening biomarker. For example, recent reports have assessed the test performance of DCP for detection of HCC and found the AUROC to be approximately 0.7 (10,11). Another biomarker, Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), has also been explored as a biomarker. In a large-scale Phase-II study, the AUROC of AFP-L3 was found to be 0.72 (7). Indeed, most of the biomarkers explored till date as standalone modality for HCC detection in the background of CLD have exhibited sub-optimal accuracy (12).

Received: March 2, 2026
Accepted: August 11, 2026
Published online: September 18, 2026

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To overcome these limitations, studies started focusing on a combinatorial approach using multiple parameters for improving diagnostic accuracy. For instance, sex, age, AFP-L3, AFP, and DCP have been combined for obtaining a score called the GALAD score. In one study, GALAD achieved a sensitivity of 54.8% at a specificity of 90% (13). More recently, in a Phase 3 biomarker validation study evaluating the GALAD score for HCC detection in cirrhotic patients, the area under the curve (AUC) within 12 months prior to HCC diagnosis was reported as 0.78 for GALAD (14). At a specificity of 82%, GALAD demonstrated a sensitivity of 62% for detecting HCC. Furthermore, a recent meta-analysis of 15 studies involving approximately 20,000 patients assessed the diagnostic performance of GALAD (15). The pooled AUC across all cancer stages, as classified by the Barcelona Clinic Liver Cancer (BCLC) staging system, was 0.82 (95% CI: 0.78-0.85), with a sensitivity of 73% (95% CI: 66-79%) for early-stage HCC. In comparison, the sensitivity of AFP alone for early-stage detection was significantly lower at 38%. As such, while the GALAD score represents an important advancement in HCC risk prediction, its variability across settings portrays the need for further validation and establishment of standardized threshold cut-off values. To improve upon the GALAD model, a recent retrospective study developed two alternate diagnostic models, GAAP and ASAP, based on sex, age, AFP and DCP (10). The sensitivity of both models to detect HCC was approximately 70% with specificity of approximately 80%.

With the growing acceptance of liquid biopsies, the absolute quantification of circulating cell-free DNA (cfDNA) is increasingly being explored as a promising alternative biomarker. For screening, modalities must offer the advantages of being rapid, simple, and cost-effective. Total concentrations of cfDNA have been extensively studied for early detection of various carcinomas, including lung, prostate, breast and gastrointestinal (GI) tract (16-20). In addition, total cfDNA has also been useful for prognosis, for surveillance and recurrence monitoring. For HCC, studies on the use of total cfDNA as a diagnostic marker have reported a sensitivity of approximately 60% with specificity ranging from approximately 80 to 90% for detecting HCC, with AUROC ranging between 0.7 and 0.8 (21,22). Another study showed that the amount of cfDNA was higher in hepatitis C virus (HCV) related HCC than in HCV-related non-HCC patients (23). However, like AFP and DCP, total cfDNA as a standalone biomarker for HCC detection did not yield sufficient diagnostic accuracy. Hence, we sought to utilize a combinatorial approach. This gap shows a critical need for affordable, minimally invasive, and scalable blood-based combinations that improve diagnostic accuracy beyond individual markers. Recently, liquid biopsy strategies that analyze the genetic and epigenetic alterations of HCC, including long non-coding RNA (lncRNA) signatures, have shown promise for early-stage detection of HCC, reflecting a broader shift from conventional protein-based markers (24).

AFP and DCP are already widely available in clinical laboratories and inexpensive to measure, while cfDNA adds a liquid biopsy component without requiring complex mutation profiling or high-cost sequencing. Using these three together offers a practical, scalable alternative to models like GALAD or AFP-L3–based assays, making the approach better suited for routine surveillance in resource-constrained or high-burden settings. In this prospective case-control study, we investigated AFP, DCP, and cfDNA and developed statistical models to determine whether the combination of these three biomarkers could significantly enhance diagnostic accuracy.

Materials and methods

Study design and participants

This study was a monocentric, prospective, case–control investigation conducted at Dr. Rela Institute and Medical Centre (RIMC). Institutional ethics committee approval was obtained prior to study initiation, and informed consent was obtained from all participants at the time of blood sample collection. All methods were performed in accordance with the relevant guidelines and regulations. Standard clinical practices involving radiological imaging such as US, computed tomography (CT), and magnetic resonance imaging (MRI) were employed as the clinical reference standard (CRS). The study included adults aged 18-75 years (inclusive) who were diagnosed with CLD. In addition, they were willing to provide informed consent. Individuals were excluded if they had contraindications to imaging, were outside the specified age range, could not tolerate contrast agents, had a history of HCC-related interventions, or had other cancer types. Further exclusion criteria included a history of liver transplantation, resection, or locoregional therapies, as well as refusal or withdrawal of consent.

The presence or absence of HCC lesions in the participants was confirmed based on the CRS. This study evaluated the performance of AFP, DCP, and cfDNA tests, both as standalone diagnostic tools and in combination. The key metrics reported included sensitivity, specificity, area under the receiver operating characteristic curve (AUROC), positive predictive value (PPV), negative predictive value (NPV), and overall diagnostic accuracy. Regression models were utilized for the training and validation of diagnostic approaches. A detailed flow diagram depicting participant selection is shown in Figure 1.

Target condition

Patients clinically diagnosed with CLD were recruited for this study. The etiologies of CLD include metabolic dysfunction-associated steatohepatitis (MASH) or metabolic dysfunction-associated steatotic liver disease (MASLD), hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease (ALD), cryptogenic steatotic liver disease (cryptogenic-SLD/undetermined causes), and other conditions contributing to CLD. The study cohort comprised both cirrhotic and non-cirrhotic patients.

Clinical assessment

Comprehensive clinical data were collected from all participants at the study center. The diagnosis of liver disease, including the presence or absence of lesions and cirrhosis, was confirmed at a centralized facility using radiological imaging. Key parameters recorded for each participant

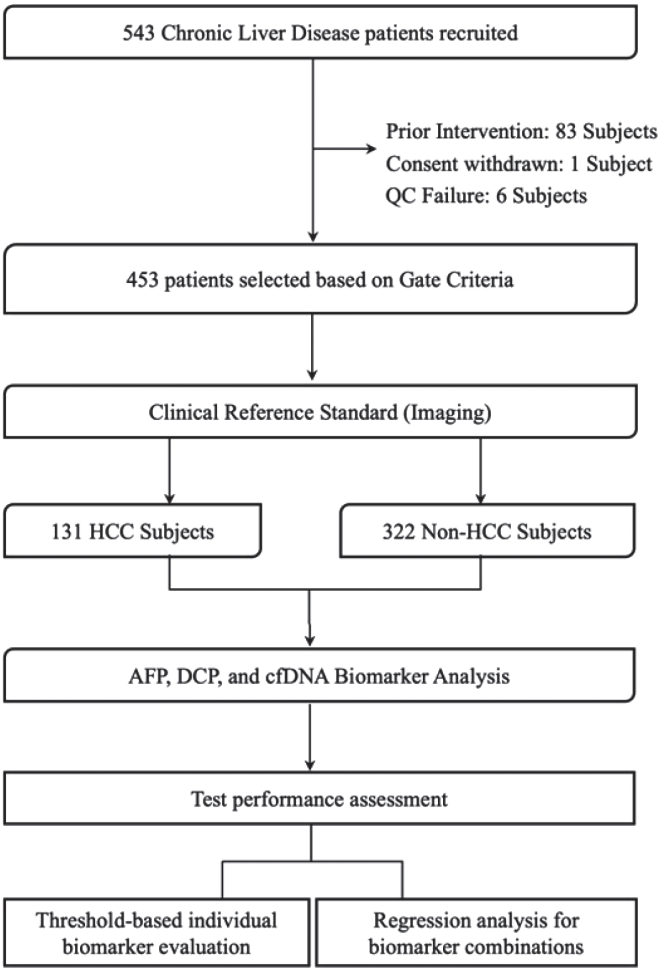


FIGURE 1 - The flowchart outlines the study design for evaluating biomarkers in CLD patients.

included age, sex, etiology of liver disease, AFP levels, DCP levels, cfDNA amount, radiological findings, and BCLC stage.

The primary objective of this study was to evaluate the diagnostic performance of blood-based biomarkers, both individually and in combination. Secondary objectives included assessment of biomarker performance stratified by BCLC stage and subgroup analyses based on age, sex, and disease etiology.

Cell-free DNA quantification

Peripheral blood (20 mL) was collected in cfDNA blood collection tubes (Streck cfDNA BCT). The tubes were processed at room temperature via a two-step centrifugation process, at 1600 × g for 15 minutes and at 1600 × g for 10 minutes, resulting in approximately 10 mL of plasma free of cell debris. cfDNA was extracted from the plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) as per the manufacturer's instructions. Briefly, plasma was combined with proteinase K and Buffer ACL (containing carrier RNA) and incubated at 60 °C for 30 min to release free-circulating cfDNA. Buffer ACB was then added, and the mixture was incubated on ice for 15 min before being drawn through a QIAamp Mini column mounted on a vacuum manifold. The membrane with cfDNA bound was washed sequentially with Buffer ACW1, Buffer ACW2, and ethanol, and residual wash buffer was removed by centrifugation at 15,000 × g for 3 mins. Purified cfDNA was eluted in 70 µL of Buffer AVE at 15,000 × g for 2 mins. The eluted cfDNA was quantified on a Qubit fluorometer using the dsDNA High-Sensitivity (HS) assay, and the total plasma cfDNA concentration was calculated.

Statistical analysis and model construction

The dataset included five features: AFP, DCP, cfDNA, Age, and Sex, with a binary target variable (HCC Status) indicating the presence or absence of HCC. Sex was encoded numerically, and all features were standardized to have a mean of 0 and a standard deviation of 1. Logistic regression analysis was used to predict the likelihood of developing HCC. The model estimates the probability of HCC by modelling the log-odds of the target variable as a linear combination of predictors.

For Model training and evaluation, the dataset was split into training (50%) and testing (20%) subsets, maintaining the class balance across both splits. Model performance was evaluated on the test set using the following metrics: (a) accuracy, the proportion of correctly classified cases; (b) AUROC for the measure of the model's ability to distinguish between classes; and (c) precision and recall assessed to evaluate the model's performance in identifying HCC cases. We employed a 2-fold stratified cross-validation strategy, in which the dataset was randomly partitioned into two equal (50–50) subsets while preserving class distribution. In the first fold, one subset was used for model training and the other for independent testing. In the second fold, the roles of the subsets were reversed. Model performance was averaged across the two folds. This approach was chosen to maximize data utilization while preventing information leakage and to closely mimic real-world deployment scenarios. All analyses were performed using Python (version 3.10.16) and scikit-learn (version 1.6.1). For the logistic regression model, the coefficients and intercept were extracted to present the final predictive equation, which was subsequently used for assessing the test performance. A standard logistic regression model with default parameters and L2 regularization was used.

For standalone biomarker evaluation, the Youden Index was applied to establish optimal thresholds, which were subsequently used to determine the diagnostic accuracy of each biomarker individually. Additionally, the diagnostic accuracy was assessed using pre-established thresholds for AFP (20 ng/mL) and DCP (40 mAU/mL).

Results

A total of 453 participants were enrolled in this study, categorized into two groups: the HCC group (n = 131; 116 males [88.5%], 15 females [11.5%]) and the non-HCC CLD group (n = 322; 258 males [80.1%], 64 females [19.9%]). Within the HCC group, 26% were classified as early stage (BCLC 0 and A), 25.2% as intermediate stage (BCLC B), and 32.8% as late stage (BCLC C and D). The remaining 16% of the participants were non-cirrhotic and therefore not assigned a BCLC

stage. Participants in both groups presented with a variety of underlying etiologies, including MASH/MASLD, HBV, HCV, ALD, cryptogenic-SLD, and other forms of CLD. Notably, 85% of individuals in both groups showed evidence of cirrhosis. The key biological, clinical, and pathological characteristics of the participants are summarized in Table 1.

TABLE 1 - Demographic characteristics, underlying liver disease causes, cirrhosis status, and tumor staging in HCC versus Non-HCC patients

	HCC (131)		Non-HCC (322)	
	n		n	
1. AVAILABLE DATA	131	(28.9%)	322	(71.1%)
2. SEX				
Male	116	(88.5%)	258	(80.1%)
Female	15	(11.5%)	64	(19.9%)
3. AGE				
18-50	22	(16.8%)	146	(45.3%)
51-60	42	(32.1%)	101	(31.4%)
61-75	59	(45.0%)	75	(23.3%)
75 and above	8	(6.1%)	0	(0.0%)
4. ETIOLOGY				
NASH/NAFLD	40	(30.5%)	129	(40.1%)
HBV/HCV	45	(34.4%)	48	(14.9%)
Alcohol	7	(5.3%)	72	(22.4%)
Others	4	(3.1%)	33	(10.2%)
Cryptogenic-SLD	35	(26.7%)	40	(12.4%)
5. CIRRHOSIS	112	(85.5%)	273	(84.8%)
6. NON-CIRRHOSIS	19	(14.5%)	49	(15.2%)
7. TUMOR STAGE (BCLC)				
Early (0/A)	34	(26.0%)	-	
Intermediate (B)	33	(25.2%)	-	
Late (C/D)	43	(32.8%)	-	
Non-Cirrhotic	21	(16.0%)	-	

Diagnostic performance: threshold optimization and comparative analysis

The diagnostic performance of AFP and DCP was initially evaluated using thresholds reported in the literature, set at 20 ng/mL and 40 mAU/mL, respectively. AFP demonstrated a sensitivity of 0.53 (95% CI: 0.45-0.62) and a specificity of 0.97 (95% CI: 0.94-0.99), with an AUROC of 0.84 (95% CI: 0.79-0.88). For DCP, sensitivity and specificity were 0.86 (95% CI: 0.79-0.92) and 0.43 (95% CI: 0.38-0.49), respectively, with an AUROC of 0.76 (95% CI: 0.71-0.81). Subsequently, the thresholds were optimized using the Youden index. The calculated cut-off for AFP was 11.3 ng/mL, resulting in a marginal increase in sensitivity to 0.62 (95% CI: 0.53-0.70), accompanied by a marginal decrease in specificity. For DCP, the optimized cut-off was 230 mAU/mL, leading to notable changes in performance. The sensitivity decreased to 0.65, whereas the specificity increased to 0.74, reflecting a substantial shift in diagnostic capability. Interestingly, the absolute quantification of cfDNA emerged as the least effective biomarker among the three evaluated markers, as shown in Figure 2. It achieved an AUROC of 0.64 (95% CI: 0.59-0.70). At a threshold of 327 ng, cfDNA demonstrated a sensitivity of 0.59 (95% CI: 0.51-0.68) and a specificity of 0.64 (95% CI: 0.59-0.69). Table 2 shows the diagnostic performance of individual biomarkers.

Using logistic regression, we investigated whether combinations of biomarkers could improve the diagnostic accuracy. In this study, we investigated the combinations of AFP, DCP, cfDNA, sex, and Age (Table 2). For logistic regression, the combination of AFP and DCP achieved an AUROC of 0.83 (95% CI: 0.78-0.87), with sensitivity and specificity of 0.63 (95% CI: 0.55-0.71) and 0.9 (95% CI: 0.86-0.93), respectively, demonstrating the highest specificity among all combinations. When AFP was combined with cfDNA, the AUROC slightly decreased to 0.77 (95% CI: 0.72-0.82), accompanied by a notable increase in sensitivity to 0.78 (95% CI: 0.73-0.84), although specificity dropped to 0.65 (95% CI: 0.61-0.68). The combination of DCP and cfDNA yielded an AUROC of 0.73 (95% CI: 0.68-0.78), with sensitivity and specificity values of 0.71 (95% CI: 0.66-0.77) and 0.64 (95% CI: 0.60-0.67). Adding cfDNA to AFP and DCP improved sensitivity to 0.85 (95% CI: 0.81-0.89) but reduced specificity to 0.62 (95% CI: 0.58-0.68), with an AUROC of 0.80 (95% CI: 0.75-0.84). A combination of AFP, DCP, cfDNA, age, and sex resulted in a similar AUROC of 0.79 (95% CI: 0.75-0.84) but did not enhance specificity. Similar results were obtained using Ridge regression analysis and Lasso regression analysis.

Diagnostic performance of cfDNA, AFP, and DCP in HCC across etiologies

The test performances of the three biomarkers with respect to etiology are detailed in Table 3. With an overall sensitivity of 62%, AFP demonstrated a similar sensitivity profile across all etiologies, ranging from 50% to 67%, except for the Others category, owing to its low sample size evidenced by the wide CI range (95% CI: 40-100%). In contrast, specificity was consistently high across etiologies and was comparable to the overall performance. ALD cases exhibited the highest specificity at 99%, followed closely by metabolic (96%) and cryptogenic-SLD (95%). Viral cases showed reduced specificity (79%) compared to the overall performance. PPV ranged from 50 to 92%. NPV was lowest in viral cases (72%, 95% CI: 62-80%). ALD cases demonstrated the highest accuracy (95%), whereas viral cases showed the lowest (73%) compared to the overall data.

DCP demonstrated the highest sensitivity for viral etiology (76%) and the lowest for ALD (43%). With an overall sensitivity of 65%, significant variability among subgroups was observed. The lower sensitivity in Metabolic and ALD cases indicates that DCP may have limited effectiveness in detecting HCC within these etiologies compared to viral or cryptogenic-SLD cases. DCP's overall specificity was 74%. It was notably higher for viral cases (90%) and the Others category (85%), while specificity dropped significantly for ALD (57%)

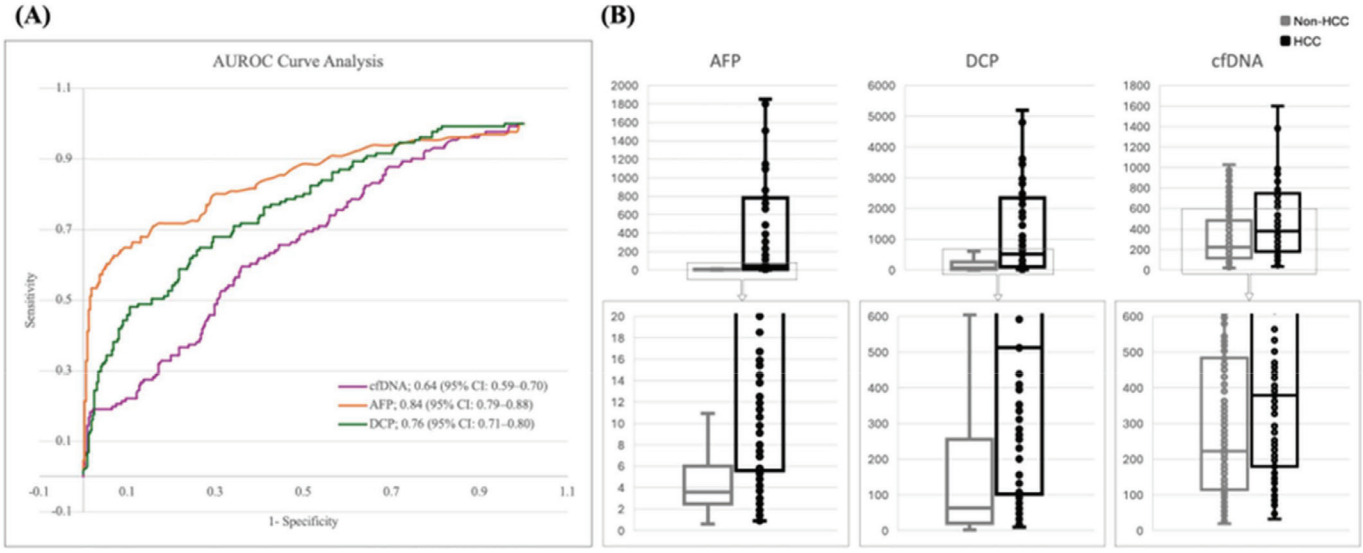


FIGURE 2 - The figure presents the performance evaluation of three biomarkers - AFP, DCP, and cfDNA in diagnosing HCC. **(A)** AUROC Curve Analysis with Sensitivity (y-axis) vs. 1 - Specificity (x-axis) compares the diagnostic accuracy of the three biomarkers. **(B)** Biomarker Distribution using Box plots for AFP, DCP, and cfDNA displaying overall data distributions as well as the zoomed-in versions for better visualization of overlapping quartiles. The zoomed-in box plots show that only for AFP, the third quartile of non-HCC patients slightly overlaps with the first quartile of HCC patients. In contrast, for both DCP and cfDNA, there is substantial overlap between the quartiles of HCC and non-HCC groups, indicating that these markers alone are insufficient to distinguish HCC from non-HCC patients.

and cryptogenic-SLD (55%) cases. Metabolic cases showed moderate specificity at 71%, aligning closely with the overall performance. PPV was highest in viral cases (87 %) and lowest in ALD (9%). Accuracy ranged from 56% in ALD to 84% in others.

The performance of cfDNA in detecting HCC varies across different etiologies of CLD. Sensitivity was highest in cryptogenic-SLD cases (69%), followed by metabolic (60%), viral (56%), and the Others category (50%), with ALD having the lowest sensitivity (43%). The overall sensitivity was 60%. Specificity was highest in viral cases (94%), significantly surpassing the overall specificity (64%), whereas metabolic cases (65%) aligned closely with the overall data. ALD (51%) and cryptogenic-SLD (53%) had lower specificities, indicating a higher likelihood of false positives. Accuracy ranged from 51% in ALD to 75% in viral cases, reflecting the variability in cfDNA performance across different etiologies.

To assess whether biomarker performance varied significantly across clinical subgroups, we performed z-tests comparing subgroup-specific sensitivity and specificity values against the overall cohort estimates. Sensitivities and specificities, along with confidence intervals, were used to calculate standard errors. p-values were computed for each group by comparing with the overall value for both sensitivity and specificity. While most subgroup performances were comparable to the overall cohort, statistically significant differences were observed in a few cases. The p-value of AFP, DCP and cfDNA in the ALD group was found to be less than 0.05, suggesting statistically significant differences vis-à-vis overall performance. In addition, the specificity of AFP in viral etiology and DCP in cryptogenic-SLD was found to have a p-value less than 0.05.

Sensitivity of biomarkers across BCLC stages

The sensitivity of cfDNA, AFP, and DCP biomarkers, based on the Youden Index-derived thresholds and the reported thresholds (AFP: 20 ng/ml and DCP: 40 mAU/ml), was analyzed across different BCLC stages and in non-cirrhotic patients, to assess their ability to identify true positives. Analysis based on Youden Index-derived thresholds for early-stage HCC (BCLC 0/A) demonstrated a sensitivity of 53% for cfDNA, followed by AFP (50%) and DCP (41%). For intermediate-stage HCC (BCLC B), AFP showed the highest sensitivity (70%), closely followed by DCP (67%), whereas cfDNA had a lower sensitivity of 52%. In advanced stages (BCLC C/D), DCP outperformed the other biomarkers with a sensitivity of 79%, followed by cfDNA (70%) and AFP (67%). Using literature-reported thresholds, the sensitivity of AFP was found to decrease significantly in early-stage HCC. For BCLC 0/A, the sensitivity was only 32%. In contrast, for intermediate (BCLC B) and advanced stages (BCLC C/D), the sensitivities were 58% and 67%, respectively. These findings suggest that the AFP thresholds are primarily relevant in later stages of the disease, with limited diagnostic utility in early-stage HCC. In the case of DCP, the sensitivity increased significantly as expected across stages. However, it is important to note that when a threshold of 40 mAU/mL was used, over 50% of results were false positives in the overall population, highlighting the limitations of this threshold in clinical applications. Among non-cirrhotic patients, based on the derived thresholds, DCP demonstrated the highest sensitivity (71%), outperforming cfDNA (62%) and AFP (57%). The details of the sensitivity based on BCLC stages are provided in Table 2.

Evaluation of blood-based biomarkers as treatment response markers

We then investigated the potential of blood-based biomarkers as indicators of treatment response in HCC. Ten patients who underwent HCC-related treatment—surgical resection, liver transplantation, or locoregional therapy (SBRT/TACE/MWA)—were included in this analysis. Blood samples were collected four to six weeks post-treatment, coinciding with the routine post-treatment follow-up schedule. This interval also helped minimize the influence of acute treatment-related tissue injury and transient changes in circulating cfDNA, allowing the biomarker measurements to reliably reflect post-treatment disease status. Changes in AFP, DCP, and cfDNA levels between pre- and post-treatment samples are shown in Figure 3, along with the corresponding treatment modality, mRECIST response, and biomarker status.

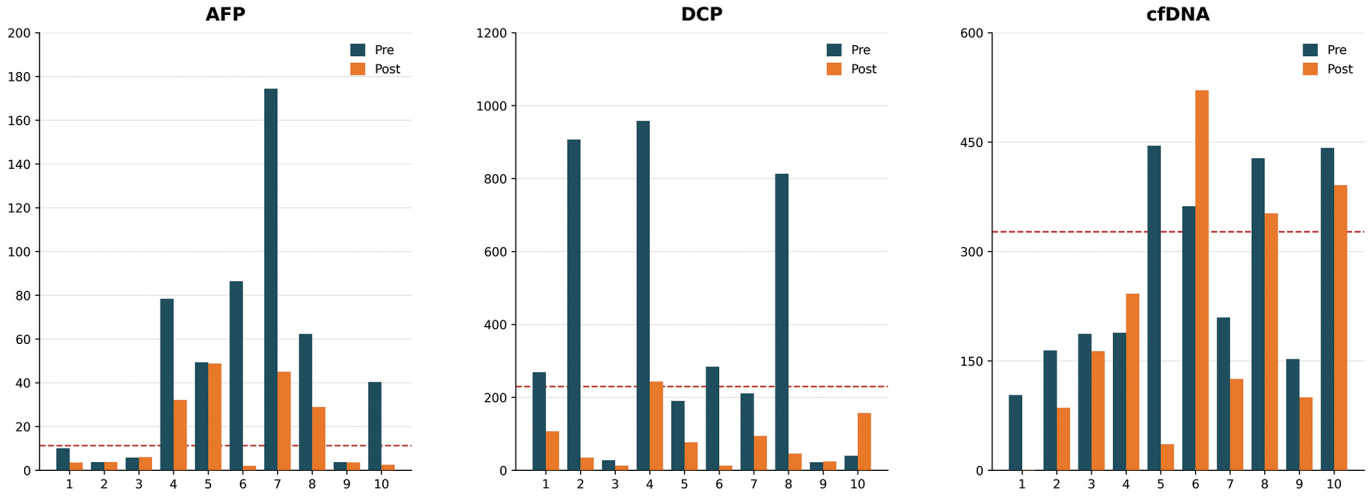
Overall, biomarker levels declined following treatment in most patients, although the magnitude and consistency of change varied by biomarker and treatment modality. Of the 6 AFP-positive cases at baseline, 2 fell below the cut-off post-treatment, 3 remained above the cut-off but showed a clear decline, and 1 remained essentially unchanged. All 4 AFP-negative cases remained negative. For DCP, 4 of the 5

TABLE 2 - Overall test performance

Diagnostic Performance			
1. Threshold Optimization			
	Predefined Thresholds		
Biomarker	**AFP - 20 ng/ml***	**DCP - 40 mAU/ml***	**cfDNA - 327 ng****
AUROC (95% CI)	0.84 (0.79 - 0.88)	0.76 (0.71 - 0.81)	-
Sensitivity (95% CI)	0.53 (0.45 - 0.62)	0.86 (0.79 - 0.92)	-
Specificity (95% CI)	0.97 (0.94 - 0.99)	0.43 (0.38 - 0.49)	-
PPV (95% CI)	0.88 (0.79 - 0.93)	0.38 (0.36 - 0.41)	-
NPV (95% CI)	0.84 (0.81 - 0.86)	0.89 (0.83 - 0.92)	-
Accuracy (95% CI)	0.84 (0.81 - 0.88)	0.56 (0.51 - 0.60)	-
Sensitivity - BCLC 0/A	0.32 (0.17 - 0.51)	0.76 (0.59 - 0.89)	-
Sensitivity - BCLC B	0.58 (0.39 - 0.75)	0.88 (0.72 - 0.97)	-
Sensitivity - BCLC C/D	0.67 (0.55 - 0.81)	0.91 (0.78 - 0.97)	-
Sensitivity - Non-Cirrhotic	0.52 (0.3 - 0.74)	0.9 (0.69 - 0.99)	-
	Optimized Thresholds		
Biomarker	**AFP - 11.3 ng/ml****	**DCP - 230 mAU/ml****	**cfDNA - 327 ng****
AUROC (95% CI)	0.84 (0.79 - 0.88)	0.76 (0.71 - 0.81)	0.64 (0.59 - 0.70)
Sensitivity (95% CI)	0.62 (0.53 - 0.70)	0.65 (0.56 - 0.73)	0.59 (0.51 - 0.68)
Specificity (95% CI)	0.93 (0.90 - 0.96)	0.74 (0.68 - 0.78)	0.64 (0.59 - 0.69)
PPV (95% CI)	0.79 (0.71 - 0.85)	0.50 (0.45 - 0.56)	0.40 (0.35 - 0.45)
NPV (95% CI)	0.86 (0.83 - 0.88)	0.84 (0.80 - 0.87)	0.80 (0.76 - 0.83)
Accuracy (95% CI)	0.84 (0.80 - 0.87)	0.71 (0.67 - 0.75)	0.63 (0.58 - 0.67)
Sensitivity - BCLC 0/A	0.5 (0.32 - 0.68)	0.41 (0.25 - 0.59)	0.53 (0.35 - 0.7)
Sensitivity - BCLC B	0.69 (0.51 - 0.84)	0.67 (0.49 - 0.82)	0.52 (0.34 - 0.69)
Sensitivity - BCLC C/D	0.67 (0.51 - 0.81)	0.79 (0.62 - 0.88)	0.69 (0.54 - 0.83)
Sensitivity - Non-Cirrhotic	0.57 (0.34 - 0.78)	0.71 (0.44 - 0.86)	0.61 (0.38 - 0.82)
2. Logistic Regression Analysis - Combinations of the Biomarkers**			
Biomarker Combinations	**AUROC (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
AFP+DCP	0.83 (0.78 - 0.87)	0.63 (0.55 - 0.71)	0.90 (0.86 - 0.93)
AFP+cfDNA	0.77 (0.72 - 0.82)	0.78 (0.73 - 0.84)	0.65 (0.61 - 0.68)
DCP+cfDNA	0.73 (0.68 - 0.78)	0.71 (0.66 - 0.77)	0.64 (0.60 - 0.67)
AFP+DCP+cfDNA	0.80 (0.75 - 0.84)	0.85 (0.81 - 0.89)	0.62 (0.58 - 0.68)
AFP+DCP+cfDNA+Age+Gender	0.79 (0.75 - 0.84)	0.84 (0.80 - 0.88)	0.62 (0.57 - 0.67)

TABLE 3 - Test performance of different biomarkers across different HCC etiologies

	AFP	DCP	cfDNA
Metabolic			
Sensitivity (95% CI)	0.50 (0.34-0.66)	0.58 (0.41-0.73)	0.60 (0.43-0.75)
Specificity (95% CI)	0.96 (0.91-0.99)	0.71 (0.63-0.79)	0.65 (0.56-0.73)
PPV (95% CI)	0.80 (0.62-0.91)	0.38 (0.30-0.48)	0.35 (0.27-0.43)
NPV (95% CI)	0.86 (0.82-0.89)	0.84 (0.79-0.89)	0.84 (0.78-0.89)
Accuracy (95% CI)	0.85 (0.79-0.90)	0.68 (0.60-0.75)	0.64 (0.56-0.71)
Viral			
Sensitivity (95% CI)	0.67 (0.51-0.80)	0.76 (0.60-0.87)	0.56 (0.40-0.70)
Specificity (95% CI)	0.79 (0.65-0.90)	0.90 (0.77-0.97)	0.95 (0.83-0.99)
PPV (95% CI)	0.75 (0.62-0.84)	0.87 (0.74-0.94)	0.89 (0.73-0.96)
NPV (95% CI)	0.72 (0.62-0.80)	0.80 (0.70- 0.87)	0.69 (0.62-0.76)
Accuracy (95% CI)	0.73 (0.63-0.82)	0.83 (0.74-0.90)	0.75 (0.65-0.84)
ALD			
Sensitivity (95% CI)	0.57 (0.18-0.90)	0.43 (0.10-0.82)	0.43 (0.10-0.82)
Specificity (95% CI)	0.99 (0.93-1.00)	0.57 (0.45-0.69)	0.51 (0.39-0.63)
PPV (95% CI)	0.80 (0.34-0.97)	0.09 (0.04-0.19)	0.08 (0.03-0.17)
NPV (95% CI)	0.96 (0.91-0.98)	0.91 (0.84-0.95)	0.90 (0.82-0.95)
Accuracy (95% CI)	0.95 (0.88-0.99)	0.56 (0.44-0.67)	0.51 (0.39-0.62)
Others			
Sensitivity (95% CI)	1.00 (0.40-1.00)	0.75 (0.19-0.99)	0.50 (0.07-0.93)
Specificity (95% CI)	0.88 (0.72-0.97)	0.85 (0.68-0.95)	0.58 (0.39-0.75)
PPV (95% CI)	0.50 (0.29-0.71)	0.38 (0.18-0.62)	0.13 (0.05-0.29)
NPV (95% CI)	1.00 (0.88-1.00)	0.97 (0.84-0.99)	0.90 (0.77-0.96)
Accuracy (95% CI)	0.89 (0.75-0.97)	0.84 (0.68-0.94)	0.57 (0.39-0.73)
Cryptogenic-SLD			
Sensitivity (95% CI)	0.66 (0.48-0.81)	0.63 (0.45-0.79)	0.69 (0.51-0.83)
Specificity (95% CI)	0.95 (0.83-0.99)	0.55 (0.38-0.71)	0.53 (0.36-0.68)
PPV (95% CI)	0.92 (0.74-0.98)	0.55 (0.44-0.65)	0.56 (0.46-0.65)
NPV (95% CI)	0.76 (0.67-0.83)	0.63 (0.50-0.74)	0.66 (0.52-0.77)
Accuracy (95% CI)	0.81 (0.71-0.89)	0.59 (0.47-0.70)	0.60 (0.48-0.71)
Cirrhotic			
Sensitivity (95% CI)	0.61 (0.51-0.70)	0.63 (0.54-0.72)	0.58 (0.48-0.67)
Specificity (95% CI)	0.95 (0.91-0.97)	0.71 (0.65-0.76)	0.63 (0.57-0.68)
PPV (95% CI)	0.82 (0.73-0.88)	0.47 (0.42-0.53)	0.39 (0.34-0.44)
NPV (95% CI)	0.85 (0.82-0.88)	0.83 (0.79-0.86)	0.78 (0.74-0.82)
Accuracy (95% CI)	0.85 (0.81-0.88)	0.69 (0.64-0.73)	0.61 (0.56-0.66)
Overall			
Sensitivity (95% CI)	0.62 (0.53-0.70)	0.65 (0.56-0.73)	0.60 (0.51-0.68)
Specificity (95% CI)	0.93 (0.90-0.96)	0.74 (0.68-0.78)	0.64 (0.58-0.69)
PPV (95% CI)	0.79 (0.71-0.85)	0.50 (0.44-0.56)	0.40 (0.35-0.45)
NPV (95% CI)	0.86 (0.83-0.88)	0.84 (0.80-0.87)	0.80 (0.76-0.83)
Accuracy (95% CI)	0.84 (0.80-0.87)	0.71 (0.67-0.75)	0.63 (0.58-0.67)



Patient	Treatment	mRECIST	AFP (11.3 ng/mL)	DCP (230 mAU/mL)	cfDNA (327 ng)
Patient-1	Right Posterior Segmental Resection	CR	10 (-) → 3.5 (-)	269 (+) → 107 (-)	103 (-) → 0.68 (-)
Patient-2	Living Donor Liver Transplant	CR	3.7 (-) → 3.8 (-)	907 (+) → 34.8 (-)	164.2 (-) → 85.7 (-)
Patient-3	Transarterial Chemoembolization and Microwave Ablation	CR	5.8 (-) → 6 (-)	27.4 (-) → 12.6 (-)	187 (-) → 163.3 (-)
Patient-4	Stereotactic Body Radiation Therapy	CR	78.3 (+) → 32.1 (+)	958 (+) → 243 (+)	188.4 (-) → 242.1 (-)
Patient-5	Stereotactic Body Radiation Therapy	PR	49.3 (+) → 48.8 (+)	190 (-) → 76.8 (-)	445 (+) → 35.8 (-)
Patient-6	Living Donor Liver Transplant	CR	86.4 (+) → 2 (-)	284 (+) → 12.6 (-)	362 (+) → 520.9 (+)
Patient-7	Living Donor Liver Transplant	CR	174.4 (+) → 45 (+)	211 (-) → 94.1 (-)	209.4 (-) → 125.1 (-)
Patient-8	Living Donor Liver Transplant	PR	62.3 (+) → 28.9 (+)	813 (+) → 45.8 (-)	427.7 (+) → 352.2 (+)
Patient-9	Dead Donor Liver Transplant	CR	3.7 (-) → 3.6 (-)	22 (-) → 24.2 (-)	152.3 (-) → 100 (-)
Patient-10	Living Donor Liver Transplant	CR	40.3 (+) → 2.5 (-)	39.4 (-) → 157 (-)	442 (+) → 391 (+)

CR, complete response; PR, partial response

FIGURE 3 - The figure presents a comparative analysis of **AFP, DCP, and cfDNA biomarkers** before (**Pre**) and after (**Post**) an intervention or treatment for HCC. The red line indicates the derived threshold (AFP: 11.3 ng/mL; DCP/PIVKA-II: 230 mAU/mL; cfDNA: 327 ng). Among the biomarkers, DCP shows the most consistent decline and normalization after intervention, making it the most robust for monitoring HCC treatment response amongst the 3 biomarkers. AFP also tends to decrease post-treatment but is less reliable in those with low baseline levels. cfDNA has variable trends and limited consistency, reducing its utility as a standalone indicator in this cohort. The corresponding table shown below summarizes the treatment received, mRECIST response, and patient-level changes in AFP, DCP and cfDNA, including the transition in biomarker status (+/- indicating positive/negative) based on the derived cut-offs. Together, the graphs and table illustrate the heterogeneity of biomarker responses following treatment.

positive cases fell below the cut-off post-treatment, and the remaining case showed a marked decline but stayed marginally above the threshold; all 5 DCP-negative cases remained negative. For cfDNA, of the 4 positive cases, 1 fell below the cut-off, 2 remained above the cut-off but declined, and 1 increased post-treatment; among the 6 cfDNA-negative cases, 4 showed a further reduction of more than 25%, while the remaining 2 showed little change or a slight increase.

Collectively, these findings show that among biomarker-positive patients, treatment produced substantial reductions across all three markers. DCP-positive cases showed the greatest and most consistent decline (median −94.4%, range −60.2% to −96.2%), followed by AFP-positive cases (median −66.6%, range −1.0% to −97.7%), often remaining above the derived cut-off despite the decline. cfDNA-positive cases showed a more modest and variable response (median −14.6%, range +43.9% to −92.0%), including one case with a post-treatment increase. Notably, patients who were biomarker-negative at baseline (4 of 10 for AFP, 5 of 10 for DCP, and 6 of 10 for cfDNA) largely remained below their respective cut-offs at follow-up, supporting the specificity of these markers and indicating that observed reductions in positive cases reflect a genuine treatment response rather than assay drift or non-specific decline. Given the limited sample size and heterogeneity of treatment modalities, these observations should be considered exploratory and warrant validation in larger prospective cohorts.

Discussion

The diagnostic utility of blood-based biomarkers in this study provides critical insights into their potential for HCC detection. Our study evaluated the diagnostic performance of AFP, DCP, and cfDNA in detecting HCC, both individually and in combination. The test performance was assessed by comparing with radiological imaging such as CECT or MRI

as the CRS. It is noteworthy to mention that although these radiological imaging modalities were used as the CRS in this study, the sensitivity and specificity of these modalities in our cohort were not independently assessed. By analyzing these biomarkers across diverse patient populations and using thresholds derived from literature and optimized through statistical approaches, we explored their strengths, limitations, and combined utility.

Using established thresholds from the literature, AFP (20 ng/ml) exhibited a strong specificity (97%), although its sensitivity (53%) was comparatively lower. This result aligns with prior studies that emphasize AFP's robust specificity but limited sensitivity, reflecting its status as a traditional but imperfect biomarker for HCC detection. On the other hand, DCP demonstrated higher sensitivity (86%) but significantly lower specificity (43%). This finding is consistent with the understanding that DCP, while more sensitive than AFP (at 40mAU/ml cut-off), is influenced by underlying liver function abnormalities, which can lead to increased false positives in non-HCC cases. The clinical implications of a high false positive rate are significant. Use of DCP at the predefined cut-off threshold may result in unnecessary diagnostic imaging for confirmation, potentially increasing healthcare burden. Moreover, false positive results may cause undue psychological distress and anxiety for patients. Hence, use of DCP as an HCC screening tool is highly questionable. These findings reaffirm the challenges of relying on a single biomarker for HCC diagnosis, for which the balance between sensitivity and specificity remains elusive. The combination of DCP and AFP yielded minimal to no improvement in diagnostic accuracy, which is in concordance with an earlier study (25).

Thresholds were adjusted using the Youden index to optimize the diagnostic utility of these biomarkers. For AFP, reducing the cut-off to 11.3 ng/mL slightly improved sensitivity but at the expense of specificity. Conversely, the optimized cut-off for DCP, set at 230 mAU/mL, enhanced specificity but reduced sensitivity, showing the inherent limitations of singular biomarkers, as no single threshold can reliably distinguish HCC from non-HCC cases across diverse patient populations. Interestingly, cfDNA, when evaluated as an independent biomarker, emerged as the least effective of the three, with an AUROC of 0.64. Since AUC values above 0.8 are generally accepted as clinically relevant, use of cfDNA as a standalone modality for HCC detection becomes irrelevant (26). This finding conforms to the earlier reported findings (21, 22). The poor diagnostic accuracy is primarily due to the fact that patients with CLD have significant liver damage, which leads to elevated levels of circulating cfDNA, even in the absence of carcinoma. This relatively poor diagnostic performance reflects the variability of cfDNA levels in liver diseases and highlights the need for further refinement of its use as a biomarker. The limited performance of cfDNA as a standalone modality necessitated an evaluation of its potential to enhance diagnostic accuracy when used as a complementary tool.

The combination of biomarkers demonstrated minimal improvement in diagnostic accuracy. Specifically, the combination of AFP and DCP achieved a specificity of 90% but with an AUROC comparable to AFP alone, highlighting the insignificant complementary role of DCP in enhancing diagnostic robustness. However, adding cfDNA to this combination improved sensitivity (85%), albeit at the expense of specificity (62%), suggesting incorporation of cfDNA into a biomarker panel may improve early detection rates in high-risk populations, where sensitivity is often prioritized over specificity. Similar diagnostic trends were observed in Ridge and Lasso regression analyses, validating the robustness of these findings.

Interestingly, the inclusion of demographic factors such as age and sex did not provide additional diagnostic value. This finding reflects the limited role of demographic modifiers in influencing the accuracy of biomarkers. These results emphasize the limitations of these biomarkers and highlight the need for alternative strategies. While AFP and DCP remain central to diagnostic algorithms, their suboptimal overall performance indicates the need for additional biomarkers to fill gaps, particularly in early-stage detection.

The performance of AFP, DCP, and cfDNA was further evaluated across various CLD etiologies. These findings revealed significant variability in sensitivity, specificity, and predictive values, underscoring the need for tailored diagnostic approaches based on specific patient populations. AFP demonstrated consistently high specificity across etiologies. However, its sensitivity remains low, with a relatively poor performance in ALD and metabolic cases. These findings are consistent with those of a previous study, which suggested that AFP sensitivity is influenced by both tumor characteristics and the underlying etiology of liver disease (27). In addition, susceptibility of DCP to false positives in specific subgroups limits its diagnostic utility as a standalone biomarker. However, DCP's high specificity and positive predictive value (PPV) in viral cases indicate that it could be a particularly valuable diagnostic marker in populations with a high prevalence of viral hepatitis-associated HCC.

The variability in biomarker performance across CLD etiologies highlights the importance of personalized approaches for HCC diagnosis. High specificity of AFP makes it a reliable first-line marker, but its inconsistent sensitivity necessitates supplementation with additional biomarkers such as DCP or cfDNA. High performance of DCP in viral cases and potential utility of cfDNA in metabolic cases suggest that these markers could be integrated into etiology-specific diagnostic algorithms. Combining these biomarkers into a multi-analyte panel in combination with advanced algorithms may enhance diagnostic performance, addressing the limitations of individual markers.

The sensitivity of biomarkers was further assessed across different stages of HCC using the BCLC staging system, which aligns with the established role of DCP in advanced HCC, where it correlates with a larger tumor burden and vascular invasion. However, its high false-positive rate across the overall population highlights the need to refine DCP thresholds to improve specificity, particularly for early-stage diseases. Among patients without cirrhosis, DCP demonstrated the highest sensitivity, followed by cfDNA and AFP. The diagnostic potential of DCP as a biomarker for HCC detection may hold particular value in non-cirrhotic patients. However, the high false-positive rate in the overall population necessitates cautious interpretation.

Recognizing the limitations of these biomarkers for diagnosis, we evaluated their relevance as indicators of treatment response. AFP demonstrated limited utility, whereas DCP showed greater promise, with all five true-positive patients having post-treatment levels below the threshold. Performance of cfDNA was inconsistent, with five patients showing reductions exceeding 25%. These findings suggest that cfDNA and DCP may serve as better markers for assessing treatment response rather than for screening or diagnostic purposes. A further limitation concerns the case-control design itself. As patients with and without HCC were enrolled as separate groups rather than followed prospectively within a surveillance population, the study is subject to spectrum bias and is expected to overestimate diagnostic accuracy relative to real-world screening, in which HCC prevalence is lower, and lesions are more often early stage. The sensitivity, specificity and AUROC values reported here should therefore be regarded as upper-bound estimates and cannot be directly extrapolated to HCC surveillance, which is typically of prospective cohort design. Validation in an adequately powered prospective cohort is required. It is also acknowledged that while this study was conducted at a single center, patient recruitment was consecutive and inclusive, encompassing a wide spectrum of CLD etiologies and stages. This approach minimizes selection bias and helps ensure that the cohort broadly represents the target patient population. Patients confirmed to have HCC could undergo these biomarker tests, and true positive cases identified through these markers may be further monitored to evaluate treatment response. It is important to note that the findings from this pilot study on treatment response, which involved only 10 patients, are a limitation of this study and require validation in a larger, independent cohort to confirm their clinical relevance. It is also noteworthy to mention that the imaging modalities such as CECT, MRI and US were used as the CRS for computing the test performance and the possibility of operator bias with respect to imaging data interpretation is acknowledged.

Conclusion

In summary, this study reports the importance of combining biomarkers for HCC detection while emphasizing the need to refine diagnostic and treatment response strategies based on biomarker performance across patient subgroups and disease stages. However, despite the improvement in overall test performance with combined blood-based markers, their diagnostic utility remains limited owing to suboptimal results. These findings suggest that to enhance the efficiency of HCC screening and diagnosis, future research should move beyond traditional protein-based biomarkers and focus on advancements in genetic and epigenetic approaches such as ctDNA methylation signatures, microRNA panels, and long non-coding RNA (lncRNA) signatures, which have shown early promise for HCC detection.

Disclosures

Conflict of Interest: The authors declare no potential conflicts of interest.

Financial support: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors

Data Availability Statement: All analysis scripts, model code, and data supporting the findings of this study have been deposited in Figshare and are accessible (DOI: 10.6084/m9.figshare.30327724) to ensure reproducibility of the results.

Due to ethical and privacy considerations, the de-identified raw dataset is available for research purposes only. Access is granted to qualified researchers upon approval through a formal data-sharing agreement, in compliance with institutional and ethical guidelines.

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