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Thieme

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Der GALAD-Score, ein AFP-, AFP-L3- und DCP-basierter Diagnosealgorithmus verbessert die Detektionsrate des hepatozellulären Karzinoms im BCLC-Frühstadium signifikant

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Schlüsselwörter
- hepatozelluläres Karzinom
- HCC
- diagnostische serologische Tests
- Prognosemodelle
- Tumormarker
- AFP
- AFP-L3
- DCP
- GALAD
- BALAD-2

Key words
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Zusammenfassung

Hintergrund: Das hepatozelluläre Karzinom (HCC) zählt weltweit zu den häufigsten Todesursachen bei Patienten mit Leberzirrhose. Die HCC-Detektion in frühen Stadien ist weiterhin zu selten, weshalb nur bei einem Bruchteil der Patienten kurativ intendierte Therapien durchführbar sind. Das Ziel dieser großen monozentrischen Studie ist die Optimierung der HCC-Frühdetektion mittels additivem Einsatz der neuen Biomarker AFP-L3 und DCP zusätzlich zur AFP-Bestimmung im neuen Diagnosealgorithmus GALAD.

Material und Methoden: Von 2007 bis 2008 sowie von 2010 bis 2012 wurden sowohl 285 Patienten mit der Erstdiagnose eines HCCs als auch 402 Patienten mit chronischen Lebererkrankungen in diese Studie eingeschlossen. Die Bestimmung von AFP, AFP-L3 und DCP erfolgte mit dem automatisierten µTASWako-i30-Immunoanalyzer. Die Leistungsfähigkeit der Biomarker wurde sowohl für Einzelparameter als auch im logistischen Regressionsmodell getestet. Zudem wurde der Diagnosealgorithmus GALAD validiert, der unter Einbeziehung von Geschlecht, Alter und der genannten Biomarker berechnet wird.

Ergebnisse: Einzeln erzielten AFP, AFP-L3 und DCP jeweils vergleichbare Sensitivitäten und Spezifitäten in der HCC-Detektion. Die höchste Sensitivität wurde in der Kombination aller drei Marker erzielt bei allerdings verminderter Spezifität. Im Gegensatz dazu zeigte der GALAD-Score eine deutlich überlegene Spezifität von 93,3 % bei einer Sensitivität von 85,6 %. Bei HCC-Frühstadien (BCLC 0/A) konnte GALAD eine AUROC von 0,9242 erzielen und war auch in dieser Subgruppe allen o. g. Markern und -kombinationen signifikant überlegen.

Abstract

Background: Hepatocellular carcinoma (HCC) is one of the leading causes of death in cirrhotic patients worldwide. The detection rate for early stage HCC remains low despite screening programs. Thus, the majority of HCC cases are detected at advanced tumor stages with limited treatment options. To facilitate earlier diagnosis, this study aims to validate the added benefit of the combination of AFP, the novel biomarkers AFP-L3, DCP, and an associated novel diagnostic algorithm called GALAD.

Material and methods: Between 2007 and 2008 and from 2010 to 2012, 285 patients newly diagnosed with HCC and 402 control patients suffering from chronic liver disease were enrolled. AFP, AFP-L3, and DCP were measured using the µTAS-Wako i30 automated immunoanalyzer. The diagnostic performance of biomarkers was measured as single parameters and in a logistic regression model. Furthermore, a diagnostic algorithm (GALAD) based on gender, age, and the biomarkers mentioned above was validated.

Results: AFP, AFP-L3, and DCP showed comparable sensitivities and specifities for HCC detection. The combination of all biomarkers had the highest sensitivity with decreased specificity. In contrast, utilization of the biomarker-based GALAD score resulted in a superior specificity of 93.3 % and sensitivity of 85.6 %. In the scenario of BCLC 0/A stage HCC, the GALAD algorithm provided the highest overall AUROC with 0.9242, which was superior to any other marker combination.

Conclusions: We could demonstrate in our cohort the superior detection of early stage HCC with the combined use of the respective biomarkers and in particular GALAD even in AFP-negative tumors.

Schlussfolgerung: Für unsere Kohorte konnten wir demonstrieren, dass der kombinierte Einsatz der o. g. Biomarker und insbesondere der GALAD-Score die HCC-Detektion insbesondere in Frühstadien selbst bei AFP-negativen Tumoren signifikant verbessern konnte.

Introduction

The worldwide annual incidence of hepatocellular carcinoma (HCC) almost parallels its mortality rate and therefore is still a leading cause of cancer-related death [1]. This clearly indicates that new strategies in detection of HCC at early stages are urgently required, when curative treatment is still possible. Until recently, HCC has been diagnosed histologically from tumor tissue, putting patients at risk of hemorrhage and tumor seeding along the biopsy tract. Presently, several guidelines allow HCC diagnosis based on imaging modalities such as contrast-enhanced CT-scan, MRI, and contrast-enhanced ultrasound (CEUS). Characteristic radiological features such as arterial hypervascularisation and wash out in the portal and late phase are used as diagnosis criteria [2 – 4]; besides CT and MRI, also CEUS can assess those features. Thus, following detection of suspicious nodules by ultrasound, CEUS is widely available and can further discriminate between benignancy and malignancy and even potentially differentiate between HCC and metastases [5]. Currently, HCC surveillance usually encompasses ultrasound, and only in some guidelines additional determination of serum alpha-fetoprotein (AFP) levels is included. In the recent German S3 level guideline, patients at risk should receive ultrasound surveillance and facultative AFP level determination every 6 months [6]. Even when AFP levels above 400 ng/mL have been considered to be diagnostic of HCC in cirrhosis together with 1 imaging technique [7], such high cutoff values have limited sensitivity in detection of smaller HCC lesions because AFP levels correlate with the extent of tumor burden.

The Japanese guidelines recommend 2 additional tests for HCC surveillance: des-gamma-carboxy prothrombin (DCP), an abnormal prothrombin molecule derived from an acquired defect in the posttranslational carboxylation of the prothrombin precursor in malignant cells, and AFP-L3, an isoform of AFP characterized by the presence of a 1 – 6–linked residue on the AFP carbohydrate side chain [8 – 10]. In the past, AFP-L3 was measurable only in cases of elevated AFP. Since 2011, an automated immunoassay utilizing microchip technology enables the determination of AFP-L3 even at very low levels of total AFP [11].

The triple combination of the above mentioned biomarkers demonstrated a superior detection of HCC with no significant decrease in specificity in Asian patient cohorts [12, 13]. For further optimization, the GALAD score was developed, encompassing patients' gender (G), age (A), AFP-L3 (L), AFP (A), and DCP (D). Using this model, early HCC at stage BCLC 0/A was detected with a sensitivity of 86 % and specificity of 89 % in a British cohort [14].

AFP- L3 and DCP are well-described prognostic markers for HCC. DCP is related to tumor angiogenesis and portal vein invasion, and AFP-L3 indicates pathological characteristics such as the extent of metastases or poor tumor differentiation [13, 15]. Both parameters are good predictors of overall survival (OS). A recently introduced scoring model assesses prognosis using operator independent variables, including liver function tests (Bilirubin [B], Albumin [A]), and the biomarkers AFP-L3 (L), AFP (A), and DCP (D). This BALAD-2 model stratifies patients into 4 different risk groups with significantly different OS [16].

The experience with these novel biomarkers in Western countries is still limited. Therefore, the current study aims to (1) investigate the diagnostic efficacy of the biomarkers AFP, AFP-L3, and DCP for HCC detection, either alone or in combination or as part of the GALAD score, and (2) to assess OS prediction of each marker and as part of the BALAD-2 score.

Material and methods

Patients

In this monocentric study, 285 HCC patients and 402 controls were enrolled from February 2007 to November 2008 and from July 2010 to February 2012 at the University Hospital Essen in Germany. HCC was diagnosed according to the EASL guidelines via histology or by 2 different imaging modalities. The Barcelona Clinic Liver Cancer (BCLC) staging system was used for determination of disease stage. Patients with viral hepatitis, nonalcoholic steatohepatitis (NASH), autoimmune hepatitis (AIH), liver cirrhosis, and other chronic liver diseases served as the control group. Liver cirrhosis was diagnosed by histology or typical findings such as portal hypertension in known chronic liver diseases.

Measurements of serological biomarkers

Common biochemical parameters were determined by standard assays of clinical chemistry (ADVIA Centaur®, Siemens Healthcare, Erlangen, Germany). AFP, AFP-L3, and DCP were measured in the same serum sample using the µTASWakoTM i30 fully automated immunoanalyzer (Wako Chemicals GmbH, Neuss, Germany). Liquid-phase binding assays followed by capillary electrophoresis and fluorescence detection in microchips were used for analysis [11]. Assay sensitivities were 0.3 ng/mL for AFP and 0.1 ng/mL for DCP. The percentage of AFP-L3 was determined in samples where both subfractions (AFP-L1 and AFP-L3) were > 0.3 ng/mL.

Statistical analysis

Statistical analyses were done with R (http://www.r-project.org). The serum parameters were compared with regard to their medians, and standard deviations and p-values were calculated via Mann-Whitney-U tests. Next, we analyzed the performance on subsequent prediction of HCC based on the serum parameters AFP, AFP-L3, and DCP in 2 ways: (1) by analyzing their performance as single parameters and (2) in a logistic regression model. Several studies show that combining parameters into a multivariate model can increase the overall prediction performance [17, 18]. Additionally, we analyzed the prediction capabilities of the GALAD score.

The GALAD score was calculated according to the following equation [14]:

$$\text{GALAD} = -10.08 + 0.09 \times \text{age} + 1.67 \times \text{gender} + 2.34 \times \log10(\text{AFP}) + 0.04 \times \text{AFP-L3} + 1.33 \times \log10(\text{DCP})$$

(Gender is set as 1 for male and 0 for female.)

Statistical analyses on prediction performance were carried out as described elsewhere [19, 20]. We calculated sensitivity and specificity for each serum parameter (AFP, AFP-L3, and DCP) as well as for the GALAD score.

▶ Sensitivity = TP/(TP + FN)
▶ Specificity = TN/(TN + FP)

We used cutoffs of 10 ng/mL and 20 ng/mL for AFP, 10 % for AFP-L3, 7.5 ng/mL for DCP, and −0.63 for the GALAD score. For direct comparison of the resulting models, we calculated the diagnostic odds ratio (DOR) as described by Riemenschneider et al. [21]. The DOR is defined as follows:

▶ DOR = (TP/FP)/(FN/TN)

For the regression models as well as for the GALAD score, we also calculated the receiver operating characteristics (ROC) curve and the area under the curve (AUC). The AUC values were compared with the method of DeLong et al. [22].

The BALAD-2 score was calculated using the following equation [14]:

BALAD-2 = 0.02 × (AFP-2.57) + 0.012 × (AFP-L3 – 14.19) + 0.19 × (ln (DCP)-1.93) + 0.17 × ((Bil µmol/l)0.5)–4.50) − 0.09 × (Alb g/l)-35.11)

AFP was capped at 50 000 units. Both AFP and DCP are modelled as per 1000 units.

Four prognostic groups were generated using the following ranges for BALAD-2: > 0.24 (risk group 4, high), 0.24 to >−0.91 (risk group 3), −0.91 to > −1.74 (risk group 2), and ≤ −1.74 (risk group 1, low).

Kaplan Meier survival curves were designed with GraphPad-Prism® software. P-values were calculated with the Mantel-Cox test.

Results

Patient characteristics

Of 697 patients enrolled, 10 were excluded from analysis because of warfarin medication. Of the remaining individuals, 285 were HCC patients; 402 were controls without HCC. As shown in **Table 1**, the predominant liver disease in the HCC group was viral hepatitis (22.3 % HCV, 15.1 % HBV, and 0.7 % HCV/HBV coinfection). The distribution in the control group was 27.1 % HCV, 23.1 % HBV, and 2.5 % HCV/HBV coinfection. Among the other etiologies, NASH was the most frequent predisposition for HCC (27.0 %) followed by cryptogenic liver diseases (16.1 %), alcohol (14.4 %), and others (3.9 %). One case of primary biliary cholangitis (PBC) and AIH was documented in this group. In the control group, various other diseases accounted for 26.1 %, following chronic viral infection. Cryptogenic diseases (8.2 %), NASH (3.2 %), and alcohol (2.7 %) were less frequent. The majority of HCC patients (86.7 %) suffered from cirrhosis whereas only 19.9 % in the control group were cirrhotic, predominantly in a Child Pugh A disease stage (67.6 % in HCC; 70.0 % in the controls). Males dominated the HCC group (3.52:1) and females the control group (1.15:1).

As shown in **Table 3**, **Fig. 1**, the majority of tumors was diagnosed at an intermediate or advanced stage (BCLC B or C). Only 22.1 % of the tumors were classified as BCLC stage 0 or A, and 15.2 % had tumors ≤ 2 cm in diameter. In only 16.4 % of HCC cases, curative therapies like liver resection or ablation were deemed applicable. About half of the HCC cases were classified as BCLC B (48.6 %) and/or had lesions of more than 5 cm in diameter (46.4 %).

Biomarker levels and distribution pattern

Median serum marker levels of AFP, AFP-L3, DCP and the resulting GALAD score were significantly higher in HCC patients compared to controls (AFP: 39.35 ± 12 329.26 vs. 2.7 ± 115.92; p = < 0.0001; AFP-L3: 16.15 ± 21.29 vs. 0.1 ± 3.22; p < 0.0001;

characteristics	HCC (n = 285)	control group (n = 402)	p-value
gender f/m (ratio)	63/222 (1:3.52)	215/187 (1.15:1)	< 0.0001
median age at blood drawn (± SD)	66.8 (± 10.8)	48.4 (± 14.7)	< 0.0001
etiology n (%)	HCV 63 (22.3 %)	HCV 109 (27.1 %)	p = 0.1528
	HBV 43 (15.1 %)	HBV 93 (23.1 %)	p = 0.0113
	HBV/HCV 2 (0.7 %)	HBV/HCV 10 (2.5 %)	p = 0.1361
	Alcohol 41 (14.4 %)	Alcohol 11 (2.7 %)	p < 0.0001
	NASH 77 (27.0 %)	NASH 13 (3.2 %)	p < 0.0001
	PBC 1 (0.3 %)	PBC 12 (3.0 %)	p = 0.0191
	autoimmune 1 (0.3 %)	Autoimmune 16 (4.0 %)	p = 0.0019
	others 11 (3.9 %)	Others 105 (26.1 %)	p < 0.0001
	cryptogenic 46 (16.1 %)	Cryptogenic 33 (8.2 %)	p = 0.0016
cirrhosis %	86.7 %	19.9 %	p < 0.0001
child pugh stages n (%)	A 167 (67.6 %)	A 56 (70.0 %)	p = 0.7827
	B 64 (25.9 %)	B 15 (18.8 %)	p = 0.2301
	C 16 (6.5 %)	C 9 (11.3 %)	p = 0.2234
	n = 247	n = 80	

Table 1 Patient characteristics of HCC and control group, including gender, median age at blood drawn, etiology, underlying cirrhosis, and Child Pugh score.

biochemical parameters	HCC (n = 285)	control group (n = 402)	p-value
GGT (U/L)	186.5 ± 208.24	45 ± 91.02	< 0.0001
AST (U/L)	63 ± 56.1	35 ± 33.45	< 0.0001
ALT (U/L)	47.5 ± 33.77	39 ± 41.5	0.0059
bilirubin (µmol/L)	15.4 ± 15.5	10.3 ± 9.38	< 0.0001
albumine (g/dL)	3.8 ± 0.43	4.2 ± 0.37	< 0.0001
creatinine (µmol/L)	92.8 ± 28.36	87.5 ± 19.08	0.0999
AFP (ng/mL)	39.35 ± 12 329.26	2.7 ± 115.92	< 0.0001
AFP-L3 (%)	16.15 ± 21.29	0.1 ± 3.22	< 0.0001
DCP (ng/mL)	13.82 ± 1769.55	0.34 ± 43.07	< 0.0001
GALAD	3.69 ± 3.93	−4.17 ± 1.76	< 0.0001

Table 2 Biochemical parameters and biomarker levels of HCC and control patients. Values are expressed as medians and range. Mann-Whitney test was used to calculate significance.

Table 3 Tumor characteristics. Assignment according to the Barcelona Clinic Liver Cancer (BCLC) staging system, number of lesions, tumor size, option for curative treatment. Curative therapies include liver resection and radiofrequencyablation; non-curative therapies encompass: TACE, SIRT, sorafenib, best supportive care.

characteristics	HCC (n = 285)
tumor stages (BCLC n/%) n = 276	0: 2 (0.7 %) A: 59 (21.4 %) B: 134 (48.6 %) C: 53 (19.2 %) D: 28 (10.1 %)
tumor number (n/%) n = 275	solitary: 92 (33.5 %) multiple: 183 (66.5 %)
tumor size (n/%) n = 250	≤ 2 cm: 38 (15.2 %) > 2 to ≤ 3 cm: 41 (16.4 %) > 3 to ≤ 5 cm: 55 (22.0 %) > 5 cm: 116 (46.4 %)
therapy curative/non-curative (n/%) n = 201	curative: 33 (16.4 %) non-curative: 168 (83.6 %)

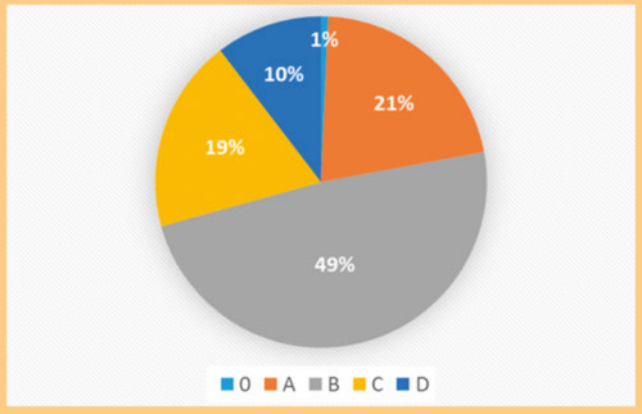


Fig. 1 Pie chart distribution of BCLC stages. Within the HCC cohort (n = 285) in percentages.

DCP: 13.82 ± 1769.55 vs. 0.34 ± 43.07; p = < 0.0001; GALAD: 3.69 ± 3.93 vs. −4.17 ± 1.76; p < 0.0001; **Table 2**, **Fig. 2a–d**).

Fig. 3A exhibits the total number of patients with HCC with increased levels of total AFP, AFP-L3, and DCP. About one-third (32.6 %) of the cohort was positive for all markers. Between 6.7 % and 11.9 % of the patients showed increased levels either for only 1 or for 2 out of the 3 biomarkers. **Fig. 3B** shows the corresponding pattern of control patients (n = 402). None of them had increased levels of AFP and DCP exclusively, and only 1 patient was positive for all 3 markers. Between 1.2 % and 4.2 % of cases were positive for either 1 or a combination of 2 markers.

Sensitivity and specificity in total cohort and early HCC

In the entire cohort, total AFP had a sensitivity of 58.2 % and a specificity of 94.0 % for detection of HCC at the commonly used cutoff of 20 ng/mL. By reducing the cutoff to 10 ng/mL, the sensitivity increased to 68.8 % whereas the specificity decreased to 88.1 %. Employing the cutoffs used in Europe for AFP-L3 (10 %) and DCP (7.5 ng/mL), those markers had comparable sensitivities (64.2 % and 57.2 % respectively) and specificities (91.5 % and 95.0 % respectively). When the markers were combined, sensitivities increased sequentially and specificities decreased slightly. The highest sensitivity of 89.1 % was observed for the combination of 3 markers at a cutoff of 10 ng/mL for AFP. However, in this combination, the specificity was reduced to 80.6 %. In contrast, utilization of the GALAD score resulted in a superior specificity of 93.3 % and sensitivity of 85.6 % (**Fig. 4A**).

When only (very) early stage HCC cases were examined (BCLC stages 0 and A, n = 61) sensitivities for the single markers were reduced to 40.0 % (AFP for 20 ng/mL), 53.3 % (AFP for 10 ng/mL), 31.7 % (DCP), and 48.3 % (AFP-L3). Combination of markers resulted in a sequential increase in sensitivity up to 76.7 % for the triple marker approach and 68.3 % for GALAD (**Fig. 4B**). The AUROCs for all marker combinations and GALAD are displayed in **Fig. 5**. GALAD provided the highest overall AUROC with 0.9242 (95 % CI, 0.8925 – 0.9559) which was superior to any other combinations and differed significantly (p < 0.0001).

Detection rates in subgroups

Patients were classified by tumor stages according to BCLC (0/A, B, C, and D), tumor size (≤ 2, > 2 to ≤ 3, > 3 to ≤ 5, and > 5 cm), low AFP (< 20 ng/mL), and low AFP associated with BCLC 0/A and tumor sizes of ≤ 2 cm. Sensitivities by these characteristics are shown for the markers alone, in any combination and GALAD (**Table 4**). Sensitivities of AFP in BCLC stages 0 and A were 54.1 % using the cutoff of 10 ng/mL and 41.0 % using 20 ng/mL as cutoff. AFP-L3 had a higher sensitivity of 47.5 % with 31.1 % sensitivity for DCP. In the various combinations, higher sensitivities were observed. In the triple marker approach, the highest sensitivity was calculated using the cutoff of 10 ng/mL for AFP (77.0 %).

By analyzing the groups classified by tumor sizes, a similar trend was observed: using 1 single marker, AFP at a cutoff 10 ng/mL had the highest sensitivity (71.1 %) for the detection of small tumors ≤ 2 cm followed by AFP-L3 (63.2 %), AFP at a cutoff 20 ng/mL (60.5 %), and DCP (36.8 %).

Focusing on patients with low AFP (< 20 ng/mL), sensitivity of AFP using the cutoff at 10 ng/mL was 25.6 %, AFP-L3 47.0 %, and DCP 42.7 %. In the combinations, all 3 markers together exhibited the highest sensitivity of 73.5 % and GALAD 67.5 %.

Performance of the markers in viral and non-viral etiology

The total cohort was separated into 2 groups, characterized by the etiology. The group with viral liver disease consisted of 108 HCC and 212 non-HCC patients (the proportions of HCV, HBV, and HCV/HBV coinfected patients are listed in **Table 1**). In the group of patients with non-viral liver disease, 177 HCC and 190 non-HCC patients were compared. Specificities for the markers alone using commonly employed cutoff levels were all above 90 % in both groups (AFP: 90.1 %; AFP-L3: 90.6 %; DCP 98.1 % in the viral group; AFP: 98.4 %; AFP-L3: 92.6 %; DCP 91.4 % in the non-viral group). Using the lower cutoff of 10 ng/mL, AFP showed a much lower sensitivity of 81.6 % in the viral group. The respective sensitivities ranged from 44.4 % for DCP in the viral to 69.5 % for AFP-L3 in the non-viral etiology group. In both groups, the sensitivities increased sequentially by combining markers with highest values using the triple marker approach and 10 ng/mL as cutoff for AFP (84.3 % for the viral and 92.1 % for the non-viral cohort), however with decreased specificities (77.3 % and 84.2 % respectively). For GALAD, slightly lower sensitivities were calculated in both groups compared to the triple marker approach (79.6 % in the viral; 89.3 % in the non-viral group). The specificity for GALAD was higher than 90 % in both groups. Accordingly, the DOR was highest for GALAD (58.5 in the viral; 87.5 in the non-viral group) **Table 5**.

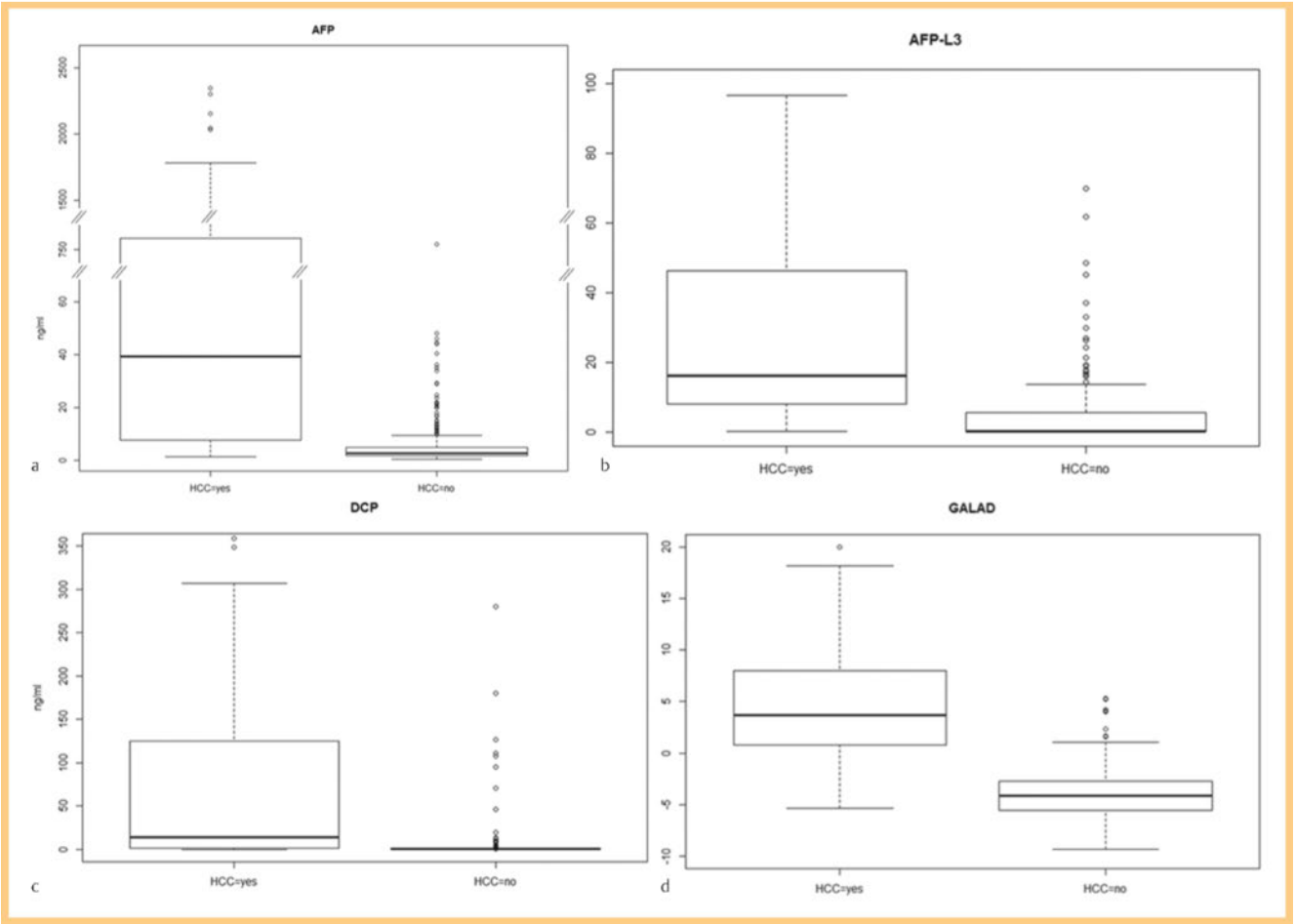


Fig. 2 Tumor marker levels in the HCC and non-HCC cohort. The median is expressed as central bar; **a**: AFP (ng/mL); **b**: AFP-L3 (%); **c**: DCP (ng/mL); **d**: GALAD (calculated values). Mann-Whitney test was used to calculate significance.

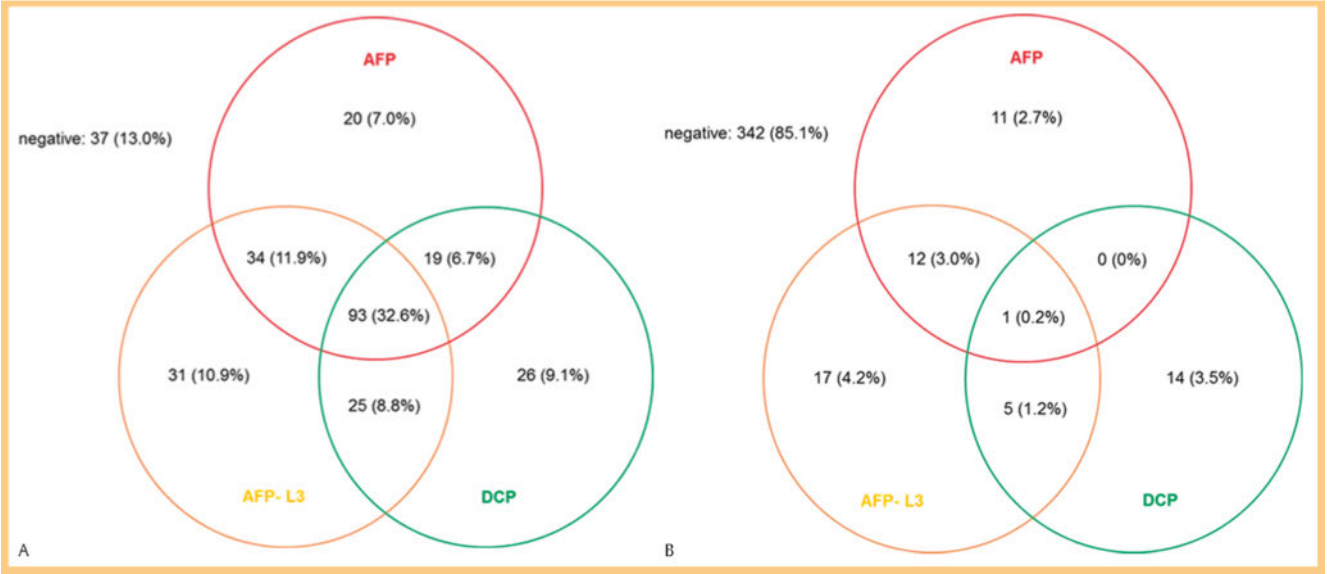


Fig. 3 Pattern of increased markers. Marker positivity according to the following cutoffs are shown: AFP ≥ 20 ng/mL, AFP-L3 ≥ 10 %, DCP ≥ 7.5 ng/mL. Circle overlap means that 2 or 3 markers are positive. A: 285 HCC patients; B: 402 non-HCC patients.

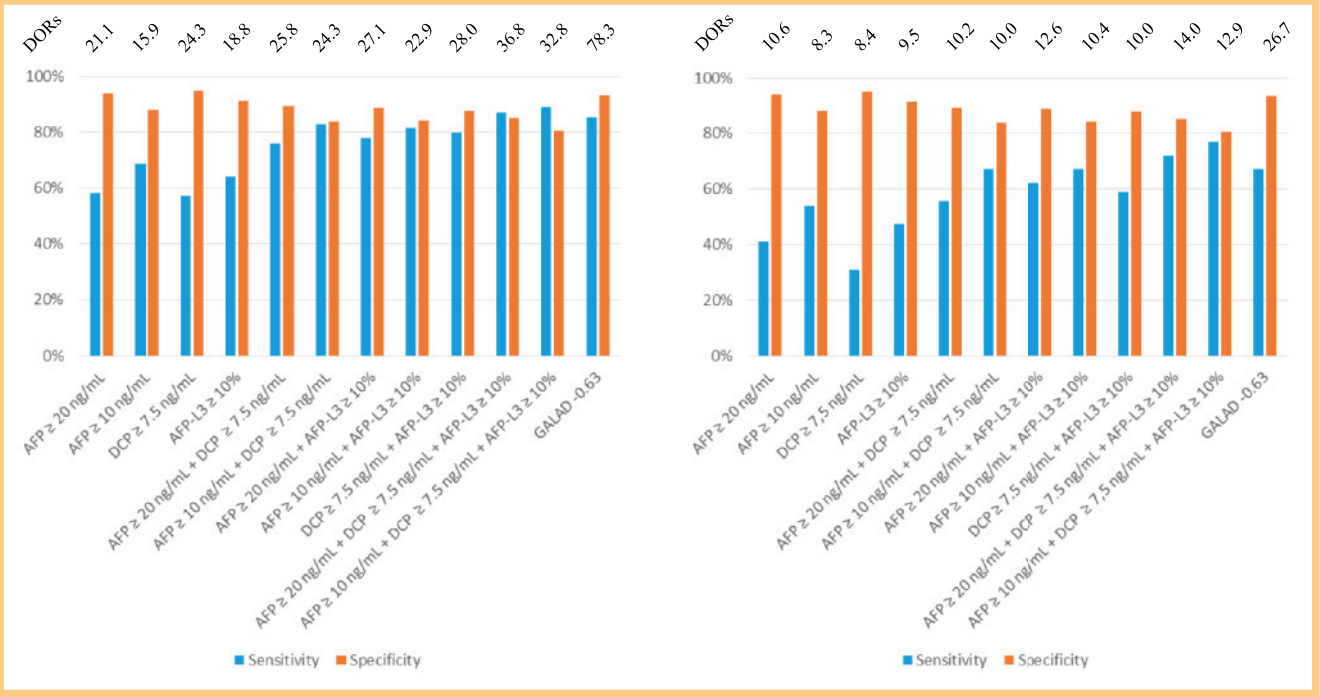


Fig. 4 Sensitivity and specificity for single markers and various combinations. Diagnostic odds ratio (DOR) additionally included for each marker combination. **A** for all 285 HCC patients vs. 402 controls and **B** 61 BCLC 0/A HCC patients vs. 402 non-HCC patients.

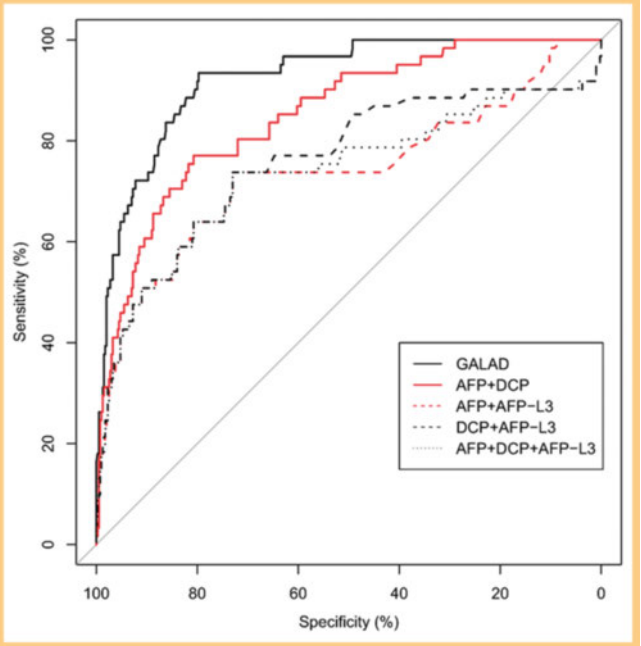


Fig. 5 ROC curves comparing the overall performance of the GALAD model with all marker combinations. The AUC values (95 % CI) for different biomarker combinations and GALAD are as follows (the 95 % confidence intervals are shown in parentheses): AFP+DCP: 0.852 [0.8008, 0.9032]; AFP+AFP-L3: 0.7367 [0.6551, 0.8183]; DCP+AFP-L3: 0.7586 [0.6798, 0.8374]; AFP+DCP +AFP-L3: 0.7361 [0.6518, 0.8204]; GALAD: 0.9242 [0.8925, 0.9559]. There was a significant difference between GALAD and all other marker combinations ($p < 0.001$).

Relationship between the markers and OS

The significance of AFP, AFP-L3, and DCP in survival prediction of HCC patients is shown in **Fig. 6A–C**. Statistical significance was observed between the patient groups with elevated AFP (≥ 20 ng/mL), AFP-L3 (≥ 10 %), and DCP (≥ 7.5 ng/mL) and markers within normal range ($p < 0.0001$). Applying the BALAD-2 model to all patients resulted in 4 well-separated prognostic groups (**Fig. 6 D**). The difference between risk group 2 and 3 reached significance ($p < 0.0001$) whereas the difference between risk group 1 and 2 ($p = 0.0007$) and 3 and 4 ($p = 0.1$) did not. The median survival of the analyzed groups is listed in **Fig. 6 E**.

Discussion

In this study, we analyzed first the performance for the detection of HCC of AFP, AFP-L3, and DCP alone, in various combinations and within the GALAD model in a large German cohort of patients with chronic liver disease. We found that each of the 3 markers performed similar with sensitivities of about 60 %, specificities higher than 90 %, and comparable DORs in the total cohort at commonly used cutoff levels. For the detection of early HCC, sensitivities were less than 50 % for any single marker. Our results are in line with European experiences that the majority of early stage tumors are AFP negative, making this marker inadequate for early tumor recognition [23]. It has been proposed to lower the AFP cutoff to 10.9 ng/mL; however, slight AFP elevations occur frequently in chronic liver disease, resulting in many false positives when using lower cutoffs [24, 25]. Due to the biologic heterogeneity of HCCs, complementary markers closing the diagnostic gap beyond AFP are urgently required.

Previous studies demonstrated AFP-L3 and DCP to be independent but complementing markers in the diagnosis of HCC [26, 27]; our own study was able to confirm those findings.

Table 4 Sensitivities in subgroups. Sensitivities of AFP in cutoffs of 10 and 20 ng/mL, AFP-L3, and DCP were determined either alone or in combination or as part of GALAD score in the scenario of different BCLC stages, tumor sizes and in patients with low AFP and early stage HCC/small tumor size.

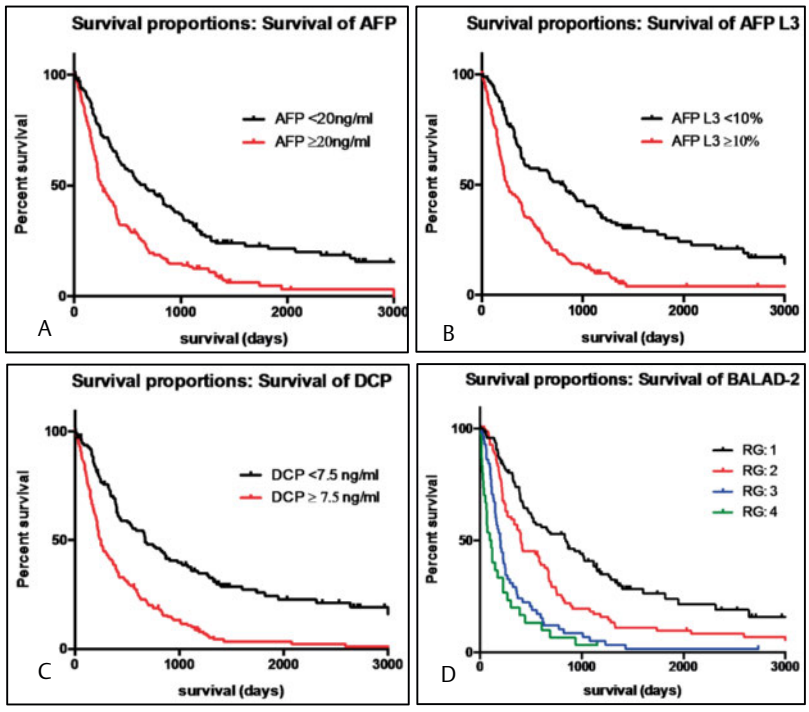
	AFP	AFP	AFP-L3	DCP	AFP + DCP	AFP + AFP-L3	AFP + AFP-L3	AFP-L3 + DCP	all markers	all markers	GALAD
cutoffs	20 ng/mL	10 ng/mL	10 %	7.5 ng/mL	AFP 20 ng/mL, 7.5 ng/mL	AFP 10 ng/mL	AFP 20 ng/mL, 10 %	AFP 10 ng/mL, 10 %	10 %, 7.5 ng/mL	20 ng/mL, 10 %, 7.5 ng/mL	10 ng/mL, 10 %, 7.5 ng/mL	−0.63
tumor stage, n = 276												
BCLC 0/A, n = 61; 22.1 %	41.0 %	54.1 %	47.5 %	31.1 %	55.7 %	67.2 %	62.3 %	67.2 %	59.0 %	72.1 %	77.0 %	67.2 %
BCLC B, n = 134; 48.6 %	60.4 %	70.9 %	64.9 %	58.2 %	77.6 %	84.3 %	81.3 %	84.3 %	82.1 %	88.8 %	90.3 %	89.5 %
BCLC C, n = 53; 19.2 %	71.7 %	81.1 %	79.2 %	69.8 %	88.7 %	90.6 %	86.8 %	90.6 %	92.5 %	96.2 %	96.2 %	96.2 %
BCLC D, n = 28; 10.1 %	67.9 %	71.4 %	71.4 %	89.3 %	96.4 %	96.4 %	85.7 %	85.7 %	92.9 %	96.5 %	96.5 %	92.9 %
tumor size, n = 250												
≤ 2 cm, n = 38; 15.2 %	60.5 %	71.1 %	63.2 %	36.8 %	68.4 %	79.0 %	81.6 %	84.2 %	73.7 %	81.6 %	84.2 %	71.1 %
> 2 and ≤ 3 cm, n = 41; 16.4 %	58.5 %	65.9 %	58.5 %	43.9 %	65.9 %	73.2 %	80.5 %	80.5 %	70.7 %	85.4 %	87.8 %	75.6 %
> 3 and ≤ 5 cm, n = 55; 22.2 %	54.5 %	69.1 %	63.6 %	54.5 %	69.1 %	78.2 %	74.5 %	76.4 %	78.2 %	83.6 %	83.6 %	85.5 %
> 5 cm, n = 116; 46.4 %	56.9 %	68.1 %	64.7 %	66.4 %	84.5 %	88.8 %	76.7 %	81.9 %	81.9 %	88.8 %	91.4 %	94.0 %
low AFP and early stage/small tumor size												
AFP < 20 ng/mL, n = 123	N/A	25.6 %	47.0 %	42.7 %	NA	59.0 %	NA	55.6 %	68.4 %	NA	73.5 %	67.5 %
AFP < 20 ng/mL, BCLC 0/A, n = 37	N/A	22.2 %	36.1 %	25.0 %	NA	41.7 %	NA	41.7 %	52.8 %	NA	58.3 %	50.0 %
AFP < 20 ng/mL, tumor ≤ 2 cm, n = 16	N/A	26.7 %	46.7 %	13.3 %	NA	40.0 %	NA	53.3 %	46.7 %	NA	53.3	33.3 %

Most patients in our HCC cohort were either positive for only 1, a combination of 2, or all 3 markers. In consequence, the sensitivity increased gradually by adding the new markers AFP-L3 and DCP to the commonly used AFP. In the past, the addition of AFP-L3 to AFP only marginally increased sensitivity, since the fucosylated subfraction could only be measured in moderately elevated AFP levels. Since the highly sensitive measurement of AFP-L3 by microchips is available, this marker gained significant additional clinical relevance. In the first trial, AFP-L3 detected 43.5 % of all HCC cases with tumors of ≤ 2 cm compared to 22.9 % using the older assay [28]. In our study, this marker was able to detect 64.1 % of tumors ≤ 2 cm. The detection rate did not differ significantly from those of larger lesions, indicating the particular potential of AFP-L3 to detect the development of small tumors. It has been shown previously that AFP-L3 can be elevated before a lesion is detectable by cross-sectional imaging. In a Japanese cohort of 104 patients who developed HCC during surveillance, AFP-L3 was significantly elevated 1 year prior to the diagnosis based on contrast-enhanced imaging, while AFP and DCP remained within normal range [29]. For DCP we observed a gradually increasing detection rate of HCC from 36.8 % for tumors ≤ 2 cm to 66.4 % for tumors > 5 cm. This is in accordance with previous investigations that found DCP being dependent on tumor size and to be less sensitive than AFP for tumors ≤ 3 cm [30]. The production of DCP is affected by various factors. An excessive elevation in serum may be related to more aggressive tumor biology (i. e., vascular invasion and intrahepatic metastases, which could explain the higher sensitivity in detection of large and advanced stage HCCs) [31].

The combination assay of DCP and AFP-L3 resulted in a detection rate of 68.4 % of HCC that was AFP-negative (< 20 ng/mL). Even in (very) early stage (BCLC 0 or A) with tumors of ≤ 2 cm, more than half of HCC patients were recognized, confirming the benefit of an additional utilization of DCP and AFP-L3 for the diagnosis of early stage HCC even in AFP-negative tumors [32].

The vast majority of studies on HCC biomarkers has been conducted in Asian countries, where patients differ from European populations in terms of demography and underlying liver disease. Most notably, viral hepatitis is by far the leading cause of chronic liver disease and associated HCC in this area, whereas in Western countries other etiologic factors like alcoholic and non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NAFLD/NASH) play a pivotal role in hepatocarcinogenesis [1]. We therefore investigated the diagnostic performance of the markers separately in the scenario of viral and non-viral etiology. This revealed some remarkable differences: AFP was proven to be more specific in the non-viral versus viral background (98.4 % vs. 90.1 %, cutoff 20 ng/mL) with a similar sensitivity (59.9 % vs. 55.6 %), resulting in a much higher DOR (69.8 vs. 10.9). Hepatic parenchymal inflammation (e. g., in the context of viral hepatitis) can cause an increase in AFP with false-positive screening results for HCC [33]. In contrast, AFP-L3 did not show a comparable deviation in specificities but improved sensitivity in patients with non-viral background of liver disease (69.5 % vs. 55.6 %).

In contrast, DCP showed superior specificity (98.1 %) in the viral cohort compared to the non-viral group (91.6 %). On the other hand, sensitivity was much lower in the viral cohort (44.4 %) compared to the non-viral group (65.0 %), suggesting that DCP is elevated in metabolic disorders. Elevation of DCP levels in the absence of HCC are common in several scenarios such as vitamin K deficiency, acute hepatic failure, malnutrition, alcoholic liver diseases, or antibiotic treatment [34]. Conflicting results on DCP in



E

Parameter	Group	n d = deaths c = censored	median surivial days	p
AFP	< 20 ng/mL	d: 93 c: 23	669	< 0.0001
	≥ 20 ng/mL	d: 144 c: 17	253	
AFP-L3	< 10%	d: 77 c: 23	819	< 0.0001
	≥ 10%	d: 160 c: 17	251	
DCP	< 7.5 ng/mL	d: 86 c: 31	674	< 0.0001
	≥ 7.5 ng/mL	d: 151 c: 9	250	
BALAD-2	Risk group1	d: 72 c: 27	843	RG 1 vs. RG 2 p < 0.0007
	Risk group2	d: 79 c: 8	403	RG 2 vs. RG 3 p < 0.0001
	Risk group3	d: 57 c: 2	202	RG 3 vs. RG 4 p = 0.1
	Risk group4	d: 29 c: 3	113	

Kaplan Meier survival rates according to
A: AFP
B: AFP-L3
C: DCP
D: BALAD-2 risk groups
E: Group sizes, median survival and p-values

Fig. 6 Kaplan Meier survival rates. According to **A**: AFP; **B**: AFP-L3; **C**: DCP; **D**: BALAD-2-risk groups; **E**: table of median survival time.

Table 5 Sensitivities, specificities, and DORs for viral and non-viral etiologies. Determined for AFP in cutoffs of 10 and 20 ng/mL, AFP-L3, and DCP either alone or in combination or as part of GALAD.

		viral etiology			non-viral etiology		
biomarker/combination/model	**cutoff value**	**sensitivity**	**specificity**	**DOR**	**sensitivity**	**specificity**	**DOR**
AFP	10 ng/mL	69.4 %	81.6 %	9.7	68.4 %	95.3 %	39.0
AFP	20 ng/mL	55.6 %	90.1 %	10.9	59.9 %	98.4 %	69.8
AFP-L3	10 %	55.6 %	90.6 %	11.4	69.5 %	92.6 %	26.6
DCP	7.5 ng/mL	44.4 %	98.1 %	33.6	65.0 %	91.6 %	19.0
AFP + DCP	10 ng/mL, 7.5 ng/mL	79.6 %	80.2 %	15.0	84.7 %	87.9 %	37.8
AFP + DCP	20 ng/mL, 7.5 ng/mL	68.5 %	88.2 %	15.5	80.8 %	90.5 %	37.5
AFP + AFP-L3	10 ng/mL, 10 %	78.7 %	78.8 %	13.1	83.1 %	90.5 %	43.5
AFP + AFP-L3	20 ng/mL, 10 %	74.1 %	85.4 %	15.9	80.2 %	92.6 %	46.9
AFP-L3 + DCP	10 %, 7.5 ng/mL	68.5 %	89.2 %	17.0	87.0 %	86.3 %	39.5
AFP + AFP-L3 + DCP	10 ng/mL, 10 %, 7.5 ng/mL	84.3 %	77.3 %	17.2	92.1 %	84.2 %	56.8
AFP + AFP-L3 + DCP	20 ng/mL, 10 %, 7.5 ng/mL	80.6 %	84.0 %	20.5	91.0 %	86.3 %	58.2
GALAD	−0.63	79.6 %	94.3 %	58.5	89.3 %	92.1 %	87.5

comparison to AFP in Western countries [35] may therefore be explained by determinants such as tumor size and composition of the cohorts with regard to underlying etiology. Regardless of etiology, combinations of biomarkers resulted in improved sensitivities coupled with minor decreases in specificity demonstrating the benefit of such synergisms like in the entire cohort.

The GALAD model enters new paths by using the following approach: by logistic regression analyses, independent variables associated with HCC were determined to establish a diagnostic algorithm. This formula calculates the measured absolute values of AFP, AFP-L3, and DCP instead of defining cutoff levels with the limitations discussed above. Gender and age information is also included, since older age and male sex are well-known risk factors for HCC [33]. Using the cutoff level −0.63, GALAD showed a high sensitivity of 85.6 % at an excellent specificity of 93.3 % in the entire cohort. For BCLC 0/A stages, GALAD performed significantly better than any marker combination in logistic regression analyses with an AUROC value of 0.924. In a recent global validation including datasets from 6834 patients from Germany, Hong Kong, Japan and the UK [36], AUROC values ranged between 0.85 and 0.95 for small and unifocal tumor lesions. Neither etiology nor ethnicity of patients influenced the diagnostic performance. Correspondingly, we observed superior specificities for both viral (94.3 %) and non-viral (92.1 %) etiology in our cohort, whereas the sensitivity was lower in the viral group than in the non-viral group (79.6 % vs. 89.3 %). In an Italian study with predominant viral etiologies, GALAD performed superior with an AUROC value of 0.976 [37].

The goal of HCC surveillance programs is to detect tumors at an early stage, when curation is still possible. Western guidelines re-

commend the use of ultrasound alone. The widely available marker AFP is considered being inadequate for surveillance, because meta-analyses demonstrated that AFP combined with ultrasound identified only 6 – 8 % additional HCC cases. The same investigation revealed that ultrasound suffers from a limited sensitivity of 63 % for early stage HCC detection [38]. The quality of ultrasound varies with ultrasonographic apparatus used and the patient's condition (obesity) but mainly with the skills and specific education of the investigator. Retrospective analyses on incidentally diagnosed HCC after transplantation demonstrated that ultrasound detected only 21 % [39] and 35 % [40] of cases with lesions ≤ 2 cm. Thus, it can be expected that sensitivity of ultrasound is much lower for early HCC in real-world setting. In German cohorts, typically about 20 % of HCC cases are diagnosed in BCLC stages 0/A with median OS rates of about 15 months [41, 42]. In contrast, in Japan more than 60 % of patients are diagnosed at early stages, resulting in superior median OS rates of more than 3 years [43]. There, the 3 biomarkers are used routinely to enhance the detection rate of ultrasound surveillance. In our cohort we clearly demonstrate that the combination of these markers and in particular GALAD improved the detection of early BCLC stage and small tumors even in AFP-negative tumors. The serological approach of early HCC detection is very attractive since it is operator independent, all biochemical parameters are available on 1 analytical platform, and the GALAD algorithm can easily be implemented in laboratory information management systems.

Secondly, we analyzed the prognostic characteristics of the markers regarding the prediction of OS. As reported frequently from Asia, all 3 markers could significantly discriminate groups in terms of survival [44]. More recent studies indicated that elevated pretreatment tumor markers or their total number do not always predict survival, when curative treatments such as hepatectomy could be applied [27]. In early stage and frequently in AFP negative tumors, AFP-L3 predicted recurrence and survival much better than AFP [45]. In our cohort, curative treatments were only applied to 16.4 % of the patients, which could explain the capability of AFP to predict OS as well. The BALAD-2 model was developed to include determinants of tumor biology (the 3 markers) and the severity of liver disease (bilirubin and albumin), which both influence prognosis. In contrast to other prognostic approaches, it is entirely objective as it does not include subjective criteria like "being symptomatic" (BCLC) or "presence/absence" of ascites (Child Pugh) [16]. Our results confirmed the utility of this model; 4 distinct prognostic groups could be determined. The lowest risk group showed a longer and the highest risk group showed a shorter median survival than any group generated by using only 1 single marker. Interestingly, the recent global validation demonstrated in European and Asian HCC patients that 4 distinct prognostic groups are calculated irrespectively of the treatment applied [36].

The major limitations of our study are the differences in patient age, etiology, and proportion of patients with cirrhosis between the 2 groups. However, the HCC patients were enrolled around the time of first diagnosis, and the chronic liver disease patients were all candidates for HCC surveillance. Therefore, the investigated cohort should reflect the real-life situation in a European treatment center. The prevalence of HCC in the context of viral hepatitis is decreasing due to vaccination programs for hepatitis B and new highly effective interferon-free treatment strategies against HCV. In contrast, the incidence of HCC in patients suffering from metabolic diseases is dramatically increasing. This epidemiologic shift will prospectively culminate in overall increasing HCC prevalence in Western countries. Recent literature provides evidence that patients suffering from chronic hepatitis B but particularly NASH in the absence of cirrhosis are at dramatically increased risk to develop HCC [46]. Therefore, preferably multicenter studies on the usefulness of the HCC markers as well as GALAD and BALAD-2 in NAFLD and NASH are urgently needed.

In conclusion, we demonstrate the effectiveness of the biomarkers AFP, AFP-L3, and DCP in combination and in particular GALAD for detection of early stage HCC in a German cohort. Increased levels of GALAD reliably predict HCC and should trigger dynamic imaging to facilitate diagnosis of HCC at curative stages. BALAD-2 can be applied after diagnosis to predict OS.

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