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The diagnostic performance of PIVKA-II in metabolic and viral hepatocellular carcinoma: a pilot study

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Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is a primary liver tumor derived from metabolic or viral chronic hepatitis, with few treatment options in advanced cases. New biomarkers that allow improving diagnosis and staging are widely desired. Here, we aim to evaluate the performance of Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA-II) in combination with α-fetoprotein (AFP), in the diagnosis of HCC in patients with metabolic or viral hepatitis.

PATIENTS AND METHODS: We enrolled 60 HCC patients (20 metabolic and 40 viral) and 20 healthy subjects (HS) as negative controls. PIVKA-II, AFP, Matrix metalloproteinase-9 (MMP-9) and Fibroblast growth factor (FGF) serum levels were assessed by immunoassays.

RESULTS: AFP and PIVKA-II levels were obviously higher in patients than in HS. AFP displayed a better diagnostic performance than PIVKA-II for viral HCC while PIVKA-II was better for metabolic HCC. The combination of the two biomarkers did not improve the discriminating ability.

CONCLUSIONS: PIVKA-II may be considered an independent predictor of macrovascular invasion from HCC cells and it can be used to better stratify HCC patients and should be evaluated in prospective studies for early detection of advanced HCC in metabolic subjects.

Key Words:
Viral Hepatocellular carcinoma, Metabolic Hepatocellular carcinoma, α-fetoprotein, PIVKA-II.

Abbreviations

Hepatocellular carcinoma (HCC), Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA-II), European Association for the Study of the Liver (EASL), α-fetoprotein (AFP), non-alcoholic fatty liver disease (NAFLD), Matrix metalloproteinase-9 (MMP-9) Fibroblast growth factor (FGF), Hepatitis C Virus (HCV), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP) and Gamma-Glutamyl Transferase (GGT).

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and represents the third most common cause of cancer-related death in the world[1]. Currently, the diagnosis of HCC requires noninvasive radiological dynamic contrast-enhanced imaging that may be followed by invasive biopsy; in parallel, laboratory investigations for the detection and analysis of all serum biomarkers that allow to evaluate liver function[2], according to the current guidelines proposed by the European Association for the Study of the Liver (EASL)[3].

To date, the most widely used and broadly known as biomarker of HCC is the α-fetoprotein (AFP), a glycoprotein produced in early fetal life

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by the liver and by a variety of tumors, which is elevated in only 40-60% of HCC and in only 10-20% of the initial phase of HCC. Unfortunately, lacking a tumor-specific correlation, it is considered inadequate (due to low sensitivity and specificity) for the screening of HCC[4,5] mostly in non-alcoholic fatty liver disease (NAFLD) subjects[6].

Recently, the Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA-II) has been assessed as a new marker for HCC management[7]. It consists in abnormal prothrombin, also known as Des-γ-carboxy prothrombin, induced either by the absence or by the antagonist-II of vitamin K; moreover, it may result from an acquired defect of post-translational carboxylation of prothrombin's precursor[8]. Thanks to its anti-inflammatory properties, vitamin K concurs to regulate blood pressure and prevents hypertension, mainly avoiding calcification of the arterial walls, ameliorating blood circulation and thus preventing cardiovascular disease[9]. A high level of PIVKA-II in at-risk patients has been reported as reliable biological marker of developing HCC in 2 years[10]. PIVKA-II can be tested together with AFP in order to improve the sensitivity and specificity for detection of HCC especially in its initial phase[7,11]. PIVKA-II has been recommended as one of the surveillance biomarker for HCC in at-risk populations and added into the guidelines of the Japanese Society of Hepatology (JSH)[10,12]. It has been shown that the diagnostic sensitivity of AFP, at the early stage of HCC, may increase from 20%, when used alone, up to 55% when considered in combination with PIVKA-I[13]. Moreover, in addition to AFP, PIVKA-II increases the detection rate for HCC in cirrhotic HBV patients under treatment[14].

PIVKA-II did not appear to be superior to AFP in screening for HCC in patients with HCV-related cirrhosis, showing moderate diagnostic accuracy. Nevertheless, the combination of PIVKA-II and AFP at the cut-off values of 36 mAU/ml for PIVKA-II and 12 ng/mL for AFP, may be considered as a screening test for HCC due to its high negative predictive value[11].

Interestingly, in a case-control study on cirrhotic patients undergoing surveillance for HCC, in patients who developed HCC both AFP and PIVKA-II were estimated to increase over time, confirming their potential usefulness during periodic surveillance. On the other hand, among patients that not developed HCC the time-related changes of PIVKA-II were more stable than those of AFP. The different trajectories of PIVKA-II in patients with or without HCC are likely to reflect the mechanism of PIVKA-II production, which, contrarily to AFP, is not affected by liver disease activity[15]. The use of serological biomarkers may represent a valid complement to imaging methods (Ultrasound/MRI/CT) in HCC surveillance since HCC is frequently characterized by a clinically silent onset and by a rather aggressive evolution; for this reason, it's crucial to detect this neoplasm in the early onset phase, when local invasiveness or metastasis are absent or still in the initial phase.

To date, reliable biomarkers able to discriminate metabolic from viral-derived HCC and to identify advanced HCC with macrovascular invasion in patients with metabolic syndrome are still lacking; moreover, there is no evidence of the best methodology to perform active surveillance in subjects with metabolic cirrhosis. In addition, in patients with HCC arising on a metabolic background, two novel candidate biomarkers, Matrix metalloproteinase-9 (MMP-9) and Fibroblast growth factor (FGF), have been investigated. A significant increase in the expression of MMP-9 and FGF-2 have been reported in human and animal samples of HCC[16]. Here, we aimed to analyze serological levels of PIVKA-II in different stages of HCC in patients with metabolic or viral liver disease through the employment of accurate measuring methods[10]. Our purpose was to identify a putative multi-marker panel to predict macrovascular invasion in order to ameliorate clinical patients' management. We compared the diagnostic efficacy of the different tumor biomarkers in diagnosing HCC, analyzing PIVKA-II as an independent biomarker or in combinations with AFP, FGF and MMP-9.

Patients and methods

Patients

We enrolled 60 consecutive patients with metabolic (20) and viral (40) liver disease and newly diagnosis of HCC according to EASL guidelines[3], from the Gastroenterology Unit of Fondazione Policlinico Universitario A. Gemelli – IRCCS, Università Cattolica del Sacro Cuore, in Rome. For all patients, the main medical history was collected from clinical charts. Physical examination, serological testing for Hepatitis C and B Virus (HCV, HBV), laboratory exams were routinely performed.

The following exclusion criteria were applied: 1) history of previous treatment (hepatic resection, liver transplant, trans-arterial chemo-

embolization, radiofrequency, anti-angiogenetic drugs); 2) Child Pugh C; 3) obstructive jaundice; 4) estimated Creatinine Clearance <30 mL/min; 5) diagnosis of second extra-hepatic neoplasia; 6) metastasis. Twenty healthy subjects were recruited in the control group. The following inclusion criteria were applied: age greater than 18 years; no evidence of HCC during the period of enrollment; no anticoagulant therapy; no HCV or HBV infection, no presence of monoclonal component, < 24.9 BMI.

The whole study, approved by the Ethical Committee of Fondazione Policlinico Universitario A. Gemelli-IRCCS with ID: 2078, was conducted according to the Declaration of Helsinki and subsequent amendments. All samples were processed anonymously.

Clinical and Laboratory Assessments

Clinical anamnesis and laboratory parameters were recorded at the time of diagnosis. Laboratory findings included Glucose, Cholesterol, Total bilirubin, Albumin, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP) and Gamma-Glutamyl Transferase (GGT). Patients' sera were collected for quantitative measurement of AFP and PIVKA-II, performed using Lumipulse® G (Fujirebio, Tokyo, Japan), based on Chemiluminescent Enzyme Immunoassay and according to the manufacturer's instructions. Following Clinical and Laboratory Standards Institute (CLSI) guidelines, we tested serum samples from 20 healthy donors to verify adherence to CLSI EP 28A3C; values of PIVKA-II ≥38 mAU/mL, AFP ≥3.5 ng/mL were considered positive[17]. MMP-9 and FGF levels were measured by means of the ELISA kits from R&D Systems (Minneapolis, MN, USA). Vascular invasion, portal trunk and/or main portal branches, diagnosed by MRI with hepato-specific contrast medium.

For statistical analysis as far as the portal invasion is concerned, we divided the patients into two groups according to the occurrence of portal invasion. Based on the number of nodules, we divided the patients in two groups (less or more than 2 nodules).

The metabolic pathogenesis of chronic liver disease complicate by HCC was established by the exclusion viral, alcoholic, autoimmune and genetic pathogenesis. Most of patients displayed diabetes (63% metabolic and 30% viral) and 8% had a BMI over 30.

Statistical Analysis

Statistical analyses were performed by using the software package R (3.5.2 release)[18]. A total of 60 HCC patients affected by metabolic syndrome (20/60) or viral infection (40/60) were included in the analyses. Twenty healthy subjects were added as negative controls. Continuous variables were tested with a visual inspection of QQ-plot and using the Shapiro-Wilk normality test. Significant deviations from normality were detected, therefore, biomarkers were reported as median (Q2) and Inter Quartile Range (IQR). A comparison between two groups was performed with Wilcoxon–Mann–Whitney test for independent samples. For more than 2 groups, the Kruskal-Wallis test followed by post-hoc analysis was adopted. The diagnostic accuracy of selected biomarkers in discriminating between the two groups was assessed by logistic regression and ROC curves. Logistic regression is executed using the function glm from the R package stats to extract probabilities from the fitted models, either with a single biomarker or with the two biomarkers in combination. The classification performance of the different predictor was evaluated using ROC curves together with C-statistics (AUC: area under the curve) with 95% confidence intervals and Decision Curve Analysis (DCA) [18]. ROC analysis was performed with the R package pROC[19]. Correlation between variables was evaluated using Pearson's correlation coefficient with the R package corrplot[20].

For the sake of completeness, all the measured correlations were arranged in a correlation matrix for all the studied groups and were reported in the ***Supplementary materials*** section.

Continuous biomarkers were also tested against the occurrence of selected clinical conditions, including the occurrence of portal vein invasion and the number of nodules (≤2 nodule or >2) by using bootstrap methods. The absolute difference between the mean of two groups were used as a bootstrap variable, a number of B=10000 bootstrap samples were generated for the computation of the selected distributions.

Results

In Figure 1, we report a box-plot analysis of AFP (Figure 1A) and PIVKA-II (Figure 1B) levels in HS and HCC patients. Due to the large data dispersion, a logarithmic scale was adopted in both cases. Data normality was investigated by means of a visual inspection of the QQ-plot and

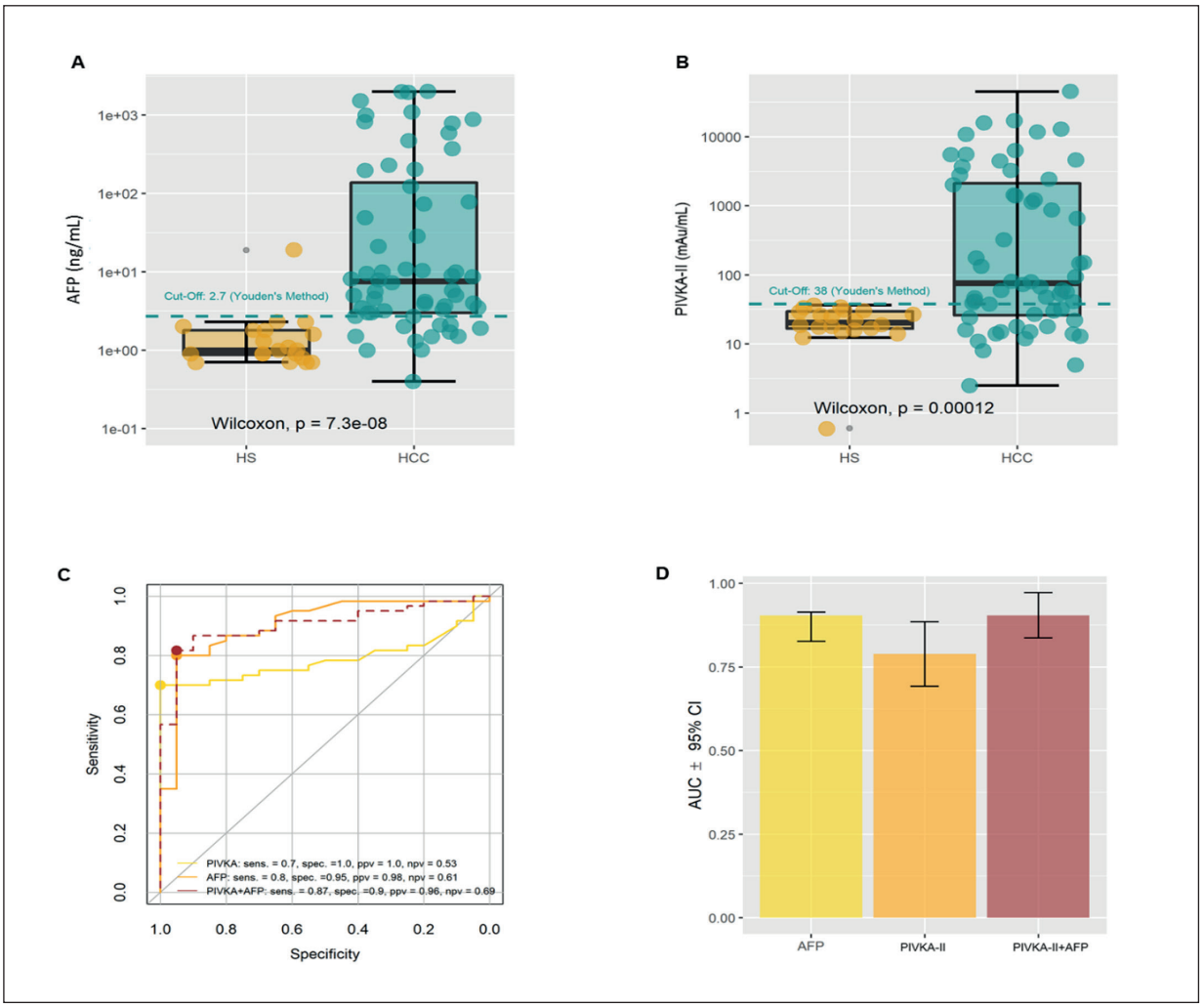

Figure 1. BOX plot analysis of AFP (**A**) and PIVKA-II (**B**) levels in HS (0) and HCC (1). ROC curve analysis for the evaluation of diagnostic performance in distinguishing between HCC and healthy subjects for PIVKA-II, AFP and their combination (**C**) with the corresponding AUC values (**D**).

with the Shapiro-Wilk test (data not shown). Significant deviation from normality was detected, in agreement with the highly skewed data distribution that can be inferred from the box-plot shape, even in logarithmic scale. Non-parametric tests were thus used to compare the two groups. Significantly raised AFP (Wilcoxon–Mann–Whitney, p=7.310^{-8}) and PIVKA-II (p=0.00012) levels were found. The two biomarkers performance in distinguishing HCC patients from controls, alone and in combination, was investigated with a logistic regression followed by a ROC curve analysis (Figure 1C). Regression coefficients are summarized in Table I. A qualitative examination of Figure 1C suggests that AFP has superior classification properties than PIVKA-II and, apparently, the combination of the two markers does not improve diagnostic performance. A quantitative comparison is shown in Figure 1D, where we report the corresponding areas under the curve (AUC) together with 95% confidence intervals (CI). AUCs and CIs are summarized in Table I. As expected, a significantly higher AUC value is observed for AFP (0.90, 95% CI: 0.88-1) compared to PIVKA-II (0.79, 95% CI: 0.69-0.86). The two markers in combination show a slightly, but not significantly, higher value than AFP alone (0.90, 95% CI: 0.84-0.97).

In Figure 2, we compare AFP (panel A) and PIVKA-II (panel B) levels between HS and HCC patients further divided in metabolic (M) and viral HCC (V). The comparison among

Table I. Logistic regression coefficients for the comparison of PIVKA-II, AFP and their combination in distinguishing between different groups.

Groups	Predictors	Parameter: Estimate Std. Error (*p*-value)	AUC (95%)
HS *vs.* HCC	PIVKA-II	Intercept: -0.61 ± 0.54 (0.290) Coeff./PIVKA-II: 0.039 ± 0.019 (0.026)	0.79 (0.69-0.89)
HS *vs.* HCC	AFP	Intercept: -0.32 ± 0.46 (0.489) Coeff./AFP: 0.33 ± 0.14 (0.023)	0.90 (0.83-0.91)
HS *vs.* HCC	AFP+PIVKA-II	Intercept: -1.30±0.70 (0.055)· Coeff./PIVKA-II: 0.037±0.021 (0.078) Coeff./AFP: 0.22±0.14 (0.606)	0.90 (0.84-0.97)
HS *vs.* M	PIVKA-II	Intercept: -6.0 ± 2.4 (0.013)* Coeff./PIVKA-II: 0.16 ± 0.07 (0.029)	0.96 (0.87-1)
HS *vs.* M	AFP	Intercept: -5.71 ± 2.33 (0.156) Coeff./AFP: 0.10 ± 0.09 (0.251)	0.83 (0.69-0.94)
HS *vs.* M	AFP+PIVKA-II	Intercept: -5.7 ± 2.3 (0.014) Coeff./PIVKA-II: -0.22 ± 0.13 (0.099) Coeff./AFP: 0.16 ± 0.07 (0.030)	0.96 (0.87-1)
HS *vs.* V	PIVKA-II	Intercept: -0.58 ± 0.54 (0.288) Coeff./PIVKA-II: 0.032 ± 0.02 (0.078)	0.71 (0.58-0.84)
HS *vs.* V	AFP	Intercept: -1.0 ± 0.5 (0.050) Coeff./AFP: 0.38 ± 0.15 (0.012)	0.94 (0.87-1)
HS *vs.* V	AFP+PIVKA-II	Intercept: -1.25 ± 0.60 (0.036) Coeff./PIVKA-II: 0.32 ± 0.15 (0.035) Coeff./AFP: 0.0068 ± 0.013 (0.60)	0.95 (0.89-1)

PIVKA-II: Protein Induced by Vitamin K Absence or Antagonist-II; AFP: α-fetoprotein; HS: Healthy subjects, M: metabolic HCC; V: viral HCC. In the last column, the AUC values together with 95% confidence intervals (CI) are reported for the corresponding ROC curves.

different groups was performed using Kruskal-Wallis test. In both cases, we can reject the null hypothesis that the mean ranks of the groups are the same. A post-hoc analysis with the Wilcoxon-Mann-Whitney test highlight the presence of statistically significant differences for all the multiple comparisons investigated. A significant increase in AFP (Figure 2A) and PIVKA-II (Figure 2B) levels are observed in both metabolic and viral HCC patients compared to healthy subjects.

A logistic regression followed by a ROC analysis was performed to evaluate the classification ability of the two investigated markers and their combination in distinguishing pathological from healthy subjects for viral HCC and metabolic HCC, separately. Regression coefficients and AUC values for each comparison are summarized in Table I. Qualitative analysis of Figure 2 (panel C and D) suggests that, while AFP has superior diagnostic ability than PIVKA-II for viral HCC subjects, the opposite can be observed for metabolic HCC, where PIVKA-II shows enhanced classification abilities. In both cases, the ROC curve of the combined markers does not seem to improve significantly compared to the most efficient biomarker alone.

A quantitative comparison is shown in Figure 3 (panel A and B), where we reported AUC values with the corresponding confidence intervals. Consistently with results of Figure 2, a higher AUC value is observed for PIVKA-II compared to AFP for metabolic HCC subjects, while the opposite is observed for viral HCC. In Figure 3C, we show the results of the decision curve analysis performed on AFP alone, which represents the gold standard, and its combination with PIVKA-II for discriminating control subjects from HCC (left), metabolic HCC (center) and viral HCC (right) patients. DCA analysis plots the net benefit against the threshold probability and it is becoming an increasingly popular approach to compare different diagnostic methods, considering the clinical utility of the specific method.

We found a statistically significant difference in PIVKA-II serum levels between patients with and without portal vein invasion independently from the etiology of HCC (Figure 4).

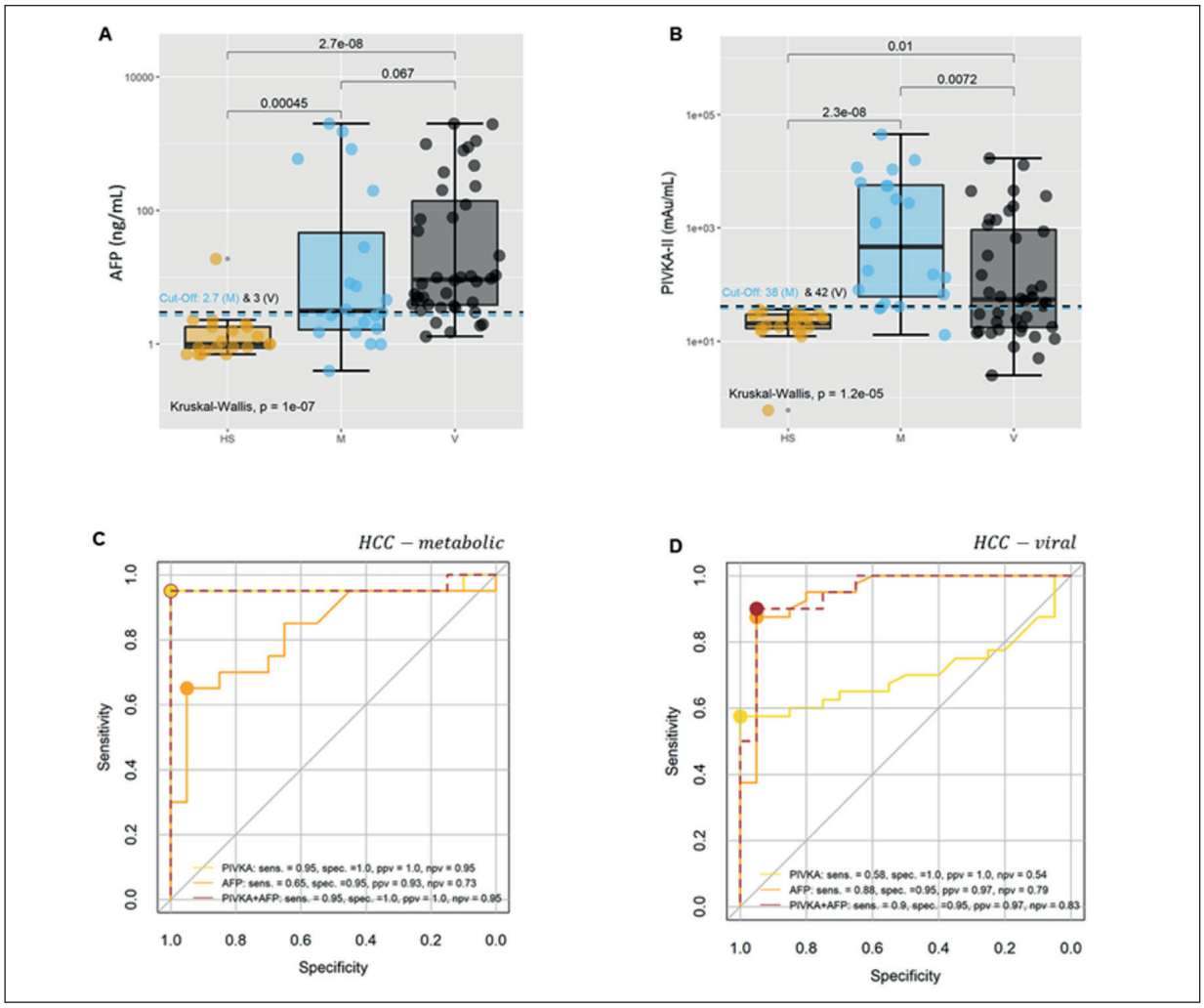

Figure 2. BOX plot analysis of AFP (***A***) and PIVKA- (**B**) levels in HS and HCC, the latter further classified in metabolic HCC (M) and viral HCC (V). ROC curve analysis for the evaluation of diagnostic performance in distinguishing between metabolic HCC (2C) and viral HCC (2D) from healthy subjects for PIVKA-II, AFP and their combination.

Discussion

Precision medicine is an emerging approach for prevention and treatment of diseases to predict the best therapeutic option and prevention strategies for different subgroups of disease patients (Precision Medicine Initiative®). It is in contrast to a one-size-fits-all approach, in which disease treatment and prevention strategies are individually tailored on the patient. The combination of multiple biomarkers may detect different aspects of tumor biology and provide additional information. The early diagnosis of HCC is essential to improve the prognosis and long-term survival of patients. Izuno et al[21] reported that AFP was better able to detect small and locally confined tumors while PIVKA-II was more sensitive for detecting more diffuse tumors. Recent studies underlined the role of PIVKA-II as independent predictor of microvascular invasiveness in HCC[22]; as reported, it seems to be correlated with a more favorable prognosis after liver transplantation than the macrovascular invasion, but it is also linked with recurrence of liver cancer after transplantation[23-26]. PIVKA-II values are considered for tumor relapse where high levels of AFP and PIVKA-II are linked[26,27]. Moreover, PIVKA-II levels are correlated with tumor size[28].

Since the importance to detect HCC at an early stage when the disease is still curable, biomarkers can make the difference and complete the diagnosis obtained with ultrasound before moving on to

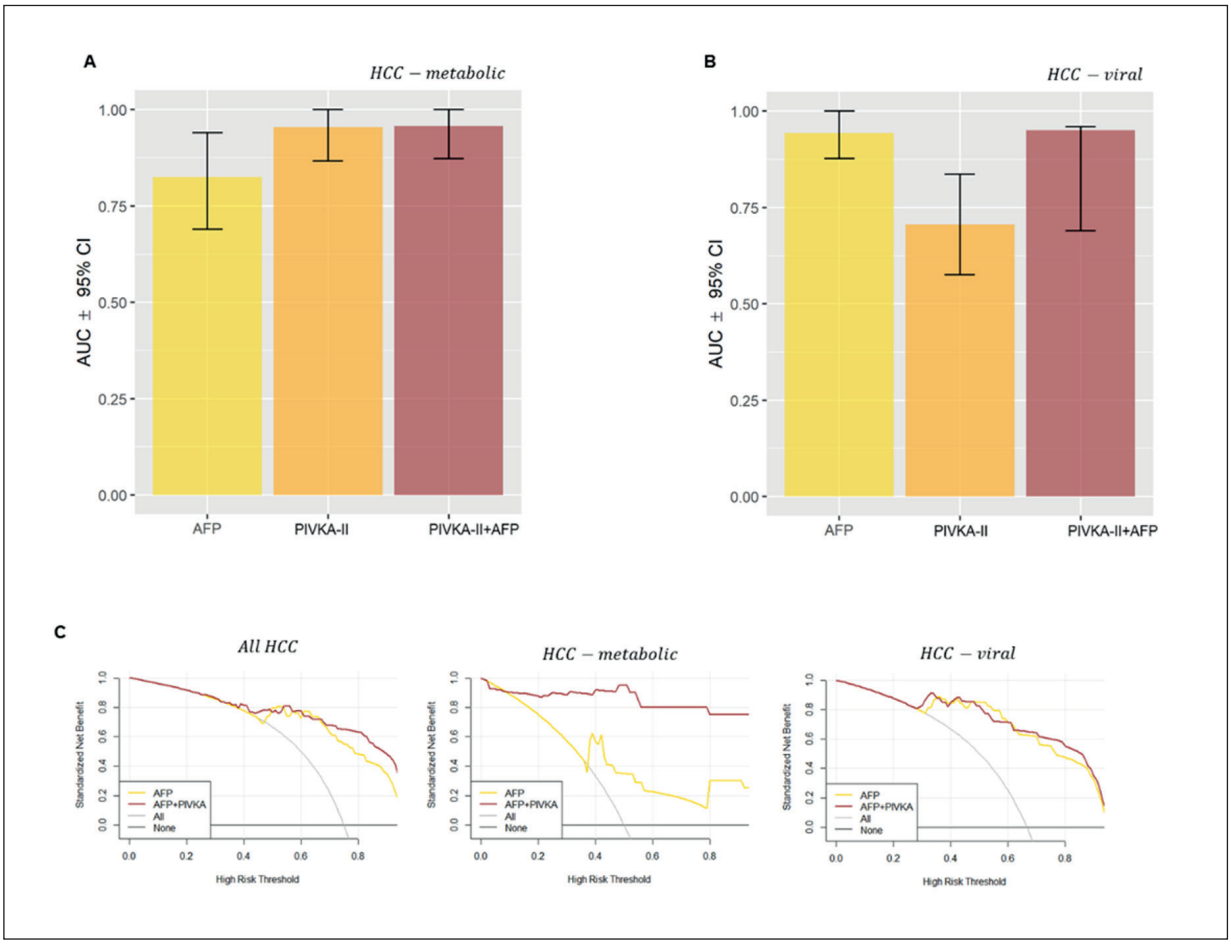

Figure 3. AUC values with the corresponding confidence intervals for AFP, PIVKA-II and their combination in metabolic HCC (**A**) and viral HCC patients (**B**). Decision Curve Analysis (DCA) performed on AFP alone and its combination with PIVKA-II for discriminating control subjects from HCC *(left)*, metabolic HCC *(center)* and viral HCC *(right)* patients (C).

the more expensive MRI. Biomarkers can also be used to better stratify patients on the liver transplant waiting list.

FGF belongs to a large family of growth factors that regulate a broad spectrum of biological pathways, including cellular proliferation, survival, migration, and differentiation. HCC is typically a hypervascular tumor and FGF plays a critical role as a stimulatory angiogenic factor of liver disease[29], sustaining angiogenesis and HCC progression.

MMPs are extracellular matrix-degrading enzymes that enhance tumor invasiveness and metastasis; high levels of MMPs are considered prognostic factors of poor overall survival of patients with HCC[30,31]. Metalloproteinases such as MMP-9 gelatinase have been shown to be involved in many steps of tumor development and progression but also of its suppression. They are generally associated with malignant phenotype, invasion, progression, and low survival[32-35]. Different studies have shown that MMP-9 is predictive of a more invasive and metastatic character of HCC and its high expression in tumor tissues is considered a prognostic factor of poor overall survival in HCC patients[36]. However, serum levels of MMP-9 do not correlate with either tumor size or serum AFP concentrations. Therefore, this result could indicate MMP-9 as a new and independent specific biomarker of HCC able to highlight some biological characteristics of the neoplasia and precisely the invasive potential and neoangiogenic abilities[37].

Serological levels of ALT and AST liver enzymes are tested routinely and automatically in current clinical settings. These biomarkers are usually elevated in patients with liver diseases and their concentrations may reflect the status

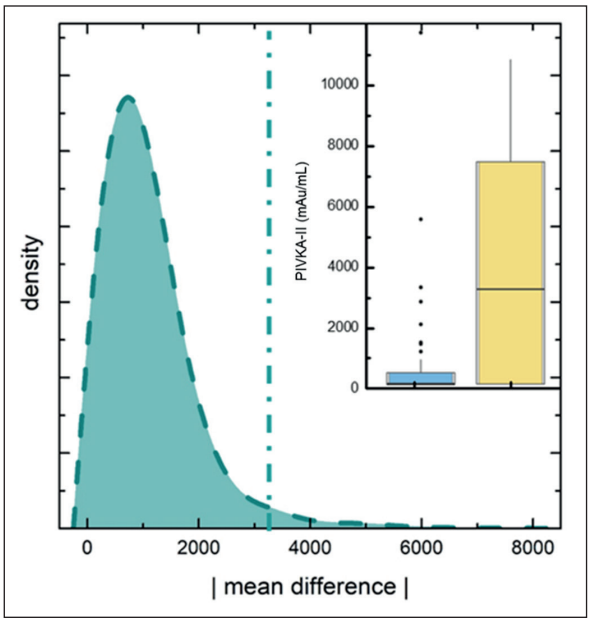

Figure 4. Main plot: bootstrap distribution of the absolute difference of the mean PIVKA levels between patient with and without portal invasion. The dashed vertical line represents the measured mean difference between the two groups. Inset plot: box plot of serum PIVKA levels expressed in mAU/mL for patients with and without portal vein invasion.

of liver injury[38]. In clinical practice high levels of ALT and AST are widely recognized suggestive of liver disease because[39] these enzymes are released from damaged hepatocytes into the circulation[40]. Therefore, the positive correlation of MMP-9 levels with ALT/AST ratio could showed that this marker is an indicator of disease severity and extent.

Our results confirmed the difference between patients and healthy individuals and displayed interesting differences between metabolic and viral HCC groups: PIVKA-II serum levels were higher in the metabolic-disease group than to patients with viral infection and it is correlated with vascular invasion; on the contrary, serum AFP, ALT, AST levels and their ratio were higher in the second group than in patients with metabolic HCC (Figure 5).

Statistical analysis of results suggests that choice of biomarker depends upon the origin of HCC: in metabolic HCC patients, PIVKA-II seems to have a better reliability than in viral HCC and therefore might be of major clinical utility in patient management. Moreover, only in metabolic HCC individuals the combination of two biomarkers is more efficient than AFP alone. AFP is not involved in the pathogenesis of metabolic liver disease, has been observed that the serum levels of AFP are not different between NAFLD patients and healthy controls and no associations was found between AFP and histological finding[41]. In clinical practice, these results indicated that PIVKA-II is a necessary complement to AFP in metabolic HCC surveillance. The different behavior observed in the two HCC groups might be of high clinical value for follow-up purposes and therefore deserves a more in-depth study.

The increased level of PIVKA-II and vascular invasion may also reflect the evidence that PIVKA-II levels are inversely correlated with vitamin K concentration and storage, which is crucial in reducing thrombus formation and others cardiovascular complications[42]. The catabolic state promoted by cancer could lead to a relative vitamin K deficiency which results in a significant increase in uncarboxylated prothrombin. The increase in PIVKA-II can represent a marker of coagulation abnormalities or inflammatory response[43,44]. These data, also considering the significant positive correlation that we found between serum PIVKA-II levels and vascular invasion, could be explained by the fact that patients were affected by HCC over a condition of metabolic liver disease[45].

It appears that the combination of PIVKA-II and AFP always outperforms AFP alone across a wide range of threshold probabilities, strengthening the idea that the use of PIVKA-II could be a valuable predictor of HCC in subjects diagnosed with metabolic hepatitis.

PIVKA-II appears as an independent predictor of microvascular invasion of HCC. It is also considered a biomarker for tumor relapse coupled with AFP. Evaluation of the other candidate biomarkers was unsatisfactory. MMP-9 and FGF do not provide any additional useful information for the improvement of clinical management. Our study is limited by the small sample size, therefore further studies are necessary to strengthen our date; however, from our results, PIVKA-II emerges as a reliable biomarker to better stratify HCC patients, and able to identify vascular invasion.

Conclusions

Monitoring of HCC both in terms of early diagnosis and during treatment remains one of the most important objectives to be achieved to

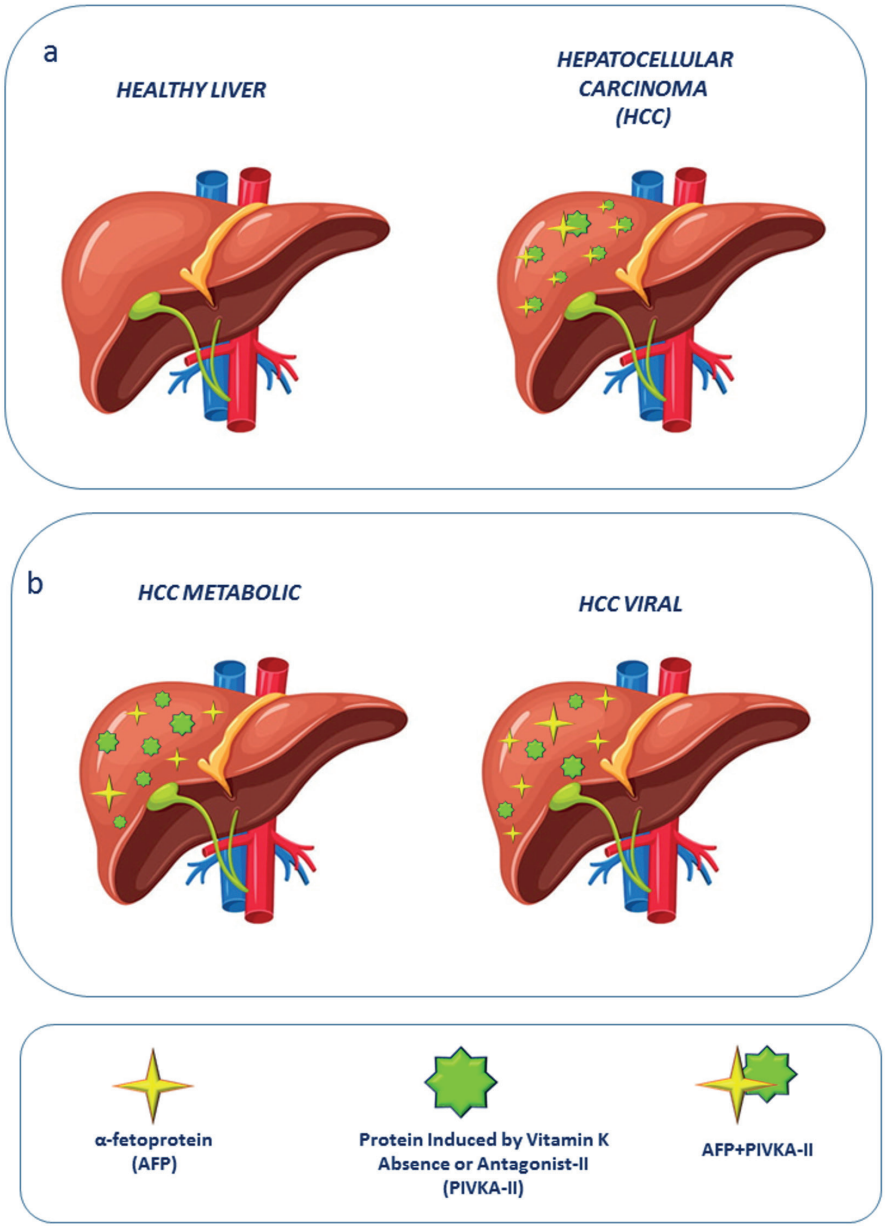

Figure 5. Graphical representation of AFP, PIVKA-II and their combination in healthy liver and in metabolic/viral HCC. **A**, The difference between healthy liver and HCC. **B**, The difference between viral HCC and metabolic HCC.

date. The difficulty for early diagnosis and careful monitoring consists in identifying suitable clinical markers that reflect disease status and response to treatment.

Developing panels with different biomarkers have been proposed in the literature for the diagnosis of liver diseases, with particular regard to proliferative disorders[46-48]; actually, most of them displayed limitations in terms of low sensitivity and specificity, and high cost[49]. To improve the therapeutic monitoring, it would be useful to know the molecular mechanisms that reside at the basis of resistance to treatment in order to use careful surveillance[50].

In the precision medicine era, the measurement of PIVKA-II could be useful in therapeutic monitoring treatment, despite further studies are needed to determine what causes the PIVKA-II increase and whether vitamin K deficiency in HCC patients may affect mortality or morbidity.

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Conflict of Interests

The Authors declare that there is no conflict of interest.

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