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Minimal increases of serum alpha-foetoprotein herald HCC detection in Caucasian HBV cirrhotic patients under long-term oral therapy

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Abstract

Background & Aims: In Caucasian patients with compensated cirrhosis caused by hepatitis B virus (HBV), the risk of hepatocellular carcinoma (HCC) developing persist despite long-term nucleos(t)ide analogs (NUC) treatment. In the surveillance of this population with persistently normal transaminases because of NUCs, the added value of serum alpha-foetoprotein (AFP) monitoring is poorly defined.

Methods: Two hundred and fifty-eight Caucasian HCC-free patients with HBV-compensated cirrhosis who started tenofovir or entecavir while having normal serum AFP levels (≤7 ng/mL) at baseline or within the first year of treatment underwent HCC surveillance by semiannual ultrasound evaluation and serum AFP determination.

Results: During 96 (18-120) months of antiviral therapy, 3947 AFP values were collected, median AFP level was 2 ng/mL. Thirty-five patients developed an HCC at an overall 8-year crude cumulative incidence of 14% (annual incidence of 2%). HCC incidence increased in parallel with increasing AFP thresholds: 24%, 36%, 64% and 92% for AFP levels after exceeding 2, 4, 6 and 7 ng/mL for the first-time. Of the 12 patients who experienced an AFP rise > 7 ng/mL, 11 developed an HCC and one had liver metastases of lung cancer. Overall, an AFP > 7 ng/mL had 99.6% specificity, 31.4% sensitivity, 91.7% PPV, 90.2% NPV, LR+ 70.1 and LR− 0.69 for HCC; this excellent specificity was maintained up to 18 months before HCC detection.

Conclusions: In Caucasian patients with HBV-compensated cirrhosis on long-term NUC, an increase in AFP over 7 ng/mL shows excellent specificity, heralding HCC development within 1 year.

KEYWORDS
Alpha-foetoprotein, diagnostic tests, hepatitis B, hepatocellular carcinoma, surveillance

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Abbreviations: ADV, adefovir; AFP, alpha-foetoprotein; ALT, alanine aminotransferase; BCLC, Barcelona Clinic Liver Cancer; CHB, chronic hepatitis B; ETV, entecavir; EV, oesophageal varices; GI, gastrointestinal; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LMV, lamivudine; LT, liver transplantation; NUC, nucleos(t)ide analogs; PCR, polymerase chain reaction; TDF, tenofovir disoproxyl fumarate; US, abdominal ultrasound.

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1 | INTRODUCTION

Alpha-foetoprotein (AFP) is a serum biomarker of hepatocellular carcinoma (HCC) that in consequence of its mainly suboptimal specificity (72%-99% depending on serum cut-off) and low sensitivity (20%-65% depending on aetiology), was dropped from the European (EASL) and American (AASLD) guidelines for HCC surveillance.[1-5] This was not the choice of the Asian Pacific Associations for the Study of the Liver which still recommends the combination of AFP with US for HCC surveillance.[6,7] More recently, the use of AFP was revamped by both AASLD and EASL, the latter, however, discouraging the use of this marker in patients with active liver inflammation.[8,9] One major obstacle to the definition of the predictive power of serum AFP, in fact, was the design of studies enrolling heterogeneous populations with liver diseases of mixed aetiologies (hepatitis C, alcohol) or, in case of hepatitis B, the study of patients unexposed to effective antiviral treatment.[10]

In patients long-term treated by effective nucleos(t)ide analogs (NUCs), with suppression of hepatic inflammation, the false positive results of serum AFP are expected to be minimized, hence the role of this marker in HCC surveillance should be re-evaluated, since the risk of hepatocellular carcinoma (HCC) is reduced but not eliminated.[11-19] Four retrospective studies in Asian patients at risk of HCC reported a diagnostic sensitivity of 38%-81% and a specificity between 80% and 100% using different AFP cut-offs spanning from 6 to 20 ng/mL.[20-23] These studies included patients who were heterogeneous in terms of liver disease stage, HBeAg status, type of NUCs, duration of follow-up and, more importantly, compliance to HCC surveillance. Further complicating the assessment of AFP accuracy is its variability in serum levels in healthy subjects according to ethnicity: 7 ng/mL stands as the 'upper normal limit' value for Caucasians.[24,25]

To shed new lights on this important topic, we therefore assessed the diagnostic performance of serum AFP as a marker of HCC in a cohort of Caucasian patients with HBV-related compensated cirrhosis who were treated for more than 8 years with tenofovir (TDF) or entecavir (ETV), with normal AFP levels at baseline or within the first year of therapy.

Keypoints

- Serum alpha-foetoprotein (AFP) levels increase as response to the regeneration stimuli owing to high necro-inflammatory activity in chronic/acute hepatitis. Moreover, AFP is associated with hepatocellular carcinoma (HCC) growth and is currently used in predictive model for HCC recurrence.
- In Caucasian-compensated cirrhotics with normal ALT levels owing to long-term effective oral therapy, an increase in AFP above the normal value of 7 ng/mL is highly specific for HCC development during surveillance, and the cumulative probability of HCC progressively increased in parallel to AFP threshold exceeded.
- AFP levels significantly differ in patients developing or not an HCC up to 24 months before HCC.
- In long-term treated HBV cirrhotics, the addition of serum AFP to 6-month ultrasound examinations may improve the surveillance strategy aimed at detecting early HCC.

2 | MATERIALS AND METHODS

2.1 | Patients' selection and study design

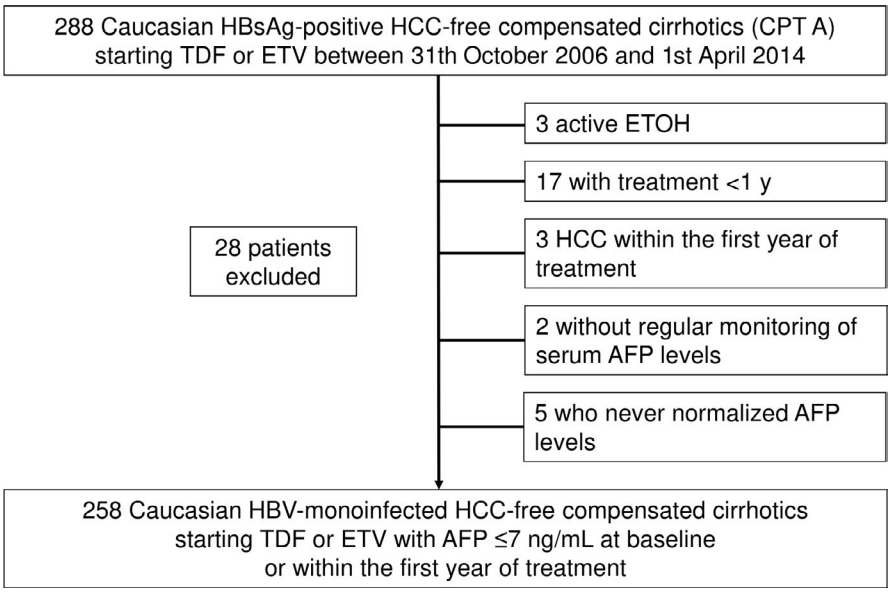
All consecutive HCC-free Caucasian HBsAg-positive monoinfected patients with compensated cirrhosis starting TDF or ETV between October 31, 2006 and April 1, 2014 at two tertiary Liver Centers in Milan, Italy, were evaluated for inclusion in this longitudinal cohort study, having a normal AFP levels at baseline or within the first year of therapy. Baseline was considered the start of TDF or ETV,[12,13,19,26] when patients were evaluated for their full medical history, comorbidities and drugs, physical and virological/biochemical parameters (HBsAg, HBV DNA, hepatitis B e antigen and antibody [HBeAg and anti-HBe], anti-HCV, anti-HDV, anti-HIV, serum AFP, liver and renal function tests, autoimmunity serum markers, serum electrophoresis), liver stiffness (Fibroscan®) and abdominal US. All patients provided written informed consent for sera sample storage and analysis; the study was approved by the Hospital Ethical Committee (Milan Area 2). Previous exposure to Lamivudine (LMV) and/or Adefovir (ADV) was allowed.

Of the 288 consecutive compensated HBV cirrhotics starting TDF or ETV, 28 were excluded from the study: 17 patients received TDF/ETV for less than one year, 3 developed HCC within the first year of treatment, 2 did not have regular monitoring of serum AFP, 3 had significant alcohol abuse (>60 g/day for men and >40 g/day for women assessed by patient's clinical interviews) and 5 who did not normalize AFP levels within the first year despite virological suppression. Finally, 258 HBV-monoinfected HCC-free compensated cirrhotics starting TDF or ETV with a treatment duration >1 year and with AFP ≤ 7 ng/mL within the first year of therapy were included in this study (Figure 1).

Monitoring was performed every 6 months with blood tests comprehensive of serum AFP, and HCC surveillance by abdominal US. Contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) techniques were performed if liver ultrasound could not carefully evaluate the whole hepatic parenchyma during surveillance.[20] As for internal protocol, whenever serum AFP increased >7 ng/mL in patients with normal ALT levels and permanent undetectable HBV-DNA, with no lesion detected by US, a CT scan or an MRI was performed within 3 months together with a new AFP determination. In patients with negative CT or MRI but still serum AFP levels persistently above the upper normal limit, a CT or MRI was repeated every 3 months.

Diagnosis of HCC was made according to international recommendations, primarily by the typical features of HCC at contrast-enhanced CT and/or MRI, whereas biopsy was employed to rule out undefined nodules.[4,5] Barcelona Clinic Liver Cancer staging system (BCLC) was applied for HCC staging and treatment.[27,28]

FIGURE 1 Patient disposition



End of follow-up was set at HCC diagnosis or at August 1, 2017. All HCC cases diagnosed after the first year of TDF or ETV therapy were included in the analysis; the corresponding AFP value was evaluated together with its trend before the event. For non-HCC cases, last serum AFP value collected at the end of follow-up was used for the analysis, but waiting for the next six-month abdominal US (until January 2018) to confirm the absence of HCC.[22]

Using prospectively collection of longitudinal samples in a cohort of patients at HCC risk, this was a phase 3 biomarker study according to National Cancer Institute's Early Detection Research Network guidelines.[29]

2.2 | Definitions

Hepatitis B virus chronic infection was defined as HBsAg positivity for >6 months. Antiviral therapy with ETV or TDF was introduced according to international guidelines,[30,31] renal function and previous NUC treatments. Persistent HBV suppression meant HBV-DNA under the lower limit of quantification of the test throughout treatment. Virological breakthrough was the serum HBV-DNA reappearance, whereas clinical breakthrough was also associated with ALT increases.

Diagnosis of cirrhosis was based on histology (Ishak score staging 5 or 6; Metavir score F4) or on clinical grounds using abdominal US features of blunted, nodular liver edge accompanied by splenomegaly (>13 cm) and platelet count <120 × 10^9/L; and/or on the presence of oesophageal varices.[32] Liver stiffness (LSM) was measured using transient elastography (Fibroscan®, Echosens, Paris, France) from 2006, when the technique became available in our centres.[33,34] Endoscopic evaluations for the detection or follow-up of oesophageal varices (EV) and treatment were performed according to international guidelines, as well as definitions of clinical decompensation (the development of at least one among these: ascites, jaundice, hepatic encephalopathy, gastro-intestinal (GI) haemorrhage from oesophago-gastric varices or Child-Pugh score ≥7).[35,36]

2.3 | Laboratory assays

Serum ALT and aspartate aminotransferase (AST) were measured by automated methods at 37°C (normal value <41 IU/L). Serum HBV DNA was measured by polymerase chain reaction assay with a lower limit of quantification (LLQ) of 71 IU/mL from 2006 until 2008, then by real-time polymerase chain reaction assay with a LLQ of 12 IU/mL until 2013, and subsequently by Abbott RealTime HBV (Abbott Diagnostics, Rome, Italy) with a LLQ of 10 IU/mL. HBV genotypes were determined by INNO-LiPA HBV genotyping (Fujirebio Europe NV). Serum AFP levels were determined by ImmunoAssay in Electrochemistry Luminescence 'ECLIA' (Roche Diagnostic GmbH, Mannheim, Germany), an immunological assay with a range of 0.605-1210 ng/mL; higher values can be diluted 1:50, normal value ≤7 ng/mL).[25]

2.4 | Statistical analysis

Patients characteristics are described by means of relative frequencies and percentage for qualitative variables, and by median and range for quantitative variables. Wilcoxon rank sum test was used to compare AFP values in two groups of patients, according to HCC occurrence. All *P*-values were two-tailed and a level lower than 0.05 (or adjusted for multiple comparisons according to Bonferroni and Sidak) was considered statistically significant.[37] Crude cumulative incidence of HCC from time to diagnosis was computed according to Kalbfleish and Prentice method taking into account the competing effect of death.[38] For the different cut-off of AFP considered, sensitivity, specificity, Youden's index, positive predictive value (PPV), negative predictive value (NPV), likelihood positive ratio (LR+), likelihood negative ratio (LR-) have been computed. The Youden's index is equal to sensitivity + specificity-1 and provides a value between 0 and 1, which represents the global ability to predict the onset of HCC. A Youden's index next to 1 means a very high sensitivity and

specificity, whereas a value close to 0 represents a condition of poor or no sensitivity and specificity. Statistical analyses were performed with R software, version 3.4.3, with package *cmprks* added.

3 | RESULTS

3.1 | Population included in the study

Baselines features of the 258 enrolled patients are summarized in Table 1. Most subjects were males, HBeAg-negative, genotype D, with normal ALT levels, undetectable HBV-DNA, previously exposed to LMV and/or ADV, without significant portal hypertension and without comorbidities. Cirrhosis was diagnosed by histology in 197 (76%) patients and on clinical grounds in 61 (24%). 215 (83%) patients received TDF and 43 (17%) ETV: all 150 patients with LMV/ADV exposure were treated by TDF. Median AFP level at the first year of TDF/ETV treatment was 2 (1-7) ng/mL. During the study period, neither virological nor clinical breakthroughs were registered.

3.2 | HCC incidence and AFP trends during long-term NUC treatment

During 96 (18-120) months of study period, HCC was diagnosed in 35 patients after a median time of 52 (18-100) months of therapy.

TABLE 1 Demographic, clinical and virological characteristics of 258 subjects included in the study at Tenofovir/Entecavir introduction (baseline)

Variables	N = 258
Age, years[†]	61 (21-83)
Male, N (%)	212 (82%)
HBeAg-negative, N (%)	226 (88%)
Genotype D [‡], N (%)	136 (80%)
ALT normal (<41 IU/L), N (%)	177 (69%)
Undetectable HBV-DNA, N (%)	141 (55%)
Previously NUC treated, N (%)	150 (58%)
BMI, Kg/m^2[†]	25 (17-40)
Diabetes, N (%)	32 (12%)
Arterial hypertension, N (%)	98 (38%)
Platelets, ×10^9/L[†]	154 (32-304)
Portal hypertension [§], N (%)	36 (14%)
Liver stiffness, kPa [†,¶]	9 (3-75)
AFP, ng/mL[†]	3 (1-167)[A]

Abbreviations: AFP, alpha-foetoprotein; ALT, alanine aminotransferase; BMI, body mass index; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.
[†]Median (range);
[‡]Available in 170 patients;
[§]Presence of oesophageal varices: F1 in 22 (61%), F2-F3 in 4 (11%), previously endoscopically treated for GI bleeding prophylaxis in 10;
[¶]Liver stiffness by Fibroscan®, value available and valid in 237 (90%);
[A]AFP 2 (1-7) ng/mL at the first year of TDF/ETV treatment.

The 8-year crude cumulative HCC incidence was 14% (95%CI: 11-18), with an estimated annual incidence of 2% (Figure 2; HCC diagnosed within the first year of TDF/ETV therapy have been excluded).

A total of 3,947 serum AFP determinations were collected during the study period: median AFP levels were 2 ng/mL (Figure S1). Comparing the means of AFP values at diagnosis (HCC group) with those collected at last visit in non-HCC subjects, AFP was higher in the former than in the latter (17.8 vs 2.6 ng/mL, $P < .0001$). Moreover, when comparing the means of the last 4 AFP values collected every 6 months in the group of patients who developed an HCC (ie the last 18 months before diagnosis), they were significantly higher compared to those of non-HCC group, and it appears that AFP levels tends to arise near the diagnosis of HCC (Table 2). Of note, features of HCC and non-HCC group at diagnosis and last visit were similar: HBV-DNA was undetectable in 100% and 99.9%, and median ALT level 25 (10-41) and 20 (3-41) IU/L ($P = .314$) respectively.

When HCC and no-HCC groups were compared after excluding the AFP value at diagnosis (that could be higher and change the mean value), AFP levels were significantly higher in the former than in the latter (3.5 vs 2.4 ng/mL, $P = .0002$) up to 24 months before the HCC (Table 3).

3.3 | Relationship between AFP thresholds and HCC incidence

To evaluate the ability of AFP to predict HCC occurrence, the cumulative incidence of HCC in patients with AFP levels exceeding for the first-time specified thresholds (AFP >2, >3, >4, >5, >6, >7 ng/mL) were estimated (Figure 3). An AFP increase over 7 ng/mL corresponded to a probability of 58% (95%CI: 33-83), 83% (95%CI: 62-105) and 92% (95%CI: 71-112) in developing an HCC within six months, 1 and 8 years respectively. The 8-year crude cumulative HCC incidence increased in parallel also for AFP thresholds < 7 ng/

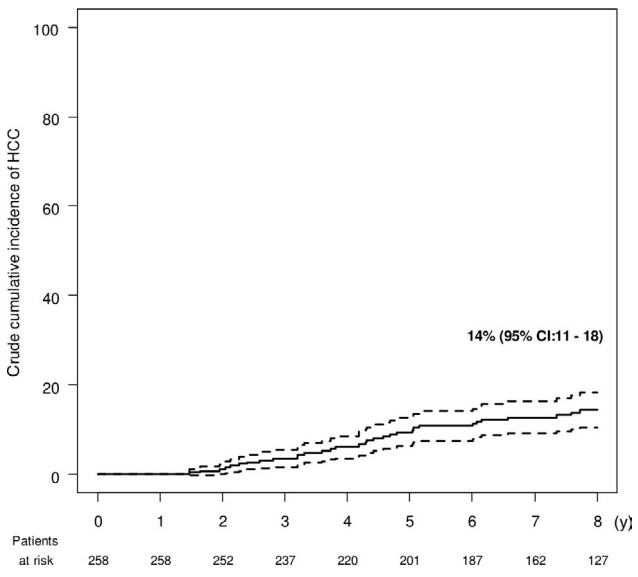


FIGURE 2 8-year crude cumulative incidence of HCC in 258 cirrhotic patients treated with Tenofovir/Entecavir

TABLE 2 Comparison of means of the last AFP values collected every 6 months in the group of patients who did or did not develop an HCC

Timing of testing	Mean AFP levels (ng/mL)			
	HCC Group (N = 35)	NO HCC Group (N = 223)	Difference between HCC and NO HCC group	*P**
Last determination†	17.8	2.6	15.2	<.0001
Last 2 determinations	11.0	2.5	8.5	<.0001
Last 3 determinations	8.5	2.5	6.0	<.0001
Last 4 determinations	7.2	2.5	4.6	<.0001

*Wilcoxon rank sum test.
†Last collection: in HCC group, AFP at diagnosis; in NO HCC group, AFP corresponds to that evaluated 6 months before the last imaging confirming the absence of HCC. AFP, alpha-foetoprotein; HCC, hepatocellular carcinoma.

TABLE 3 Comparison of means of the last AFP values collected every six months in the group of patients who did or did not develop an HCC, excluding AFP at HCC diagnosis

Timing of testing	Mean AFP levels (ng/mL)			
	HCC Group (N = 35)	NO HCC Group (N = 223)	Difference between HCC and NO HCC group	*P**
Last determination† (6 months before)	3.8	2.4	1.4	.0025
Last 2 determinations (12 months before)	3.6	2.3	1.3	.0010
Last 3 determinations (18 months before)	3.5	2.4	1.1	.0004
Last 4 determinations (24 months before)	3.5	2.4	1.1	.0002

*Wilcoxon rank sum test.
†Last collection: in HCC group, AFP six months before diagnosis; in NO HCC group, AFP corresponds to that evaluated 6 months before the last value collected.

mL: 24%, 36% and 64% for AFP levels exceeding 2, 4 and 6 ng/mL for the first-time respectively (Figure 3).

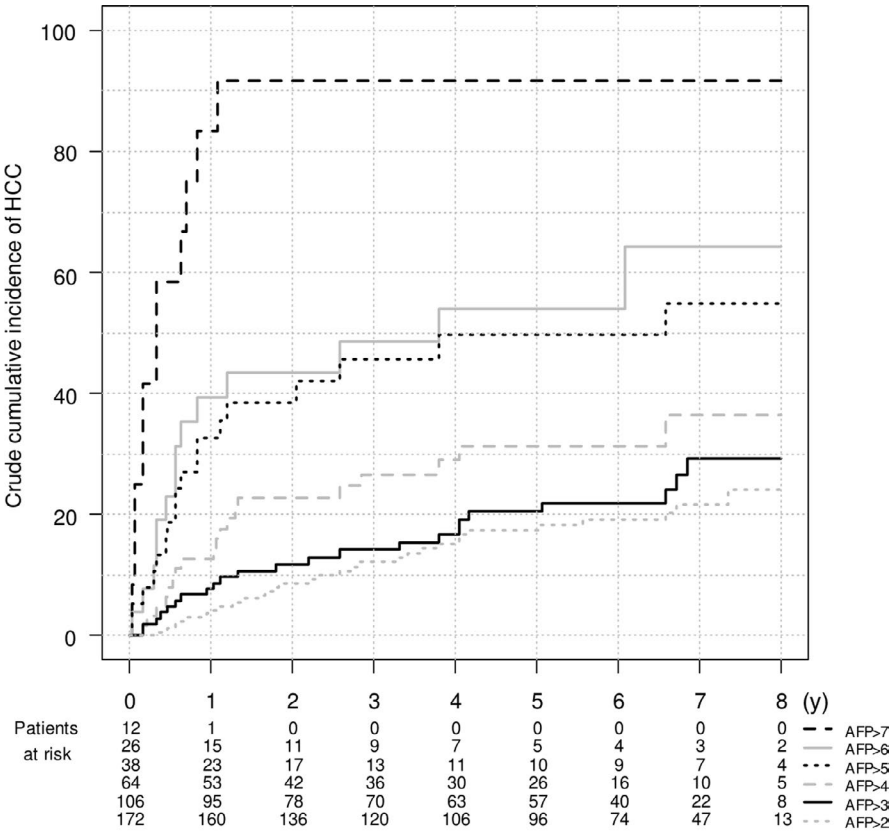
The correlation between a higher AFP level and HCC onset is also represented in Figure 4, in which X axis represents the months before the end of follow-up, Y axis different AFP thresholds, and the two lines the groups of patients with or without HCC. In the Figure are represented the proportion of patients that overcome a specific AFP threshold (from 2 to 7 ng/mL) during the follow-up. In the HCC group the proportion of patients who exceed the highest AFP thresholds is greater, as well as the slope of the line in relation to time is steeper, compared to non-HCC patients, indicating the close temporal relationship between the event 'AFP increase' and HCC development. The same time-relationship between 'AFP increase' and 'HCC onset' is represented in Figure S2, in which the AFP values collected up to 6 months before the end of follow-up were used.

3.4 | Diagnostic accuracy of AFP in HCC surveillance

When considering last AFP value collected, an AFP >7 ng/mL had a 99.6% specificity and 31.4% sensitivity for HCC occurrence, with a 91.7% PPV and 90.2% NPV (Table 4A). To evaluate the accuracy of AFP in HCC surveillance, the last four values collected every six months were considered and their mean levels were used to forecast the HCC development: the almost absolute specificity of AFP as tumour marker in this setting has been confirmed as far as 18 months before the end of follow-up, with only a slight decrease in sensitivity, up to 25.7% (Table 4B-D).

Applying only the last AFP value to predict HCC development, the AFP cut-off of 4 ng/mL had the best performance (Youden Index = 0.465), with 95% specificity and 51.4% sensitivity. However, because of its low PPV (62%), low cumulative HCC incidences at year 1 (13%, range 6-20) and year 8 (36%, range 24-49) (Figure 3), and because of a change in imaging surveillance as per internal protocol and the 'upper normal limit' of the test for Caucasian, the AFP threshold of 7 ng/mL was used for the subsequent description.

Of note, even retrospectively computing the diagnostic accuracy of AFP at 6, 12 and 18 months before the end of follow-up, the absolute specificity of higher AFP thresholds were also confirmed (sensitivity 14.7, specificity 99.6, PPV 83.3 for AFP over 7 ng/mL; Table S2.1), showing again a time-relationship between AFP levels and prediction for HCC (Tables S2.1-2.3).



	AFP >7	AFP >6	AFP >5	AFP >4	AFP >3	AFP >2
6 mo	58% (33 - 83)	23% (9 - 37)	19% (8 - 29)	8% (2 - 13)	5% (1 - 8)	1% (0 - 3)
1 y	83% (62 - 105)	39% (23 - 56)	33% (20 - 46)	13% (6 - 20)	8% (3 - 12)	4% (1 - 6)
8 y	92% (71 - 112)	64% (42 - 87)	55% (39 - 71)	36% (24 - 49)	29% (20 - 39)	24% (17 - 31)

FIGURE 3 Crude cumulative incidence of HCC since the first-time AFP is over a specific cut-off (Percentages are depicted in the Table under the Figure)

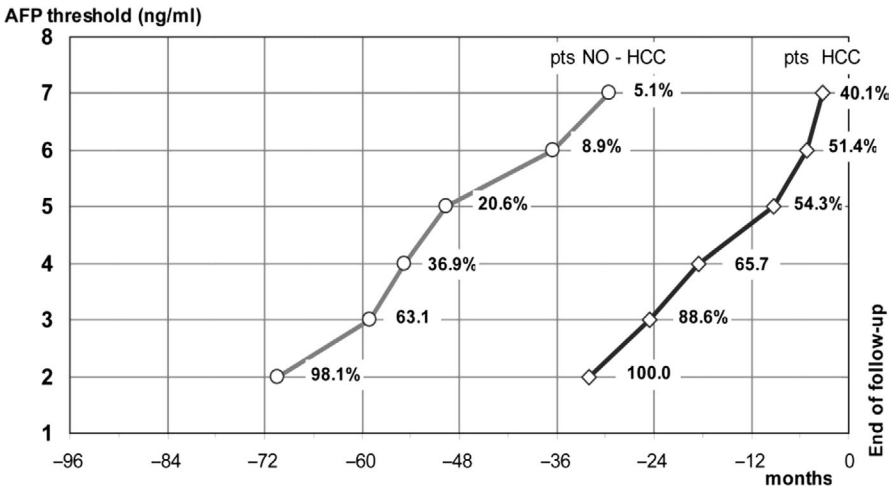


FIGURE 4 Median values of the months before the end of the observations in which the threshold of AFP has been exceeded for the first-time, according to HCC development. Percentages refer to the number of subjects who exceed each threshold in the two groups

Twelve (5%) patients showed an AFP increase >7 ng/mL 53 (13-99) months after TDF/ETV introduction: all but one developed an HCC within 13 months after the AFP threshold has been exceeded for the first-time. In six (50%) of these 12 patients, the rise of AFP occurred concurrently to the detection by US of a liver nodule that was diagnosed as HCC by contrast imaging techniques. Five (42%) patients who experienced AFP levels >7 ng/mL and negative US examination, performed II level imaging: AFP overcame the 7 ng/mL cut-off 3 to 9 months before a liver nodule could be detected by contrast-enhanced techniques. The time course of AFP levels and imaging findings is illustrated in detail in Table S1. Among these 12 patients, one with AFP levels >7 ng/mL (9 ng/mL) and no evidence of HCC had hepatic metastases of lung cancer diagnosed by CT scan 4 months later (AFP = 14 ng/mL).

TABLE 4 Sensitivity, specificity, positive predictive value and negative predictive value, according to different AFP cut-offs and different AFP values

	AFP levels (ng/mL)					
	7	6	5	4	3	2
A. Last AFP value collected.						
SN	31.43 (16.85-49.29)	34.29 (19.13-52.21)	42.86 (26.32-60.65)	51.43 (33.99-68.62)	57.14 (39.35-73.68)	74.29 (56.74-87.51)
SP	99.55 (97.53-99.99)	99.1 (96.8-99.89)	97.76 (94.85-99.27)	95.07 (91.35-97.51)	83.41 (77.86-88.04)	55.16 (48.37-61.8)
PPV	91.67 (61.52-99.79)	85.71 (57.19-98.22)	75 (50.9-91.34)	62.07 (42.26-79.31)	35.09 (22.91-48.87)	20.63 (13.94-28.75)
NPV	90.24 (85.83-93.65)	90.57 (86.19-93.93)	91.6 (87.32-94.79)	92.58 (88.38-95.62)	92.54 (87.99-95.76)	93.18 (87.45-96.84)
Youden Index	30.98 (14.38-49.28)	33.39 (15.93-52.1)	40.61 (21.17-59.91)	46.5 (25.33-66.13)	40.55 (17.22-61.72)	29.44 (5.12-49.31)
LR+	70.09 (9.34-526.15)	38.23 (8.93-163.61)	19.11 (7.41-49.29)	10.43 (5.39-20.17)	3.44 (2.28-5.19)	1.66 (1.3-2.11)
LR−	0.69 (0.55-0.86)	0.66 (0.52-0.84)	0.58 (0.44-0.78)	0.51 (0.36-0.72)	0.51 (0.35-0.76)	0.47 (0.26-0.83)
B. Mean of last two AFP values collected (mean of the last 6 months of study period).						
SN	28.57 (14.64-46.30)	31.43 (16.85-49.29)	45.71 (28.83-63.35)	51.43 (33.99-68.62)	54.29 (36.65-71.17)	77.14 (59.86-89.58)
SP	99.55 (97.53-99.99)	99.10 (96.80-99.89)	96.41 (93.05-98.44)	93.27 (89.15-96.19)	80.72 (74.92-85.68)	52.91 (46.14-59.61)
PPV	90.91 (58.72-99.77)	84.62 (54.55-98.08)	66.67 (44.68-84.37)	54.55 (36.35-71.89)	30.65 (19.56-43.65)	20.45 (13.93-28.35)
NPV	89.88 (85.42-93.34)	90.20 (85.78-93.62)	91.88 (87.61-95.04)	92.44 (88.18-95.54)	91.84 (87.08-95.26)	93.65 (87.87-97.22)
Youden Index	28.12 (12.16-46.29)	30.53 (13.65-49.18)	42.13 (21.88-61.79)	44.7 (23.14-64.80)	35.00 (11.56-56.85)	30.06 (6.00-49.19)
LR+	63.71 (8.41-482.47)	35.04 (8.11-151.48)	12.74 (5.90-27.53)	7.65 (4.26-13.73)	2.82 (1.88-4.22)	1.64 (1.30-2.06)
LR−	0.72 (0.58-0.88)	0.69 (0.55-0.87)	0.56 (0.42-0.76)	0.52 (0.37-0.73)	0.57 (0.39-0.82)	0.43 (0.23-0.80)
C. Mean of last three AFP values collected (mean of the last 12 months of study period).						
SN	28.57 (14.64-46.30)	28.57 (14.64-46.30)	42.86 (26.32-60.65)	51.43 (33.99-68.62)	57.14 (39.35-73.68)	74.29 (56.74-87.51)
SP	99.55 (97.53-99.99)	99.10 (96.80-99.89)	96.41 (93.05-98.44)	92.83 (88.61-95.84)	78.03 (72.01-83.28)	49.78 (43.03-56.53)
PPV	90.91 (58.72-99.77)	83.33 (51.59-97.91)	65.22 (42.73-83.62)	52.94 (35.13-70.22)	28.99 (18.69-41.16)	18.84 (12.69-26.37)
NPV	89.88 (85.42-93.34)	89.84 (85.36-93.31)	91.49 (87.16-94.72)	92.41 (88.13-95.52)	92.06 (87.25-95.49)	92.50 (86.24-96.51)
Youden Index	28.12 (12.16-46.29)	27.67 (11.43-46.20)	39.27 (19.38-59.09)	44.25 (22.6-64.46)	35.17 (11.37-56.96)	24.06 (−0.23-44.04)
LR+	63.71 (8.41-482.47)	31.86 (7.28-139.36)	11.95 (5.47-26.08)	7.17 (4.05-12.69)	2.60 (1.78-3.80)	1.48 (1.17-1.87)
LR−	0.72 (0.58-0.88)	0.72 (0.58-0.89)	0.59 (0.44-0.79)	0.52 (0.37-0.74)	0.55 (0.37-0.81)	0.52 (0.29-0.92)
D. Mean of last four AFP values collected (mean of the last 18 months of study period).						
SN	25.71 (12.49-43.26)	28.57 (14.64-46.30)	42.86 (26.32-60.65)	51.43 (33.99-68.62)	57.14 (39.35-73.68)	74.29 (56.74-87.51)
SP	99.55 (97.53-99.99)	99.10 (96.80-99.89)	96.41 (93.05-98.44)	91.93 (87.54-95.15)	78.48 (72.49-83.68)	47.53 (40.83-54.31)
PPV	90.00 (55.50-99.75)	83.33 (51.59-97.91)	65.22 (42.73-83.62)	50.00 (32.92-67.08)	29.41 (18.98-41.71)	18.18 (12.23-25.49)
NPV	89.52 (85.02-93.04)	89.84 (85.36-93.31)	91.49 (87.16-94.72)	92.34 (88.02-95.48)	92.11 (87.31-95.51)	92.17 (85.66-96.36)
Youden Index	25.27 (10.02-43.24)	27.67 (11.43-46.20)	39.27 (19.38-59.09)	43.36 (21.53-63.76)	35.62 (11.85-57.36)	21.82 (−2.43-41.82)
LR+	57.34 (7.49-438.81)	31.86 (7.28-139.36)	11.95 (5.47-26.08)	6.37 (3.68-11.02)	2.65 (1.81-3.89)	1.42 (1.12-1.78)
LR−	0.75 (0.61-0.91)	0.72 (0.58-0.89)	0.59 (0.44-0.79)	0.53 (0.37-0.74)	0.55 (0.37-0.81)	0.54 (0.30-0.97)

Abbreviations: LR−, likelihood negative ratio; LR+, likelihood positive ratio; NPV, negative predictive value; PPV, positive predictive value; SN, sensitivity; SP, specificity; Youden Index: sensitivity + specificity-1 (means: 1 = very high sensitivity and specificity; 0 = poor or no sensitivity and specificity);

TABLE 5 HCC characteristics at baseline, diagnosis and during follow-up according to AFP pattern at HCC diagnosis

Variables	All HCC N = 35	HCC with AFP ≤ 7 ng/mL N = 24	HCC with AFP > 7 ng/mL N = 11	*P*
At baseline				
Age, year	64 (49-77)	63 (49-77)	65 (57-75)	.634
Male, N (%)	31 (89%)	22 (92%)	9 (82%)	.395
Portal hypertension[†], N (%)	12 (34%)	8 (33%)	4 (36%)	.861
Previously NUC treated, N (%)	26 (74%)	18 (75%)	8 (73%)	.886
At HCC diagnosis				
Platelets, $x10^9$/L	143 (43-260)	143 (43-260)	133 (58-254)	.846
HCC monofocal, N (%)	28 (80%)	20 (83%)	8 (73%)	.466
HCC diameter, mm[‡]	16 (6-40)	19 (9-40)	15 (6-35)	.361
Neoplastic portal vein thrombosis, N (%)	1 (3%)	1 (4%)	0 (0%)	.507
BCLC 0-A, N (%)	32 (91%)	22 (92%)	10 (91%)	.926
Edmonson Grade 3/available[§], N (%)	6/20 (30%)	1/14 (7%)	5/6 (83%)	.001

Note: For quantitative variables, median (range) and *P*-value of Wilcoxon's test are reported; for categorical variables, number (percentage) and *P*-value of Chi-square test are reported. After Bonferroni–Sidak correction for multiple test, the new threshold with which the *P*-values have to be compared is 0.0046.

Abbreviations: AFP, alpha-foetoprotein; ALT, alanine aminotransferase; BCLC, Barcelona Clinic Liver Cancer; HCC, hepatocellular carcinoma; NUC, nucleos(t)ide analogs.

[†]Presence of oesophageal varices or previously endoscopically treated varices;

[‡]diameter of the largest lesion if multifocal HCC;

[§]if available histology from fine needle biopsy or after hepatic resection of HCC.

Out of 35 patients who developed an HCC, 24 (69%) had the diagnosis when serum AFP was ≤7 ng/mL. However, AFP showed a rising trend even in 12 (50%) such patients in the months preceding the detection by US of a liver nodule that was diagnosed as HCC thereafter (Table S3).

3.5 | Clinical features of HCC

Table 5 shows the clinical and histological features of 35 HCCs. Most patients developed a single, small HCC in BLCL stage 0 or A. Comparing HCC groups according to the AFP cut-off of 7 ng/mL, no differences were found in HCC features between the two groups with the only exception of Edmonson classification: HCC was more frequently poorly differentiated in the group with AFP >7 ng/mL (83% vs 7%, *P* = .001).

4 | DISCUSSION

The role of serum AFP in HCC surveillance has been debated for a long time and, because of its suboptimal sensitivity and specificity, it has been excluded for a long time from several national and international HCC guidelines.[2-9] However, nowadays HBV represents a unique clinical setting because third-generation NUCs maintained viral suppression, shutting down liver inflammation, thereby maximize AFP specificity for early HCC detection, as well as recently showed in HCV cirrhotics cohorts cured by direct-acting antivirals.[39-41] Only two of the four previous Asian studies evaluating AFP-HCC relationship in HBV cohorts enrolled patients treated with high genetic barrier-to-resistance NUC (ETV).[20-23] While in the first study, a cut-off of 13 ng/mL of AFP levels had a 50% sensitivity and a 98.8% specificity for the diagnosis of HCC,[20] the corresponding features in the second study varied according to the AFP thresholds, with sensitivity ranging from 36.8% to 80.7% and specificity from 80.4% to 98.9% for an AFP cut-off of 20 and 6 ng/mL respectively.[22] Limiting, however, the transferability of these findings into clinical practice was disease severity that ranged from chronic hepatitis to decompensated cirrhosis, the limited duration of studies (less than 5 years) and the tumour size ranging from 28 to 42 mm, speaking against an efficient surveillance strategy. Our study provides several new relevant information on this topic: the most important being the increase in AFP >7 ng/mL strongly associated with detection of

early HCC within one year with excellent specificity. We also demonstrated that even smaller increases of AFP <7 ng/mL could be of clinical significance in HBV suppressed patients with cirrhosis. Of note, these findings were generated in patients in whom HCC was diagnosed at early stage further highlighting the relationship that exists between AFP levels and tumour size.[42] A recent merge-study on 42 HCCs (79% HBV, 85% undetectable HBV-DNA, 86% normal ALT, 93% cirrhotics) and 168 matched controls out of 4 different prospective clinical Asian studies and with a mean follow-up of 2 years showed an optimal AFP cut-off of 5 ng/mL (62% sensitivity and 87% specificity) at HCC diagnosis.[43] Moreover, we confirmed that incorporating the history of prior AFP testing can improve accuracy for detecting HCC and has prognostic significance: in an American study on HCV cirrhotics (82 HCC patients and 885 controls; 64% Caucasian), incorporating the standard deviation of AFP and the rate of AFP increase during surveillance improved the prognostic accuracy for HCC to an AUROC of 0.81, compared with 0.76 when only the last AFP level was used.[44]

We indeed confirmed the limited sensitivity of AFP also in long-term NUC treated patients with compensated cirrhosis, as only approximately 30% of early HCC tested positive for this marker. Even if the combination of AFP and *Lens culinaris* agglutinin-reactive fraction of alpha-foetoprotein (AFP-L3) biomarker seemed to improve the sensitivity for detecting HCC at very early stage compared to prothrombin induced by vitamin K absence-II (PIVKA-II),[43,45] even using lower AFP cut-off (5 ng/mL),[43] studies on long-term NUC suppressed Caucasian patients are awaited.

Finally, we confirm the AFP role as a marker of biological aggressiveness of HCC. Recently, AFP levels have been included in several models to predict survival after resection or liver transplantation for HCC.[42,46-48] In our study, an association between high AFP levels and poor tumour differentiation according to Edmonson staging system has been show, even the limited sample size of our study prevented the identification of any survival difference.

We acknowledge that our study has some limitations as the exclusion of patients with cofactors of liver disease (ie alcohol abuse). The exclusion of five patients who maintained AFP levels above the ULN beyond one year lead these findings to be applied only to patients with normal AFP levels at TDF/ETV baseline. Being generated in tertiary referral centres, findings require to be prospectively validated in other settings. We did not perform any cost-effectiveness analysis on the HCC surveillance, and the potential impact of AFP determinations on patient survival is presently unknown.

On the other hand, the study has several strengths. To our knowledge, this is the first study to assess the time course of AFP levels and its diagnostic accuracy in HBV compensated Caucasian cirrhotics long-term treated with ETV/TDF. Second, this large cohort of patients has been followed for more than 8 years, the longest follow-up among published studies. Third, the study cohort is homogeneous for ethnicity, disease stage, clinical features, viral genotype, HBV therapy, surveillance and management of HCC. Finally, HCC was diagnosed at early stage in the vast majority of patients, in agreement with current international recommendations.

In conclusion, this study demonstrates that in HBV-monoinfected patients with compensated cirrhosis who had been long-term treated with ETV/TDF, AFP levels tend to normalize or remain very low, and any increase above 7 ng/mL significantly predicts the development of HCC within one year. Importantly, smaller increases below this cut-off were associated with increased likelihood of HCC development over long time (PPV 91.7%). While the limited sensitivity of AFP calls for the development of additional strategies of early diagnosis of HCC in these populations, its excellent specificity (99.6%) favours additional studies aimed to re-evaluate AFP as a surveillance active tool of such a selected population as HBV-suppressed patients.

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CONFLICT OF INTEREST

Massimo Iavarone: Speaking and Teaching: Bayer, Gilead Science, Janssen, BTG, Abbvie; Consultant for BTG. Mauro Viganò: Speaking and Teaching: Roche, Gilead Sciences, BMS. Mariagrazia Rumi: Speaking and Teaching: MSD, Abbvie, Gilead; Advisory board: Abbvie. Angelo Sangiovanni: speaker bureau for Bayer, Gilead Science, Janssen, BTG, Abbvie, Novartis, Advisory board for Tiziana science. Massimo Colombo: Grant and research support: BMS, Gilead Sciences; Advisory Committees: Merk, Roche, Novartis, Bayer, BMS, Gilead Sciences, Tibotec, Vertex, Janssen Cilag, Achillion, Lundbeck, GSK, GenSpera, AbbVie, Alfa Wasserman; Speaking and teaching: Tibotec, Roche, Novartis, Bayer, BMS, Gilead Sciences, Vertex, Merk, Janssen, Abbvie. Pietro Lampertico: Speaking bureau/advisory boards: BMS, Roche, Gilead Sciences, GSK, MSD, Abbvie and Janssen, Eiger, Myr pharma.

AUTHORS CONTRIBUTION

AL, MI, MV, PL: study concept and design; AL, FF, GL, FC, VO: data collection; AL, AO, IC, PL: statistical analysis of data; AL, MI and PL: interpretation of data and drafting of the manuscript; AS, MR, MC: critical revision for important intellectual content; AL, MI, MV and PL: manuscript editing. All authors approved the final version of manuscript.

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SUPPORTING INFORMATION

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